UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"
Câmpus de São José do Rio Preto

Thalles Fernando Rocha Ruiz

Efeitos tardios da exposição ao bisfenol A na mama de fêmeas de gerbilos durante o período gestacional e lactacional

São José do Rio Preto
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Financiadora: FAPESP – Proc. 20/01240-9

Orientador: Profª. Dr. Sebastião Roberto Taboga

Coorientadora: Profª. Dra. Ellen Cristina Rivas Leonel

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R934e

Ruiz, Thalles Fernando Rocha

Efeitos tardios da exposição ao bisfenol A na mama de fêmeas de gerbilos durante o período gestacional e lactacional / Thalles Fernando Rocha Ruiz. -- São José do Rio Preto, 2022

140 p. : il., tabs., fotos

Dissertação (mestrado) - Universidade Estadual Paulista (Unesp), Instituto de Biociências Letras e Ciências Exatas, São José do Rio Preto

Orientador: Sebastião Roberto Taboga

Coorientadora: Ellen Cristina Rivas Leonel

1. Morfologia. 2. Histologia. 3. Biologia da Reprodução. 4. Endocrinologia. 5. Glândula Mamária. I. Título.

Sistema de geração automática de fichas catalográficas da Unesp. Biblioteca do Instituto de Biociências Letras e Ciências Exatas, São José do Rio Preto. Dados fornecidos pelo autor(a).

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Comissão Examinadora

Profª. Drª. Sebastião Roberto Taboga
UNESP – Câmpus de São José do Rio Preto
Orientador

Prof. Dr. Alexandre Bruni Cardoso
USP - Instituto de Química

Prof. Dr. Manoel Francisco Biancardi
UFG – Câmpus de Goiânia

São José do Rio Preto
25 de fevereiro de 2022

AGRADECIMENTOS

A Deus pela oportunidade e companhia durante todo esse tempo. Até aqui o Senhor me guiou.

Aos meus pais, Rosângela e Luiz Fernando, que me instruiram e me incentivaram em sempre trilhar o caminho acadêmico. Com muito amor e esforço me formaram e me aconselharam; apesar das dificuldades, pesares e perdas, sempre houve amor.

Ao meu orientador, Professor Sebastião Taboga, que me acolheu como verdadeiro filho e me instruiu como verdadeiro cientista. Sempre tive uma admiração profissional e pessoal que só aumentaram ao vir trabalhar aqui em Rio Preto. Ao senhor só tenho agradecimentos por ter aceito o desafio de me orientar durante esses anos.

À minha coorientadora, Professora Ellen Leonel, por todos os momento vividos desde que cheguei para trabalhar com o grupo. Meu crescimento pessoal e profissional tem muito do que essa maravilhosa pessoa, professora e pesquisadora me ensinou. O meu obrigado eterno não bastará.

Ao Ibilce, pelo crescimento acadêmico, e à Faculdade de Ciências – Unesp Bauru, pelo meu início na ciência. A Unesp sempre estará pronta para sustentar seus filhos.

Aos meus orientadores de iniciação científica, Profa. Irene e Prof. Carlos Vicentini, que me ajudaram a construir parte da pessoa que eu sou hoje, além de me banharem nas águas da morfologia.

Aos meus familiares, em especial minhas tias Rossania e Tânia, que cuidaram de mim a vida toda e sempre se preocuparam com meu bem estar. À minha prima, Lilliam, pelo incentivo e longas conversas sobre pesquisa e vivências. Ao meu tio William pelas risadas e ensinamentos eternos.

Aos meus eternos amigos Lamoanos, Maira, Mário, Mateus e Diego, pela vida que compartilharam comigo e pelas chatices que aguentaram. Em especial um agradecimento à minha melhor amiga, Maira, por ser minha eterna alma gêmea; obrigado pelo caminho trilhado todo esse tempo.

Aos meus amigos da vida e de graduação, Priscila, Mariana (Xablau), Isabella, Bianca, Kayan e Stefany, por serem cúmplices das minhas loucuras diárias. Eu sempre vou amar vocês e sempre estarei aqui quando precisarem.

Aos meus amigos do LMM: Simone, por me aguentar todos os dias, e ainda não ter cansado; Carol, por sempre compartilhar risadas e alegrias; Vitor, por sempre ser parceiro nas situações do laboratório; Nayara, por me ajudar com meus desafios. Vocês são demais e eu os levarei comigo para sempre.

Ao meu amigo e técnico do laboratório, Luiz Falleiros, pelo companherismo em todo cafézinho, toda coloração e todo concurso. Você é um amigo que eu posso confiar em tudo e contar pra tudo. Obrigado pelo cuidado e zêlo.

À Professora Patrícia Vilamaior por ter me acolhido à família do laboratório e por me ensinar tantas coisas que talvez você não tenha nem ideia que me ensinou. Seu cuidado e primor são um espelho para o meu trabalho acadêmico.

Aos meus colaboradores que fizeram esse Mestrado tornar-se real. Nenhum artigo, dissertação ou resumo na ciência se faz sozinho. Todo companheirismo de vocês foram necessários para a construção científica escrita aqui.

Ao Professor Alexandre Bruni Cardoso e Professor Manoel Francisco Biancardi por aceitarem avaliar meu trabalho. Agradeço à Professora Cristiane Damas Gil e Professor Luiz Fernando Barbisan pelas contribuições durante a qualificação. Agradeço tambéms os membros suplentes de ambas etapas: Professora Fernanda Alcântara, Professora Silvana Campos e Professora Ana Paula Perez.

Ao Programa de Pós-Graduação em Biologia Animal, atual Biodiversidade, pelo apoio durante o período do Mestrado.

Agradeço à FAPESP pela concessão da bolsa de pesquisa, sob o processo nº 20/01240-9, Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP).

Por fim, agradeço às minhas mães gerbilos que possibilitaram que eu pudesse contribuir com a Ciência e me permitiram apaixonar pelo modelo e pelos resultados.

“Tudo flui, para fora e para dentro; tudo tem suas marés; todas as coisas se levantam e caem; a oscilação do pêndulo se manifesta em tudo; a medida da oscilação à direita é a medida da oscilação à esquerda; o ritmo compensa.”

Hermes Trismegisto (1978, p. 91)

RESUMO

As glândulas mamárias apresentam grande plasticidade morfológica devido ao seu remodelamento associado à modulação por hormônios durante as fases da vida. O estabelecimento do câncer de mama vem sendo relacionado com compostos chamados desreguladores endócrinos. O bisfenol A (BPA), um xenoestrógeno disseminado nos ambientes, é alvo de estudos aprofundados sobre seu potencial cancerígeno devido à sua atuação em diversos níveis dos sistemas biológicos. Ainda, a exposição à este desregulador endócrino causa efeitos persistentes nos organismos observados tardiamente. Assim, o objetivo do presente estudo foi avaliar o potencial desregulador do BPA nas glândulas mamárias de fêmeas senis expostas durante duas janelas de remodelamento mamário, a gestação e lactação. Foram utilizados gerbilos da Mongólia (*Meriones unguiculatus*) como modelo experimental devido seu potencial para o desenvolvimento espontâneo de neoplasias. Vinte fêmeas foram divididas em 4 grupos experimentais: controle (gavadas com água); veículo (gavadas com óleo de milho); BPA (50 µg/kg/dia); e BPA (5000 µg/kg/dia). As fêmeas foram expostas durante 39 dias (gestação e lactação) e eutanasiadas aos 18 meses de idade (senis). Análises histopatológicas demonstraram o início do desenvolvimento tumoral, associados à transição epitélio-mesenquimal (EMT) das células epiteliais. Ainda, a exposição ao BPA apresentou um aumento na expressão de TGF-β1, indicado como marcador do processo de EMT. Um perfil microinvasivo foi observado pela expressão de metaloproteases (MMP-2, MMP-3, MMP-9) pelas células tumorais. O BPA promoveu um microambiente estromal com aumento de fibroblastos associados ao câncer e remodelamento das fibras colágenas e elásticas. Em relação aos receptores, no carcinoma induzido pelo BPA apresentou um aumento expressivo no receptor de estrógeno ERα, e uma perda da expressão dos receptores ERβ, de progesterona e de prolactina. Nas células que expressaram ERα foi co-localizado o marcador epigenético EZH2, relacionado à instalação tumoral ERα-positivo. Ainda, o BPA modulou a localização dos receptores de andrógeno (AR) e HER2/ErbB2 nas células do epitélio mamário. Por fim, a exposição ao BPA promove um microambiente tumoral (TME) que corrobora com o desenvolvimento neoplásico mamário. O recrutamento de elementos como perfis de macrófagos e mastócitos associados ao câncer expressando meidadores inflamatórios foi observado no estroma. Ainda, a expressão de COX-2 e fosfo-STAT3 nas células neoplásicas contribuem com a sinalização epitélio-estroma para o desenvolvimento do TME. Em conclusão, o BPA apresenta-se como um indutor carcinogênico do câncer de mama durante a senilidade após a exposição na janela gestacional/lactacional.

Palavras–chave: Neoplasia. Transição epitélio-mesenquimal. Microambiente. Inflamação. Receptores.

ABSTRACT

Mammary glands present a great morphological plasticity due to their remodeling associated to hormonal modulation during the phases of life. The establishment of breast cancer has been related to compounds called endocrine disruptors. Bisphenol A (BPA), a xenoestrogen widespread in environments, is the target of in-depth studies on its carcinogenic potential due to its action on several levels of biological systems. Furthermore, exposure to this endocrine disruptor causes persistent effects in organisms observed in long term. Thus, the aim of the present study was to evaluate the deregulatory potential of BPA in the mammary glands of aged females exposed during two windows of mammary remodeling, pregnancy and lactation. Mongolian gerbils (*Meriones unguiculatus*) were used as an experimental model due to their potential for the spontaneous development of neoplasias. Twenty females were divided into 4 experimental groups: control (gavaged with water); vehicle (gavaged with corn oil); BPA (50 µg/kg/day); and BPA (5000 µg/kg/day). Females were exposed for 39 days (pregnancy and lactation) and euthanized at 18 months of age (aging). Histopathological analyses demonstrated the onset of tumor development, associated with epithelial-mesenchymal transition (EMT) of epithelial cells. Also, exposure to BPA showed an increase in the expression of TGF-β1, indicated as a marker of the EMT process. A microinvasive profile was observed by the expression of metalloproteases (MMP-2, MMP-3, MMP-9) by the tumor cells. BPA promoted a stromal microenvironment with increased cancer-associated fibroblasts and remodeling of collagen and elastic fibers. Regarding receptors, BPA-induced carcinoma showed an expressive increase in the estrogen receptor ERα, and a loss of expression of ERβ, progesterone and prolactin receptors. In cells expressing ERα, was observe co-localization of the epigenetic marker EZH2, related to ERα-positive tumor establishment. Also, BPA modulated androgen receptor (AR) and HER2/ErbB2 localization in the mammary epithelial cells. Finally, BPA exposure promotes a tumor microenvironment (TME) that corroborates with mammary neoplastic development. Recruitment of elements, such as cancer-associated phenotypes of macrophage and mast cells, that express inflammatory mediators was observed in the stroma. Also, COX-2 and phospho-STAT3 expression in neoplastic cells contribute to epithelium-stroma signaling for TME development. In conclusion, BPA presents as a carcinogenic inducer of mammary cancer during aging, especially when exposure occurs in the gestational/lactational window.

Keywords: Neoplasia. Epithelial-mesenchymal transition. Microenvironment. Inflammation. Receptors.

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LISTA DE ABREVIATURAS E SIGLAS

AKT/ERK	*Protein kinase B*/ extracellular signal-regulated kinase
AR	Receptor de andrógeno
Areg	Anfiregulina
BPA	Bisfenol A
COX-2	Cicloxigenase 2
DE	Desregulador endócrino
EDC	*Endocrine disruptor chemical*
EFSA	*European Food Safety Authority*
EGF	Fator de crescimento epidermal
EMT	*Epithelial-mesenchymal transition*/ transição epitélio-mesenquimal
ERα	Receptor de estrógeno alfa
ERβ	Receptor de estrógeno beta
EZH2	potenciador do zeste homólogo 2/ *enhancer of zeste homolog 2*
FGF	Fator de crescimento de fibroblastos
GH	Hormonio do crescimento/*growth hormone*
HER2/ErbB2	*Human epidermal receptor* 2 (humano/roedor)
HGF	Fator de crescimento hepático
IL6	Interleucina 6
MAPK	*Mitogen Activated Protein Kinases*
MC	Mastócito
MG	*Mammary gland*/glândula mamária
MMP	Metaloprotease
NF-κB	*Nuclear factor kappa-light-chain-enhancer of activated B cells*
PR	Receptor de progesterone
PRL	Prolactina
p-STAT3	*Phospho-signal transducer and activator of transcription* 3
TGFβ1	Fator de crescimento transformador beta 1
TNF-α	Fator de necrose tumoral alfa
α-SMA	*Alpha smooth muscle actin*

SUMÁRIO

1. INTRODUÇÃO

1.1. Glândula Mamária: morfologia e fisiologia

A glândula mamária constitui uma das glândulas anexas ao sistema reprodutor feminino que apresenta secreção exócrina e é influenciada pelo sistema endócrino (PAWLINA; ROSS, 2016; TOPPER; FREEMAN, 1980). Este órgão apresenta grande importância para os mamíferos uma vez que produz e secreta o leite para nutrição da prole (INMAN et al., 2015). Estruturalmente são glândulas túbulo alveolares constituídas por um epitélio simples circundado por um estroma conjuntivo (DAWSON; VISVADER, 2021; WISEMAN; WERB, 2002). As principais células presentes nos alvéolos mamários são as células secretoras, seguidas pelas células basais proliferativas e as mioepiteliais, estas apresentando função contrátil (INMAN et al., 2015; MAN; SANG, 2004). Já o estroma apresenta grandes quantidades de fibras colágenas, elásticas e proteoglicanos, assim como fibroblastos, células de defesa, adipócitos, vasos sanguíneos e linfáticos (CUNHA; COOKE; KURITA, 2004; WISEMAN; WERB, 2002). A interação entre estes compartimentos, epitélio e estroma, em indivíduos adultos e senis vem sendo estudados e divulgados na literatura para a compreensão dos mecanismos associados às glândulas anexas (NIETO; RIDER; CRAMER, 2014).

Alterações estruturais e funcionais são observadas em diferentes etapas da vida reprodutiva e se relacionam às mudanças hormonais de acordo com o ciclo estral/menstrual (INMAN et al., 2015; RUSSO; RUSSO, 1996). Ainda, a glândula mamária apresenta plasticidade morfológica relacionada ao remodelamento dos compartimentos epitelial e estromal durante a gestação, lactação e involução (RODGERS et al., 2018; TERRY et al., 2019). Vários hormônios e fatores de crescimento do sistema endócrino interferêm na dinâmica tecidual: hormônios esteróides femininos, como estrógenos e progesterona; hormônios hipofisários-hipotalâmicos, como a prolactina e ocitocina; adipocinas, como a leptina e adiponectina; e fatores de crescimento, como o fator de crescimento transformador (TGFβ). Estes são modulados em diferentes fases promovendo efeitos em janelas de remodelamento tecidual (Figura 1), também chamadas janelas de susceptibilidade, uma vez que estas drásticas alterações morfológicas propiciam microambientes pré-neoplásicos (TERRY et al., 2019).

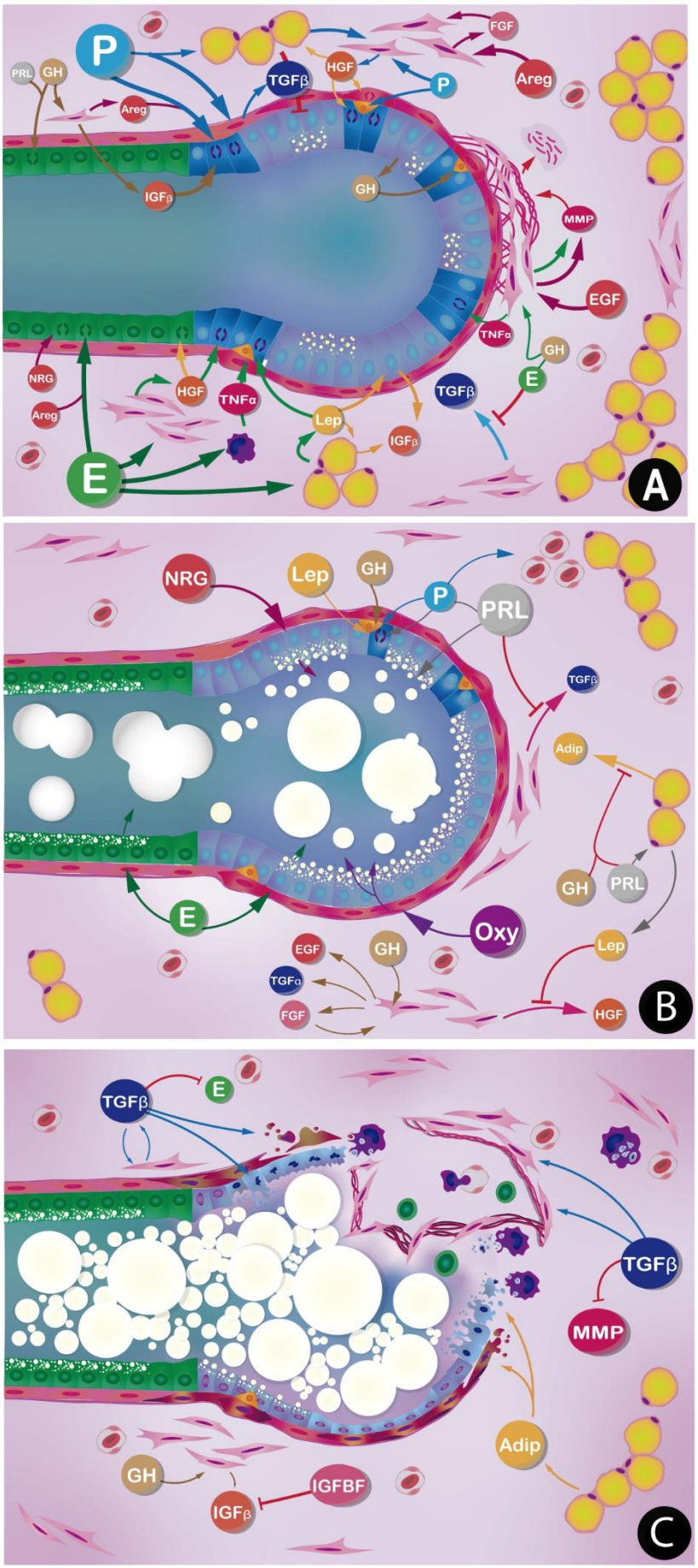
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Figura 1. *Ação hormonal no remodelamento da glândula mamária durante a gestação, lactação e involução. (A) Gestação.* O estabelecimento da estrutura alveolar durante a gestação ocorre por meio da remodelação estromal e proliferação epitelial induzida pelo estrógeno (E) e progesterona (P). Os estrógenos atuam promovendo a proliferação e modulando a síntese de MMPs. Já a progesterona promove a proliferação epitelial luminal e estimula a síntese de HGF para diferenciação de células do mesmo compartimento. A anfiregulina (Areg) e EGF apresentam imporntantes funções na diferenciação e remodelamento estromal. *(B) Lactação*. Durante a lactação, a prolactina (PRL) induz a proliferação e secreção das células epiteliais, por vias associadas à progesterona, estrógenos, leptina (Lep) e hormonio do crescimento (GH). Para expelir o leite dos alvéolos, a ocitocina (Oxy) atua nas células mioepiteliais para promover sua contração. No estroma, EGF e FGF são sintetizados pelos fibroblastos, enquanto os adipócitos são estimulados pela prolactina a produzir leptina, inibindo a ação de agentes mitóticos. *(C) Involução.* Durante a involução mamária, o TGFβ, assim como o adiponectina (Adip), atuam como promotores apoptótico, inibindo a ação proliferativa dos estrógenos. O TGFβ atua na proliferação de células estromais para aumentar este compartimento, inibindo a ação da MMP e atuando como fator quimiotático para células inflamatórias. O GH atua no compartimento estromal para a proliferação de fibroblastos. Imagens autorais adaptadas de Ruiz et al. (2021).

Na gestação, estrógenos e progesterona sinergizam para o estabelecimento das estruturas mamárias finais, os alvéolos (MACIAS; HINCK, 2012). Os estrógenos são de grande importância para o aumento da taxa proliferativa do compartimento epitelial e estromal para formação dos alvéolos (CUNHA; COOKE; KURITA, 2004; RUSSO et al., 1999). Durante a fase de amamentação/lactação, o lúmen dos alvéolos mamários altera seu tamanho de acordo com a secreção dos componentes glandulares, sendo este processo mediado principalmente pela ocitocina e progesterona (MASSO-WELCH et al., 2000). Além da ocitocina, as glândulas mamárias são controladas pela testosterona e hormônios adrenais, como o dehidroepiandrosterona (DHEA) (LABRIE et al., 2005). Assim, os receptores destes hormônios sofrem modulações durante as fases de proliferação, diferenciação e produção da glândula (LEONEL et al., 2017). Devido a tais alterações e modulações de receptores no desenvolvimento das mamas e à alta predisposição a patologias na fase senil em fêmeas, os efeitos de desreguladores endócrinos (DE) vêm sendo estudados para compreender a ação destes em diferentes fases de vida.

1.2. Biologia da Glândula Mamária: Janelas de Desenvolvimento

Durante o desenvolvimento fetal, a glândula mamária tem sua origem a partir das células epidérmicas, que invaginam para formar a árvore ductal. Na superfície da pele, o tecido mesenquimal torna-se mais espesso para se tornar o mamilo mamário (HYTTEL; SINOWATZ; VEJLSTED, 2012). Desde o nascimento até a puberdade, a MG mostra um crescimento alométrico e nenhuma diferenciação significativa ocorre

[6]. Uma vez que a puberdade chega, o eixo hipotálamo-hipófise-gonadal maduro promove a secreção cíclica de gonadotrofinas e hormônios sexuais, contribuindo para modificações dramáticas na glândula (MACIAS; HINCK, 2012).

A plasticidade morfológica do MG é baseada em um processo de ramificação e remodelação tecidual durante cada ciclo estral/menstrual, bem como durante a gestação, lactação e involução. A sinalização molecular e o controle fisiológico atuam na morfogênese da glândula por diferentes vias intra e extracelulares. No ciclo estral/menstrual, o MG sofre um processo de proliferação seguido de apoptose de suas estruturas ductais. Na gravidez, ocorre rápida proliferação do ducto e epitélio secretor estimulado pelo estrogênio e a progesterona leva à diferenciação de células epiteliais específicas para a síntese e secreção do leite (MASSO-WELCH et al., 2000). Essa estrutura epitelial, denominada alvéolo, apresenta células epiteliais luminais que sintetizam componentes do leite por estímulo de prolactina a ser liberada durante a lactação (Figura 3) (INMAN et al., 2015). Células-tronco mamárias bipotentes (MSC) proliferam e se diferenciam em lúmen e células progenitoras mioepiteliais (VISVADER; STINGL, 2014). Estes últimos estão localizados ao redor do sistema ductal e de alvéolos e, quando estimulados pela ocitocina, se contraem para expelir o leite das células luminais para os mamilos (INMAN et al., 2015). No desmame, a falta de estímulo de sucção diminui a produção de ocitocina e posteriormente dos hormônios lactantes, levando ao processo de involução da glândula mamária, com drástico rearranjo dos compartimentos celulares mediado por eventos apoptóticos (GREEN; STREULI, 2004; WANG; SCHERER, 2019).

Durante as fases de proliferação, o compartimento epitelial interage com o compartimento estromal, remodelando a matriz extracelular e permitindo o rearranjo celular, expansão e alveologênese (FEINBERG et al., 2016; MORI et al., 2013). O tecido adiposo atua como armazenamento local de energia para as células epiteliais durante a gravidez e lactação, quando a população de adipócitos diminui (DZIĘGELEWSKA; GAJEWSKA, 2019). Durante a involução, entretanto, eles se restabelecem por processo de desdiferenciação (NEVILLE et al., 1998; WANG; SCHERER, 2019). Os fibroblastos atuam secretando proteases que desmontam o arcabouço do estroma mamário e produzem fatores de proliferação durante a gravidez (DZIĘGELEWSKA; GAJEWSKA, 2019; UNSWORTH; ANDERSON; BRITT, 2014). Na involução, os fibroblastos são ativados para produzir metaloproteinases, remodelando

o compartimento estromal (MALLER; MARTINSON; SCHEDIN, 2010; WANG et al., 2010).

O desenvolvimento e a diferenciação de células epiteliais e estromais respondem a estímulos fisiológicos durante cada etapa morfológica da remodelação tecidual. Hormônios, fatores de crescimento, citocinas e vários metabólitos interagem entre vias complexas para desencadear proliferação, diferenciação e/ou apoptose em células epiteliais e estromais e na matriz extracelular (MEC). O papel de elementos-chave fisiológicos e moleculares tem sido pouco estudados para entender os mecanismos envolvidos nos processos da glândula mamária e a atuação de desreguladores endócrinos (DE) (AKERS, 2016; ENGLUND et al., 2019; FEINBERG et al., 2016; PAMARTHY et al., 2016; RUIZ; TABOGA; LEONEL, 2021).

1.3. Desreguladores Endócrinos

Os DE são substâncias químicas de origem exógena que interferem no sistema endócrino de modo a mimetizar substâncias endógenas. Isto afeta diretamente o metabolismo e desenvolvimento dos órgãos influenciados pelo sistema endócrino podendo causar danos estruturais e funcionais (BIGSBY et al., 1999; TOPPARI, 2008). Estes DE competem pelo mesmo sítio de ligação das substâncias endógenas, ligando-se aos receptores específicos nas células. Assim, estudos sobre as repercussões da ação de certos DE sobre a glândula mamária vêm sendo feitos e uma crescente compreensão das relações morfológicas e endócrinas tem sido obtida.

O potencial de desreguladores endócrinos já foi relatado e relacionado à neoplasias na próstata feminina (glândulas parauretrais de Skene) de gerbilos, onde os DE causaram um aumento na expressão de receptores de andrógenos no epitélio (SILVA et al., 2019). Também foi amplamente descrito nas glândulas mamárias e relacionado a patologias da mama em gerbilos e várias outras espécies (ACEVEDO; AMAYA; LÓPEZ-GUERRA, 2014; BROMER et al., 2010; LEONEL et al., 2020; MAFFINI et al., 2006). Recentemente, nosso grupo de pesquisa publicou uma revisão sobre a modulação dos hormônios durante as fases de remodelamento da glândula mamária e implicações do DE bisfenol A (BPA) (APÊNDICE A) (RUIZ; TABOGA; LEONEL, 2021).

1.4. BPA: Exposição e Efeitos

O BPA é um desregulador endócrino da classe dos xenoestrógenos, reconhecido por promover efeitos estrogênicos e anti-androgênicos (GOLOUBKOVA; SPRITZER, 2000; STAPLES et al., 1998; VIÑAS; JENG; WATSON, 2012). É encontrado em plásticos policarbonados e é um dos compostos mais disseminados no meio ambiente (PRINS, 2008; TIMMS et al., 2005). O BPA atua na desregulação da sinalização endócrina pelo aumento do estímulo nos sítios de ligação, além de desencadearem mecanismos moleculares e epigenéticos de regulação gênica, que podem levar a repercussões histopatológicas negativas e pró-tumorais (ACCONCIA; PALLOTTINI; MARINO, 2015; MEANEY; SZYF; SECKL, 2007). Assim, seu potencial desregulador atinge múltiplos níveis dos sistema biológicos (Figura 2), interagindo com diversos receptores celulares (SHAFEI et al., 2018) e alterando caracteres morfológicos e epigenéticos (BASAK; DAS; DUTTAROY, 2020; FERNANDEZ; RUSSO, 2010).

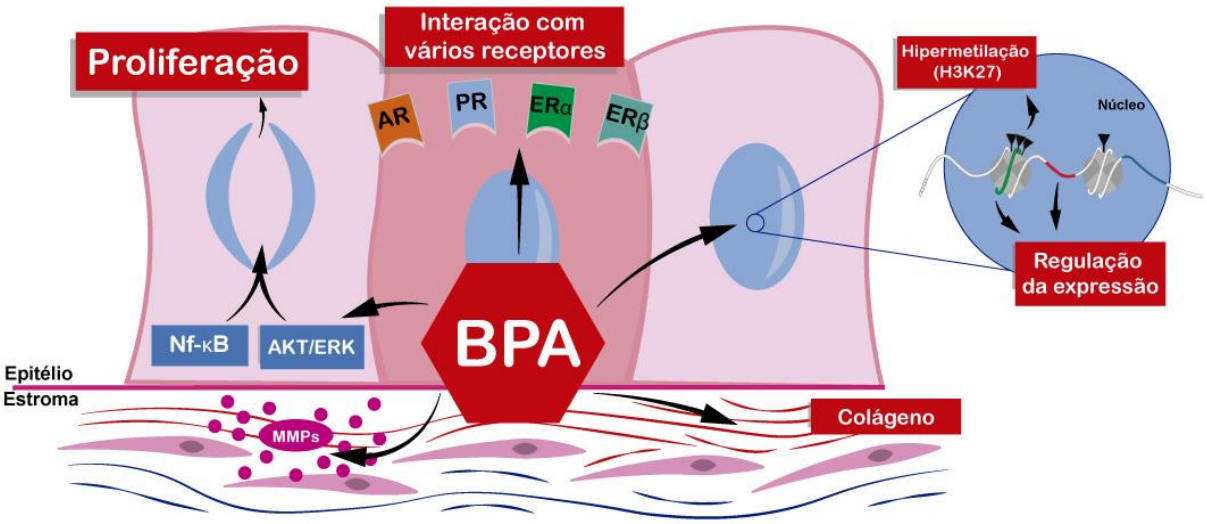


Figura 2. *Disrupção multinível nos sistemas biológicos pelo BPA*. Na glândula mamária, o BPA atua promovendo a proliferação das células epiteliais luminais, por meio das vias Nf-κB e AKT/ERK. Ainda, estas vias são ativadas pela interação do BPA com diversos receptores celulares, principalmente AR, PR, ERα e ERβ. A expressão destes receptores é modulada também por vias epigenéticas de disrupção promovidas pelo BPA. No estroma repercussões relacionadas com a síntese e degradação de matriz extracelular são observadas. Autoria da Imagem: Thalles Ruiz©.

Apesar do interesse científico dos efeitos de DE nas mães expostas durante a gestação e lactação ser limitado, alguns trabalhos descrevem as consequências desta exposição. Alguns estudos sugerem que a exposição ao BPA durante a gestação e lactação, assim como em longo prazo, podem causar danos cerebrais e mudanças na

expressão de ERα no cérebro de ratas (ALOISI et al., 2001; SETA et al., 2005). Ainda, podem causar alterações negativas relacionadas a insulina e glicose. Segundo os estudos de Alonso-Magdalena e colaboradores (2010, 2015), ratas tratadas com BPA podem apresentar a longo prazo um desbalanço no metabolismo da glicose podendo aumentar a incidência de doenças como diabetes e obesidade. Estes dois distúrbios metabólico-fisiológicos estão ligados à modulação em nível celular causada pelo BPA relacionados a resistência insulínica e à leptina (adipocina) (MELI et al., 2020), ao efeito de diferenciação dos adipócitos causado pela forma glicurosidada BPA-G (BOUCHER et al., 2015), e alterações no ciclo celular do pâncreas aumentando o nível sérico de insulina (ALONSO-MAGDALENA et al., 2015, 2016).

Efeitos como estresse oxidativo e inflamação estão ligados à exposição ao BPA. Alterações na modulação da síntese de mediadores oxidativos levam a uma desregulação nas funções de organelas celulares importantes, como as mitocôndrias, além de desencadear vias de sinalização celular que causam a expressão de proteínas apoptóticas (GASSMAN, 2017). Em mulheres na fase de pré- e pós-menopausa, a exposição ao BPA aumenta a produção de marcadores inflamatórios e de estresse oxidativo, o que pode induzir efeitos adversos nas células de modo geral e a debilidade na saúde dos indivíduos (YANG et al., 2009).

Assim, observa-se que este composto apresenta efeitos em múltiplos órgãos e um potencial cancerígeno deste composto em órgãos do sistema reprodutor (HASS et al., 2016; RUBIN, 2011; WISNIEWSKI et al., 2015). Entretanto, pouco se conhece sobre as respostas da glândula mamária após exposição gestacional/lactacional a este DE.

1.5. Modelo Experimental e Justificativa do Estudo

No modelo experimental gerbilo da Mongólia (*Meriones unguiculatus*), espécie poliéstrica (NISHINO; TOTSUKAWA, 1996), a estrutura básica das glândulas mamárias foi descrita em nosso laboratório por Leonel et al. (2017), que demonstrou as mudanças estruturais ocorridas durante os períodos de gestação, lactação e involução da glândula.

O estudo das repercusões histopatológicas ocasionadas por DE faz-se necessário em modelos experimentais como o gerbilo da Mongólia, espécie que apresenta desenvolvimento espontâneo de neoplasias (CUSTODIO et al., 2010;

NORRIS; ADAMS, 1972), o que mimetiza o ambiente natural no qual observa-se uma maior propensão ao desenvolvimento cancerígeno e inflamatório. Assim, visando à possibilidade de expansão do conhecimento para novas espécies, como o gerbilo da Mongólia, estudos que aproximem as condições experimentais às ambientais são necessários. Ainda, a avaliação do potencial desregulador do BPA nas glândulas mamárias em fêmeas senis são necessárias para compreender as possíveis associações com processos inflamatórios e cancerígenos nesta glândula.

No presente estudo, nós Hipotetizamos que a exposição das mães ao BPA nas janelas gestacional-lactacional pode ser um fator de risco para o desenvolvimento neoplásico, potencializando-se devido ao envelhecimento. Repercussões histopatológicas, expressão de receptores imortantes para o desenvolvimento mamário, e alterações inflamatórias importantes foram propostas para analizar tal hipótese

2. OBJETIVO

Este projeto teve como objetivo principal avaliar, tardiamente na fase senil, os efeitos nas glândulas mamárias de gerbilos após exposição ao BPA, durante as fases de desenvolvimento alveolar mamário (gestação) e de produção láctea (lactação), em doses seguras e elevadas.

2.1. Objetivos específicos

- Avaliar os aspectos histopatológicos e morfométricos do epitélio da glândula mamária após exposição ao BPA, bem como da manutenção de estruturas epiteliais de acordo com a identificação de células mioepiteliais, e do estroma periglandular;
- Analisar a expressão dos receptores hormonais de estrógeno (ERα e ERβ), progesterona (PR), prolactina (PRL-R), HER2/ErbB2, e andrógeno (AR) no epitélio da glândula mamária de animais expostos ao BPA.
- Comparar as repercussões inflamatórias tecido mamário, avaliando marcadores de inflamação TNFα, COX-2 e p-STAT3, assim como a população de macrófagos (F4/80+, CD68+ e CD163+) e de mastócitos (triptase e quimase);

3. MATERIAL E MÉTODOS

3.1. Delineamento Experimental

Foram utilizadas 20 fêmeas de gerbilo da Mongólia mantidas em isoladores de polisulfona equipados com comedouro e bebedouro (ração balanceada e água fresca *ad libitum*). As fêmeas de aproximadamente 3 meses de idades, ainda virgens, formaram famílias com machos da mesma idade para suas duas gestações. O primeiro dia da segunda gestação foi determinado após o nascimento da primeira ninhada, que foi descartada. Este descarte assegura que após 6 horas do nascimento da primeira prole ocorrerá cópula e nova gestação. As fêmeas, com exceção daquelas do grupo controle, foram acompanhadas e expostas ao BPA durante o período correspondente à sua segunda gestação (24-26 dias) e lactação (21 dias). Todos os animais foram eutanasiados aos 18 meses de idade (fase senil). Para os diferentes tratamentos as fêmeas foram divididas em 4 grupos experimentais (Figura 3):

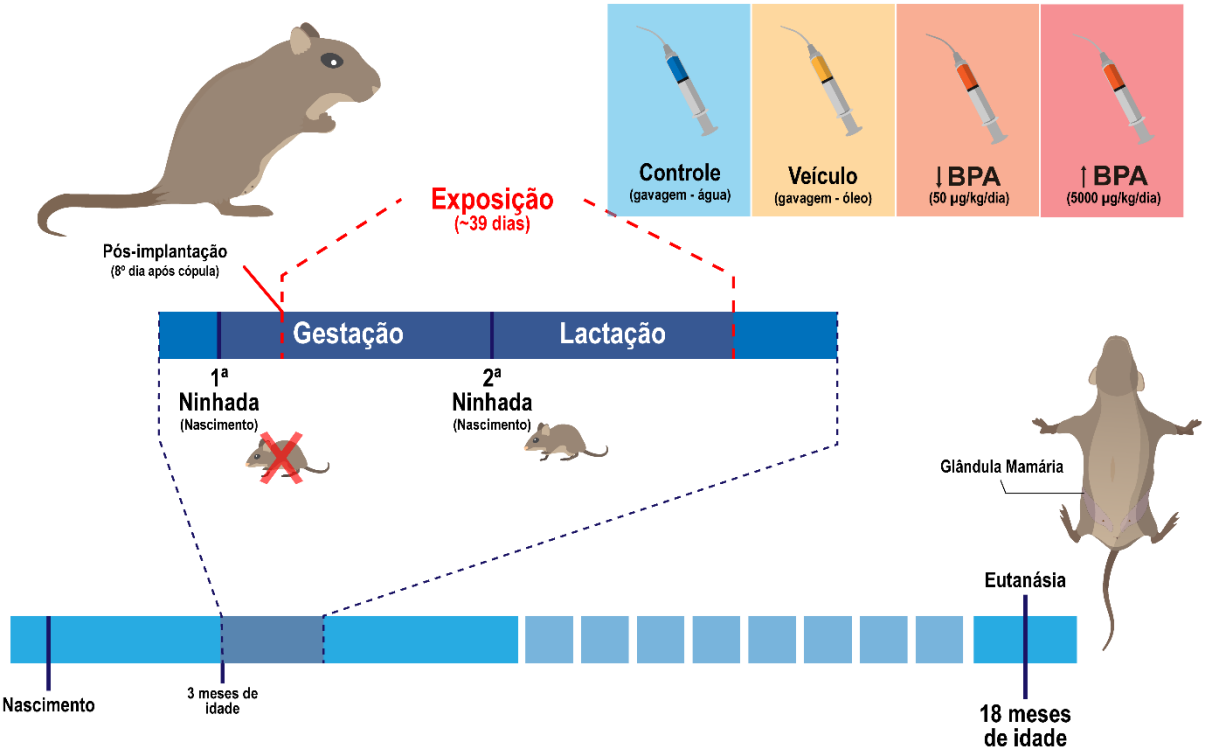


Figura 3: Delineamento dos grupos experimentais e tempo experimental dos grupos expostos ao BPA. A exposição às respectivas dosagens iniciam no 8º dia após o nascimento da primeira geração (implantação pós-cópula), finalizando no 21º dia de lactação. Ao final de 18 meses de idade, as mães foram eutanasiadas para as análises.

- **Grupo 1: Controle** (n=5) – fêmeas mantidas em isoladores até atingirem 18 meses de idade, submetidas à administração de água a partir do 8º dia da segunda gestação até o final da lactação;
- **Grupo 2: Veículo** (n=5) – fêmeas mantidas em isoladores até atingirem 18 meses de idade, submetidas à administração de óleo de milho (veículo) a partir do 8º dia da segunda gestação até o final da lactação;
- **Grupo 3: ↓BPA** (n=5) – foram administradas às fêmeas doses diárias seguras (50 µg/kg) de BPA segundo a US EPA (United States Environmental Protection Agency) (SORIANO et al., 2019), diluído em óleo de milho por gavagem a partir do 8º dia da segunda gestação até o final da lactação;
- **Grupo 4: ↑BPA** (n=5) – foram administradas às fêmeas doses diárias 100 vezes maiores do que as doses ambietais (5000 µg/kg) de BPA diluídas em óleo de milho por gavagem a partir do 8º dia da segunda gestação até o final da lactação.

Para estes grupos foram administrados seus respectivos compostos ou veículos durante 39 dias (gestação e lactação). Aos 18 meses de idade, as fêmeas foram anestesiadas com isofluorano 3% em oxigênio, pesadas e eutanasiadas por decapitação. As glândulas mamárias abdominais (direita) foram coletadas e fixadas em paraformaldeído 4% e submetidas ao processamento de rotina para histologia. As glândulas mamárias abdominais (esquerda) foram congeladas à -80ºC para análises de quantificação proteica por Western Blotting.

Todos os protocolos e procedimentos adotados no presente estudo seguiram as diretrizes do Conselho Nacional de Controle de Experimentação Animal (Concea) e devidamente registrado e aprovado pelo Comitê de Éticaa no Uso de Animais (CEUA) do Instituto de Biociências, Letras e Ciências Exatas (IBILCE, Unesp), sob Protocolo Nº 217/2019 (Anexo).

3.2. Estudos histológicos e histoquímicos

As glândulas mamárias de todos os grupos experimentais foram submetidas à inclusão em parafina no processador automático modelo Leica TP 1020 e os blocos histológicos foram obtidos a partir da central de inclusão Leica Modelo 1150HeC. Os blocos foram seccionados a 4µm de espessura em micrótomo rotativo automático Leica RM 2255.

Os cortes foram submetidos primeiramente à rotina de coloração em Hematoxilina-Eosina para avaliação da morfologa geral e controle de qualidade das glândulas mamárias. Posteriormente foram analisadas e quantificadas as alterações morfológicas, como lesões, focos hiperplásicos e estruturas neoplásicas.

A seguir, foram feitos testes histoquímicos para detecção fibras conjuntivas do estroma, como resorcina-fucsina de Weighert para elastina e picrossírius para fibras colágenas. Foram avaliados e quantificados os elementos fibrilares do estroma para comparação entre os diferentes grupos experimentais. Para tanto, campos aleatórios foram capturados e digitalizados, e quantificados por densidade de área pelo software ImageJ.

3.3. Imunohistoquímica

O procedimento contou com as seguintes etapas: desparafinização e reidratação dos cortes com banhos de xilol, álcool e água. A recuperação antigênica foi realizada em tampão citrato 10 mM ou Tris EDTA, em banho Maria a 92°C ou 98ºC. Em seguida, foi realizado o bloqueio das peroxidases endógenas com H_2O_2 10% em metanol. A inibição das proteínas inespecíficas foi feita com albumina sérica bovina ou leite desnatado 5%. Após isto, foi realizada a incubação do anticorpo primário para os anticorpos específicos (overnight) citados na Tabela 1. Após este procedimento foi realizado a incubação com anticorpo pós-primario e polímero (Novolink™ polymer detection system 1, Leica Biosystems Newcastle Ltdde acordo com as descrições do fabricante. A revelação das ligações anticorpo-proteína foi feita com 3-30 diaminobenzidine tetrahydrochlorido (DAB) (Novolink™, DAB, RE7270-CE, Leica Biosystems) e contracoloração com hematoxilina. As etapas descritas foram intercaladas com lavagens em PBS ou TBS e as lâminas foram analisadas em microscópio de luz.

Tabela 1. Anticorpos Primários – Imunohistoquímica e imunofluorescência.

MARCADORES	Anticorpo Primário	Diluição	Host	Fabricante
ESTRUTURAIS	Alfa-actina	1:100	Mouse	Santa Cruz Biotech.
	P63	1:100	Mouse	Santa Cruz Biotech.
	Pan-citoqueratina	1:100	Mouse	Santa Cruz Biotech.
	Vimentina	1:100	Mouse	Santa Cruz Biotech.

	FAP	1:100	Mouse	Santa Cruz Biotech.
REMODELAMENTO	MMP-2	1:50	Mouse	Santa Cruz Biotech.
	MMP-3	1:100	Rabbit	Invitrogen
	MMP-9	1:100	Mouse	Santa Cruz Biotech.
	TGF-β1	1:100	Rabbit	Santa Cruz Biotech.
PROLIFERAÇÃO	Fosfo-histona H3	1:75	Rabbit	Cell Signaling
APOPTOSE	Caspase 3 (ativa/clivada)	1:100	Rabbit	Novus Biological
RECEPTORES	ERα	1:50	Mouse	Invitrogen
	ERβ	1:50	Rabbit	Invitrogen
	Progesterona (PR)	1:50	Mouse	GeneTex
	Prolactina (PRL-R)	1:100	Rabbit	Cell Signaling
	HER2/ErbB2	1:50	Mouse	Abcam
	Andrógeno (AR)	1:75	Mouse	Santa Cruz Biotech.
EPIGENÉTICO	Enhancer of zeste homolog 2 (EZH2)	1:75	Rabbit	Cell Signaling
INFLAMATÓRIOS	Fosfo-STAT3	1:75	Rabbit	Cell Signaling
	TNFα	1:50	Rabbit	Cell Signaling
	COX-2	1:50	Rabbit	Cell Signaling
	Interleucina 6 (IL-6)	1:50	Mouse	Santa Cruz Biotech.
	F4/80	1:75	Rabbit	Cell Signaling
	FcεRI	1:50	Rabbit	Sigam-Aldrich

A quantificação das células positivas (marcação citoplasmática e nuclear) foi realizado aplicando o software QuPath (versão 0.1.2, an open-source pathology software platform). O total área de cortes histológicos foi considerada para o quantificação da marcação positiva e os dados foram expresso em células/mm^2.

3.4. Imunofluorescência

A imunofluorescência foi realizada para detectar a localização de alguns receptores celulares (HER2/ErbB2, AR e co-localização de ERα e EZH2), marcadores

celulares específicos (CD68, CD163, iNOS, Triptase, Quimase, FcεRI), e marcadores/mediadores inflamatórios em co-localização em células específicas (COX-2, TNFα, TGF-β1, MMP-2, MMP-9, IL-6). A recuperação do antígeno e o bloqueio de proteínas inespecíficas foram realizados conforme estabelecido acima para imuno-histoquímica. A incubação do anticorpo primário foi realizada overnight (4ºC). Em seguida, foi realizada a incubação de fluorocromos conjugados com anticorpos secundários específicos: anti-mouse FITC (sc-2010, Santa Cruz Biotechnology); anti-rabbit FITC (sc-2359, Santa Cruz Biotechnology); anti-mouse Rhodamine (sc-2092, , Santa Cruz Biotechnology); anti-goat Texas Red (sc-2783, Santa Cruz Biotechnology) e/ou anti-rabbit Texas Red (sc-2780, Santa Cruz Biotechnology), por 1 hora em temperatura ambiente. As lâminas foram montadas com DAPI (Fluoroshield ™ com DAPI, F6057, meio de montagem de histologia, Merck, Darmstadt, Alemanha) para fluorescência nuclear. As seções foram analisadas com um Microscópio de Fluorescência Zeiss AX10 (Zeiss, Oberkochen, Alemanha) acoplado ao software AxioVision (Zeiss).

3.5. Western Blotting

As glândulas mamárias armazenadas à -80ºC foram encaminhadas para análise de Western blotting. Esta análise foi aplicada para os resultados que não apresentaram diferença significativa na quantificação para imuno-histoquímica (TNFα). A extração de proteínas foi realizada com tampão de extração contendo inibidor de protease (Protease Inhibitor Cocktail, P8340, Sigma Aldrich, St. Louis, MO, EUA) em amostras maceradas, as quais foram centrifugadas (20 min; 14.000 rpm; 4ºC). A concentração de proteína foi quantificada por absorbância usando BCA Protein Assay Kit (Pierce BCA Protein Assay Kit, 23,227, Thermo Fischer Scientific, Rockford, IL, EUA) em leitor de microplaca SPECTROstar Omega (BMG Labtech, Ortenberg, Alemanha). A eletroforese foi realizada em SDS page gel (15 ug de amostra; 105 V; 90 min) e a transferência eletroforética foi realizada posteriormente para uma membrana de nitrocelulose (Amersham Protram, 10.600.003, GE Healthcare, Darmstadt, Alemanha). Para o ensaio de Western Bloting, foi realizado o bloqueio de proteínas com leite desnatado 3% em TBS + Tween (0,1%). Após isto, as membranas foram incubadas com anticorpos primários anti-TNFα (coelho monoclonal, D2D4, #11948; Cell Signaling) e anti-GAPDH (coelho monoclonal,

14C10, #2118, Cell Signaling, usado como controle positivo endógeno) overnight. A incubação do anticorpo secundário foi realizada com conjugado de peroxidase (IgG anti-coelho, anticorpo ligado a HRP, #7074). Todas as etapas foram intercaladas com lavagens com TBSt. A detecção do anticorpo foi feita com substratos quimioluminescentes - Luminol Enhancer e reagentes de solução de peróxido (Westar Antares, Cyanagen, XLS142,0250) e revelada no ChemiDoc Image System (BioRad, Hercules, CA, EUA). O software Image J (versão 1.52a, EUA) foi usado para análise densitométrica e quantificação de proteínas.

3.6. Análises estatísticas

Os dados morfométricos e quantificações das imunoistoquímicas – dados estereológicos e as porcentagens de células positivas – foram expressos em média ± erro da média (SEM). Os testes foram escolhidos de acordo com a determinação de normalidade dos valores: paramétricos (ANOVA) ou não-paramétricos (Kruskall-Wallis). As análises foram seguidas de pós-teste (Tukey ou Dunns).

4. RESULTADOS

Os resultados do presente trabalho são apresentados em forma de artigos científicos de acordo com os objetivos propostos. O primeiro capítulo apresenta os resultados já publicados na Revista *Endocrine-Related Cancer* (doi: 10.1530/ERC-21-0198.), com a caracterização do início do processo carcinogênico induzido pelo BPA nas fêmeas senis quando expostas durante a gestação e lactação. O segundo capítulo foi publicado na Revista *Life Sciences* (doi: 10.1016/j.lfs.2021.120010) e apresenta a expressão e papel dos receptores hormonais na glândula mamária durante o desenvolvimento da carcinogênese induzida pelo BPA. O terceiro capítulo apresenta dados do manuscrito submetido para Revista *International Journal of Cancer* (ISSN: 1097-0215) contendo os resultados relacionados ao microambiente tumoral instalado na glândula mamária após exposição ao BPA.

Como parte integrante dos resultados desta Pesquisa foram redigidos um artigo de revisão sobre os efeitos do BPA nas diferentes janelas de susceptibilidade da glândula mamária publicado na Revista *Reproductive Toxicology* (doi: 10.1016/j.reprotox.2021.07.011) (Anexo II). Ainda, foi publicado um trabalho sobre as repercussões histopatológicas na glândula mamária durante a involução após a exposição das mães ao BPA (Revista *Environmental Toxicology and Pharmacology* – doi: 10.1016/j.etap.2021.103785). Este trabalho complementa o objetivo do presente estudo e completa o estudo da exposição e efeito do BPA nas mães em diferentes janelas de susceptibilidade (Anexo III).

4.1. Capítulo 1: Mammary carcinoma in aged gerbil mothers after endocrine disruption in pregnancy and lactation

Revista *Endocrine-Related Cancer* (IF: 5.678)

Endocrine-Related Cancer | T F R Ruiz *et al.* | Carcinoma in aged mammary gland exposed to BPA | 28:11 | 715–730

RESEARCH

Mammary carcinoma in aged gerbil mothers after endocrine disruption in pregnancy and lactation

Thalles Fernando Rocha Ruiz[1], **Simone J Colleta**[1], **Ellen C R Leonel**[2] and **Sebastião Roberto Taboga**[1]

[1]Department of Biology, Institute of Biosciences, Humanities and Exact Sciences, São Paulo State University (UNESP), Jardim Nazareth, São José do Rio Preto, São Paulo, Brazil

[2]Department of Histology, Embryology and Cell Biology, Institute of Biological Sciences (ICB III), Federal University of Goiás (UFG), Campus Samambaia, Goiânia, Goiás, Brazil

Correspondence should be addressed to T F R Ruiz or S R Taboga: thalles.ruiz@unesp.br or taboga@ibilce.unesp.br

ABSTRACT

Compounds that trigger breast cancer onset and establishment are of great interest in biological research. Endocrine disruptors are relevant because they initiate carcinogenesis by changing endocrine pathways. Bisphenol A (BPA), as a ubiquitous xenoestrogen, is largely associated with dysfunctions in the female reproductive system and associated organs. This study proposes an investigation of the mammary gland (MG) in aged Mongolian gerbil (Meriones unguiculatus) mothers after their exposure to BPA in two windows of morphophysiological plasticity: pregnancy and lactation. A low dose (50 µg/kg), and a high dose (5000 µg/kg) were considered and results showed few differences between them. As expected, we observed contrasts among control and BPA-exposed MG. The control groups presented a regressive phase with high apoptotic activity and elastic stroma. However, BPA damaged mammary tissue and provoked multifocal carcinoma development supported by an apparent epithelial-mesenchymal transition (EMT) and reactive stroma establishment. BPA remodeled stromal fibers deposition and enhanced the recruitment of tumor-associated cells, contributing to a tumoral microenvironment. Overexpression of TGF-β1 was induced by BPA in epithelial compartment of exposed MG and increased expression of metalloproteinases (MMP-2, MMP-3, MMP-9) was present in carcinoma

cells. In conclusion, exposure of mothers to BPA during the gestational/lactational window of susceptibility leads to carcinogenic impacts with aging.

Introduction

Carcinogenesis in the breast is a complex pathological process of the female reproductive system. Due to the occurrence of different types of breast cancer, diverse onset and dynamics are attributed to its progression and the regulation of tumor growth and metastatic dissemination (Alvarado-Cabrero et al. 2020). A central feature in breast cancer is deregulation of cell homeostasis and microenvironment, associated with tumor development (Zhang et al. 2017).

Considering the risk factors, age and parity are relevant aspects for the onset and advancement of breast cancer. In addition, due to the fact that the mammary gland (MG) is one of the most plastic organs, oncogenesis in this tissue is closely related to stages of susceptibility that this gland undergoes during a woman's life (Terry et al. 2019). Pregnancy and lactation are two windows of susceptibility where MG is subjected to major morphological compartment rearrangements to perform its functionality (Faupel-Badger et al. 2013).

Several triggering factors have been attributed to carcinogenesis. Some of them are environmental chemicals that disrupt mammary tissue, leading to the development of endocrine-related cancers (Vandenberg et al. 2019), in women (Giulivo et al. 2016) and rodent models (reviewed by Seachrist et al. 2016). Endocrine disrupting chemicals (EDCs) act by different pathways in the reproductive system, causing different consequences, mainly through establishing a tumoral microenvironment (Soto et al. 2013). Exposure to EDCs has also been implicated in increasing the incidence of breast cancer (Terry et al. 2019). Among EDCs, xenoestrogens, especially bisphenol A (BPA), promote disruptive effects by estrogenic routes, interacting with other receptors, such as androgen (AR) and mitogen receptors linked to MAPK/ERK, for promoting a disruptive activity (reviewed in Shafei et al. 2018a). Studies have demonstrated the development of carcinoma and tumor progression in prostate (Huang et al. 2018), endometrium (Leung et al. 2020) and ovaries (Seachrist et al. 2016) after perinatal experimental BPA exposure.

In the MG, studies analyzing different perinatally exposed experimental models describe the impacts in offspring when mothers are exposed to BPA during pregnancy and lactation (Mileva et al. 2014; Mandrup et al. 2016; Leonel et al. 2020). These

exposure-periods are susceptible to the development of pathologies in MG, mainly neoplasia (Terry et al. 2019), being BPA a triggering agent for the incidence of breast cancer (Soto et al. 2013; vom Saal & Vandenberg 2021). In women, BPA has been associated with increase in breast stromal density (Sprague et al. 2013), in addition to provoking modifications in molecular and epigenetic pathways that can trigger or promote breast cancer installation (Goodson et al. 2011; Soto et al. 2013; Dhimolea et al. 2014; Shafei et al. 2018b; Atlas & Dimitrova 2019). In fact, BPA has non-monotonic effects in the MG (Montévil et al. 2019), which may contribute to carcinogenic effects at different doses.

Thus, in the present study we analyze MG from mothers directly exposed to BPA endocrine disruption during their gestation and lactation. We investigated long-term effects in aging phase of a rodent experimental model, the Mongolian gerbil (*Meriones unguiculatus*), which shows spontaneous development of neoplasms (Norris & Adams 1972; Custodio et al. 2010), thus mimicking a natural environment with greater propensity for tumoral and inflammatory development. Also, aging in the gerbil is a critical point to the development of hormone-related disorders (Vincent et al. 1980; Campos et al. 2008), previously studied in prostate from males and females (Skene's paraurethral glands) (Custodio et al. 2008; Biancardi et al. 2017).

The relevance of this study lies in the fact that aging is related to the establishment of a tumor favorable microenvironment (Li et al. 2020). Therefore, we hypothesize that gerbil mothers' exposure to BPA in gestational-lactational windows can be a risk factor for tumor development, increased with aging. In addition, we present relevant aspects of the MG neoplastic developmental process caused by an EDC and morphological aspects associated with tumoral microenvironment.

Materials and Methods

Experimental Design

Twenty female Mongolian gerbils (*Meriones unguiculatus*) were used. The animals were kept in polysulfone isolators with wood shaving bedding. They were fed LABMIX® commercial chow (Agromix, SP, Brazil), containing only traces of soy and corn, and fresh filtered water ad libitum. The three-month-old females were housed with fertile males of the same age. The gerbil females were divided into four experimental groups (n=5 for each group): control – subjected to daily gavage with water; vehicle – subjected to daily gavage with corn oil; treated groups: ↓BPA (50

µg/kg) – subjected to daily gavage with a dosage considered safe (not capable of provoking disruption), according to US EPA (Soriano et al. 2019) and EFSA (EFSA 2015) – and ↑BPA (5000 µg/kg) – subjected to daily gavage of 100x the safety dose/high exposure, both diluted in 0.1 ml corn oil. The experimental design is shown in Figure 1. Gerbil mothers were monitored and exposed to the treatments from the 8th day after first parturition, when second pregnancy started (first offspring was discarded), until the end of lactation. The treatment comprised 39 days of exposure in total (length of second pregnancy: 24-25 days, length of lactation: 21 days). The second offspring were separated (used in other studies) and all mothers were kept until 18 months of age (elderly females). One animal was maintained per cage to avoid social stress. Euthanasia was performed during the morning, after confirmation of the exclusive presence of cornified cells in the vaginal smear. The animals were anesthetized in 3.0% isoflurane vaporized with oxygen and euthanized by decapitation.

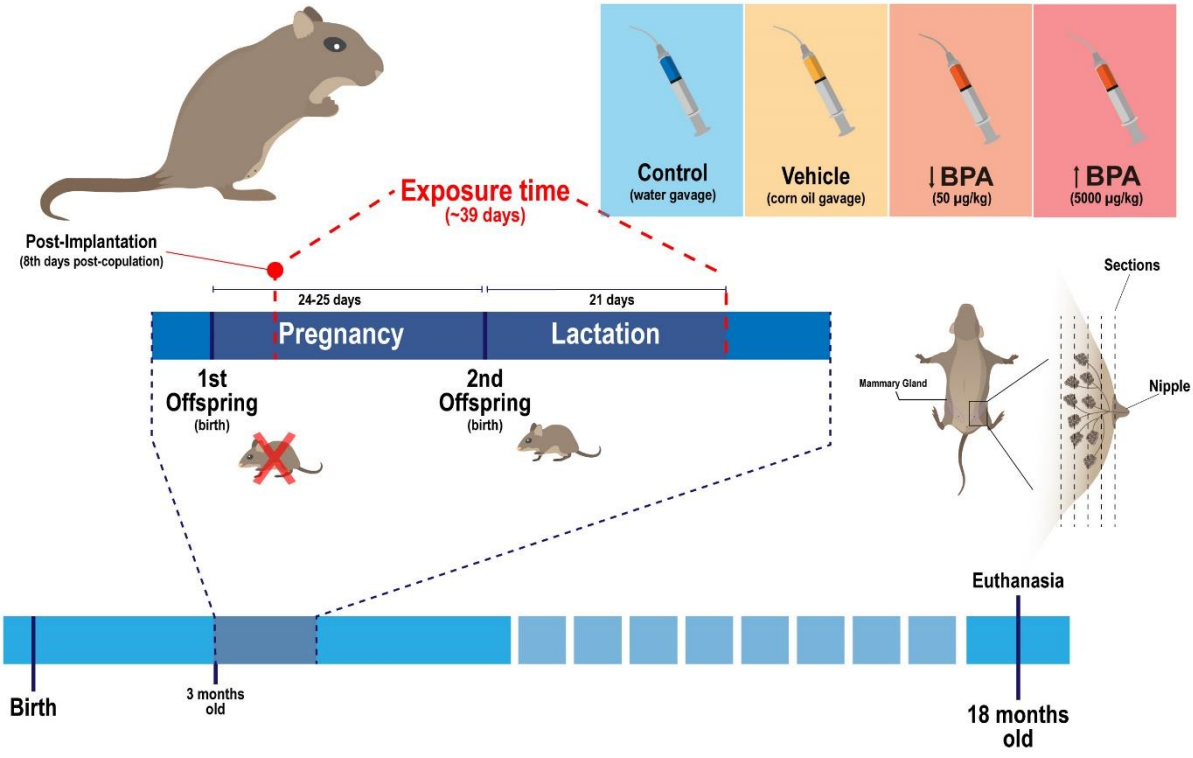


Figure 1. Experimental design. Pregnant female gerbils were divided into four experimental groups (n=5): control (water gavage); vehicle (corn oil gavage); ↓BPA (50 µg/kg); ↑BPA (5000 ug/kg). They were exposed from the 8th day after the birth of the 1st offspring (period corresponding to the pregnancy of the second offspring) until the end of lactation (total of 39 days). At 18 months of age, gerbil mothers were euthanized and MG was transversally sectioned for histopathological analysis.

Histological procedures

The abdominal MG was collected. The entire gland with the skin and nipple was placed flattened in histological cassette and fixed with paraformaldehyde 4% for 24 hours. Then, skin was removed and nipples were maintained. Samples were washed in ethanol 70% and processed by routine histology in a Leica Semi Enclosed System (TP1020, Leica Biosystems, Buffalo Grove, USA). Taking the nipple as a reference, histological sections (4.5 µm thickness – serial) were made transversally to it (Figure 1) and placed on silanized slides. All samples were collected within the MG sentinel lymph node region, visually observed during euthanasia. Hematoxylin and Eosin-Floxin (HE) staining was performed for histopathological analysis and description of tissue alterations using ordinary light microscopy and fluorescent light microscopy (Carvalho & Taboga 1996). Techniques for stromal connective fiber identification were performed: Weigert resorcin-fuchsin for elastin, and picrosirius for collagen fibers using polarized light microscopy (Junqueira et al. 1979).

Immunohistochemistry

The slides were subjected to immunohistochemistry (IHC). The procedure followed the steps described below and was adapted according to the specificity of each primary antibody. The sections were first deparaffinized and rehydrated in a gradient of xylol, alcohol, and water, followed by antigenic retrieval, in 10 mM citrate buffer or EDTA Tris, at 92 °C or 98 °C. Next, endogenous peroxidases were blocked with 10% H2O2 diluted in methanol. The inhibition of nonspecific proteins was performed in 5% bovine serum albumin or skimmed milk. Subsequently, the following primary antibodies were incubated overnight with specific dilution: MMP-2 (mouse monoclonal, 8B4, 1:50, sc-13595, Santa Cruz Biotechnology), MMP-3 (rabbit polyclonal, PA5-27936, 1:100, Invitrogen, ThermoFisher), MMP-9 (mouse monoclonal, 2C3, 1:100, sc-21733, Santa Cruz Biotechnology), α-smooth muscle actin (α-SMA) (mouse monoclonal, 1A4, 1:100, sc-32251, Santa Cruz Biotechnology), FAP (mouse monoclonal, F11-24, 1:100, sc-65398, Santa Cruz Biotechnology), TGF-β1 (rabbit polyclonal, 3c11, 1:100, sc-146, Santa Cruz Biotechnology), active/cleaved caspase-3 (rabbit polyclonal, 1:100, NB100-56113, Novus Biological), Phospho-Histone H3 (PH-H3, rabbit polyclonal, 1:75, Ser10, 9701, Cell Signaling), p63 (mouse monoclonal, D-9, 1:100, sc-25268, Santa Cruz Biotechnology), pan-cytokeratin (mouse monoclonal, AE13, 1:100, sc-57012, Santa Cruz Biotechnology), and vimentin (mouse

monoclonal, V9, 1:100, sc-6260, Santa Cruz Biotechnology). The steps described were interspersed with washes in PBS or TBS. The slides were then incubated with a post-primary antibody and Polymer kit (Novolink ™ polymer detection system 1, Leica Biosystems Newcastle Ltd., Newcastle, United Kingdom) according to the manufacturer's descriptions. Detection of positive staining was performed with 3-30 diaminobenzidine tetrahydrochloride (DAB) (Novolink TM DAB, RE7270-CE, Leica Biosystems, Buffalo Grove, USA) and counterstaining with hematoxylin. Pictures of IHC positive and negative controls are provided in Supplementary Figure 1.

Histopathological analysis

The areas of different MG compartments (epithelium, stroma and adipose tissue) were assessed by analysis of HE sections (3 sections per animal from different depths) and evaluated by the adapted M130 multipoint test system (Leonel et al. 2020). The incidence and multiplicity of microscopic lesions in the MG of aged female gerbils were also evaluated. For this, α-SMA staining was applied for myoepithelial cells identification – aiming to evaluate the maintenance of basal membrane and structure of the epithelial unit – and mammary epithelial structures were divided into 3 classifications: normal morphology (without hyperplasia or rupture of the myoepithelial layer); hyperplasia (more than two layers of luminal epithelial cells, without disruption of myoepithelial layer); and carcinoma/neoplasia (total or partial loss of myoepithelial layer and disruption of epithelial compartment). The quantification was performed taking into account the incidence of normal alveoli, hyperplasia, and carcinoma regions in relation to all the epithelial structures in the same histological section. Data were expressed as a percentage of epithelial lesions found in the same section of MG.

The area of collagen and elastic fibers was quantified by the automated system from ImageJ software (version 1.52a, Wayne Rasband, 130 NIH, USA). For these quantifications, 15 random fields (200x magnification) were selected in 3 different sections, corresponding to different depths of the MG sample, from the slides stained with Picrosirius (for collagen analysis, under polarized microscopy) and Resorcin-Fuchsin (for elastic fibers analysis, under light microscopy).

The quantification of IHC positive staining for identification of cytoplasmic and nuclear markers was performed by applying QuPath software (Version 0.1.2, an open-source pathology software platform). The total area of histological sections was

considered for quantification of the positive staining and data were expressed in cells/mm^2.

Statistical Analysis

Analyses of stereological parameters (incidence of compartments) and areas of collagen and elastic fibers were performed in 3 sections from different depths of the mammary gland sample. The mean of the 3 analyzed sections was calculated and this mean was used as the individual value for each animal in statistical analyses. For IHC analyses, one random section per animal was analyzed and values of cells/mm^2, presented as means ± SEM, were considered for the statistical analyses.

All data were checked for normality by the Kolmogorov-Smirnov test. Parametric data (epithelial area and lesions incidence, elastin area; IHC positive staining: PHH3, TGF-β1, FAP, MMP-2, MMP-3, MMP-9) were analyzed by one-way ANOVA followed by Tukey's test. For non-parametric data (collagen area, stromal and adipose tissue areas; IHC positive stains: p63, active caspase-3) we applied the Kruskal-Wallis test followed by Dunn's test. Statistical differences among groups were considered significant when $p < 0.05$. For all statistical analyses, GraphPad Prism 5.00 for Windows (GraphPad Software, San Diego, CA, USA, www.graphpad.com) was applied.

Ethical Standards

The animals were kept at the Animal Breeding Center of the Institute of Biosciences, Humanities and Exact Sciences (IBILCE) of the São Paulo State University (UNESP) throughout the experiment, under controlled temperature (24 ± 2 °C) and luminosity (12 h light / dark cycle). The procedures followed the standards according to the National Council of Animal Experiment Control (CONCEA, Brazil), and were authorized by the Ethics Committee on the Use of Animals (CEUA) from IBILCE/Unesp (Protocol number: 217/2019).

Results

Histopathological repercussions of BPA exposure in aged female MG

All tissue compartments showed differences when comparing control and vehicle groups to both BPA groups (Figure 2 A-D). The areas of epithelial and stromal compartments in both BPA groups were increased (Figure 2 C-D; E-F) in comparison

to control and vehicle groups. At 18 months of age, MG from control and vehicle groups presented an increase in adipose tissue (Figure 2 G) compared to the BPA-exposed gerbils MG. The stromal area increased in both BPA groups, being statistically different from control and vehicle groups (Figure 2 F). MG epithelium occupation in BPA groups was almost 4-fold (↑BPA) and 3-fold (↓BPA) increased compared to control (Figure 2 E). These groups also showed disorganization of the epithelial compartment with loss of differentiated luminal cells and alveolar shape, with development of multifocal carcinoma regions. These regions showed heterogeneous nuclear atypia (Figure 2 H), with no apparent cytokeratin expression (Figure 2 I), and enhanced vimentin expression (Figure 2 J).

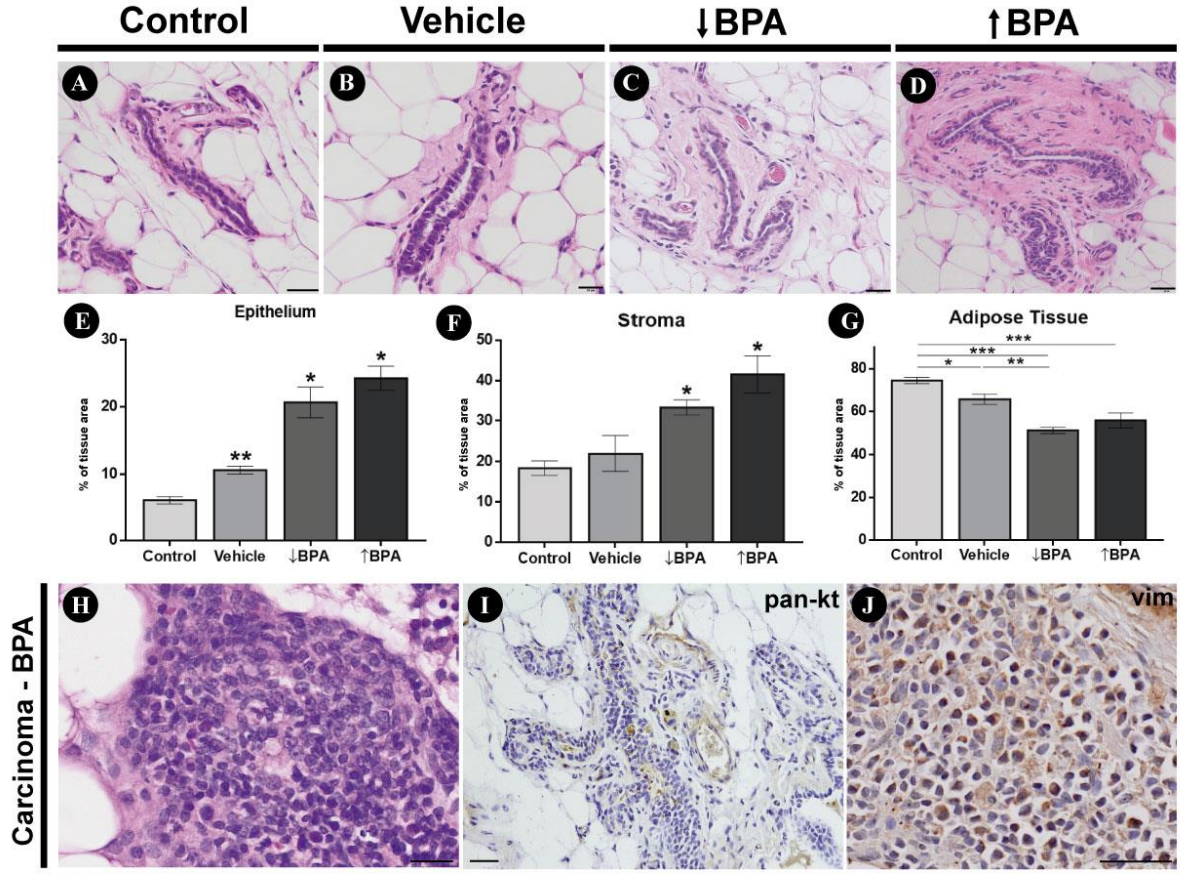


Figure 2. Tissue compartments and carcinoma features. (A-D) Morphology of normal mammary tissue in control (A), vehicle (B), ↓BPA (C), and ↑BPA (D) groups. (E-G) Incidence (percentage) of epithelial (E), stromal (F), and adipose tissue (G) compartments in different groups (n=5). Asterisks indicate statistical differences among groups, considering significance when $p<0.05$ (Statistical analysis: Parametric data: One-Way ANOVA followed by Tukey's test (epithelial percentage); non-parametric data: Kruskal-Wallis test followed by Dunn's test (stromal and adipose percentage). (H-J) Carcinoma feature in MG of BPA-exposed aged females. Nuclear atypia and nuclear irregularity were observed (H) with absence of pan-cytokeratin (I) and presence of vimentin (J) expression. Staining: (A-D, H) HE. Scale Bars: (A) 50 µm; (B-D, H, J) 20 µm; (I) 30 µm.

In comparison to control and vehicle groups (Figure 3 A, B), MG from BPA-exposed female gerbils showed an increase in hyperplastic structures and the presence of neoplastic features (Figure 3 C, D, respectively). Hyperplastic structures were identified by the presence of two (Figure 3 C) or more layers (Figure 3 D) of epithelial cells. Control and vehicle groups presented hyperplastic foci incidence (Figure 3 E) 2-fold lower than BPA groups. Neoplastic structures showed discontinuity of the myoepithelial cell layer, as shown by α-SMA immunostaining; thus, a carcinoma/neoplastic phenotype was characterized by the absence of this layer around normal alveoli (Figure 3 F). In addition, ↓BPA and ↑BPA groups presented alveoli with secretory activity (Figure 3 F-inset), which was not observed in the other groups. These secretory structures were dispersed in MG parenchyma and identified in all ↓BPA and ↑BPA samples.

The vehicle group (Figure 3 I) demonstrated a slight increase in p63 basal cell expression, but did not differ significantly from the control, which showed only nuclear staining (Figure 3 H). Contrarily, in the BPA groups, the expression increased almost 3-fold compared to the control. Furthermore, in these groups the expression of p63 was nuclear and cytoplasmic in the regions of early carcinoma (Figure 3 H, J). ↑BPA showed the highest expression rates but did not differ significantly from ↓BPA.

The proliferative and apoptotic activity was evaluated by PH-H3 (Figure 4 A-D) and active caspase-3 (Figure 4 E-H) nuclear staining, respectively. The control group presented rates of PH-H3 positive cells almost 10-fold lower compared to BPA groups (Figure 4 I). Carcinoma in BPA groups and hyperplasia in both BPA and vehicle groups induced a high proliferative rate in comparison to control group. ↓BPA and ↑BPA groups did not differ from each other and showed the highest values of PH-H3-positive cells in epithelial and stromal compartments (Figure 4 C-D). Nuclear expression of active caspase-3 was only observed in the epithelial compartment, from all groups. At 18 months of age, the control group presented a high number of apoptotic cells (caspase-3 positive cells) (Figure 4 J), while a drastic decrease in their expression was observed in ↓BPA and ↑BPA groups compared to control and vehicle. In multifocal carcinoma structures, present only in BPA exposed gerbils MG, scarce expression of caspase-3 was observed in ↓BPA (Figure 4 G) and was absent in ↑BPA (Figure 4 H).

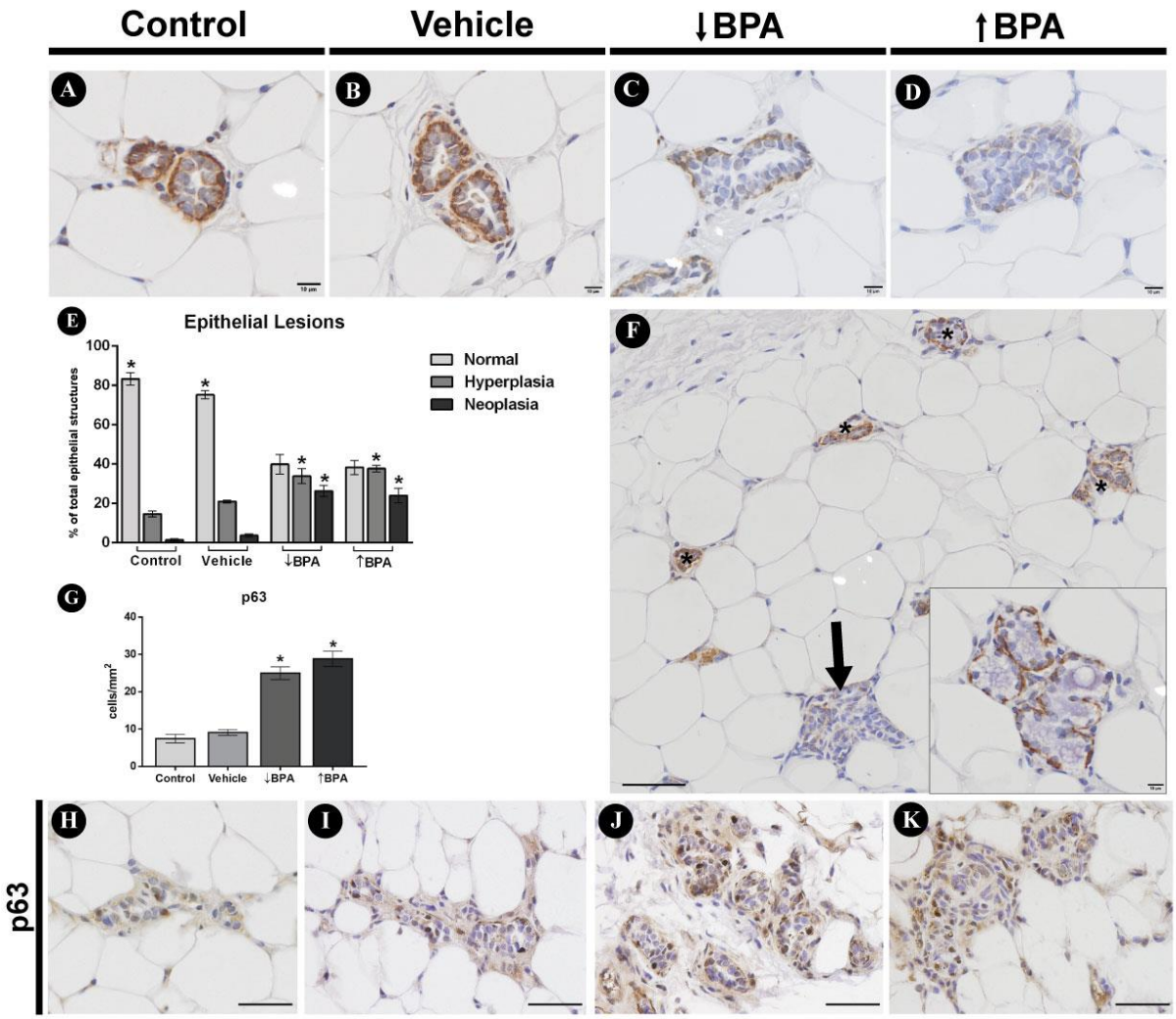


Figure 3. Mammary epithelial lesions, myoepithelial layer, and p63 expression. (A-D) Myoepithelial cell layer (α-SMA) in mammary tissues indicates continuity of myoepithelial layer in control (A) and vehicle (B), whereas in ↓BPA and ↑BPA there is discontinuity (C) or absence (D) of this layer. (E) Epithelial lesions (% of total epithelial structures): normal, hyperplasia, and neoplasia, were quantified. ↓BPA and ↑BPA groups presented a high incidence of both lesions, with multifocal carcinoma development (F, arrow) among normal alveoli (F, asterisks). Note alveoli with secretory activity (F-inset). (G) Incidence of p63 positive cells. (H-K) Expression of p63 in MG. Control (H) and vehicle (I) presented only nuclear staining, indicating basal epithelial cells. In ↓BPA and ↑BPA, in addition to nuclear staining, basal-like stemness was assigned by cytoplasmic staining in carcinomas. Asterisks indicate statistical differences among groups, considering $p<0.05$ of significance (n=5, parametric data: One-Way ANOVA followed by Tukey's test (epithelial lesions percentage); non-parametric data: Kruskal-Wallis test followed by Dunn's test (p63). Scale Bars: (A-D, F-inset) 10 µm; (F) 50 µm; (H-K) 30 µm.

Expression of TGF-β1 in MG of aging BPA-exposed females

TGF-β1 was overexpressed in BPA groups (Figure 4 K). Gerbil MG of control and vehicle groups presented low rates of TGF-β1-positive cells, which were restricted to the stromal compartment (Figure 4 L-M). However, BPA groups presented a drastic increase (3-fold) in TGF-β1-positive cells with epithelial and stromal staining (Figure 4 N-O). Epithelial staining of TGF-β1 was observed in normal and hyperplastic ducts, whereas carcinoma cells did not stain for this marker.

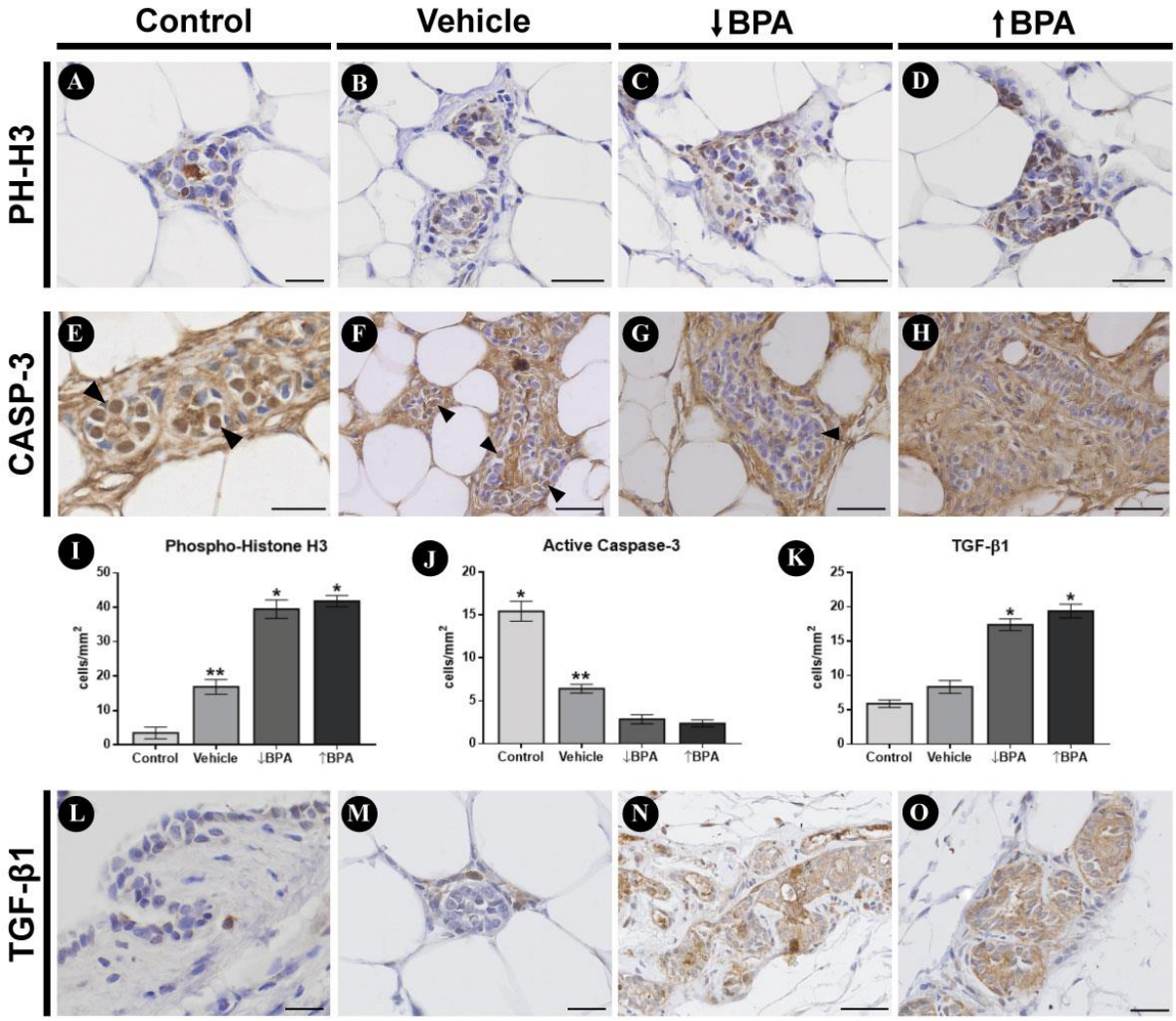


Figure 4. Proliferative and apoptotic activity, and TGF-β1 expression. (A-D) Phospho-histone H3 positive cells indicating proliferative activity and (E-H) active caspase-3 indicating apoptosis signaling (both nuclear staining - arrowheads). (I) and (J) show the proliferative/apoptotic opposite dynamics of mammary tissue among groups. Proliferation was increased in ↓BPA and ↑BPA groups, whereas in control, apoptosis was enhanced in epithelial cells. (K) TGF-β1 expression in MG of aged females. Overexpression was observed in BPA groups. Control and vehicle groups presented positive cells only in stroma (L and M, respectively), whilst BPA presented overexpression in epithelial compartment (N and O). Asterisks indicate statistical

differences among groups, considering $p<0.05$ of significance (n=5, parametric data: One-Way ANOVA followed by Tukey's test (PHH3, TGF-β1); non-parametric data: Kruskal-Wallis test followed by Dunn's test (active caspase-3). Scale Bars: (A, E, L, M) 20 µm; (B-D, F-H, N, O) 30 µm.

Stromal fiber density of BPA exposed MG in aged females

Collagen area occupation presented low values in control and vehicle groups (Figure 5 A, B), with more than 2- and 3-fold increase in ↓BPA and ↑BPA (Figure 5 C, D), respectively, compared to control group. Epithelial structures and carcinoma areas were surrounded by a collagen-rich stroma. The percentage of collagen and elastin fibers was different among groups (Figure 5 E-F). For elastin fibers, a high percentage was observed in control and vehicle, comparing to both BPA exposed groups (Figure 5 G-G'; H-H'; I-I'). Furthermore, elastic fibers surrounding stroma of intact ductal structures in BPA-exposed gerbil MG were ruptured (Figure 5 H').

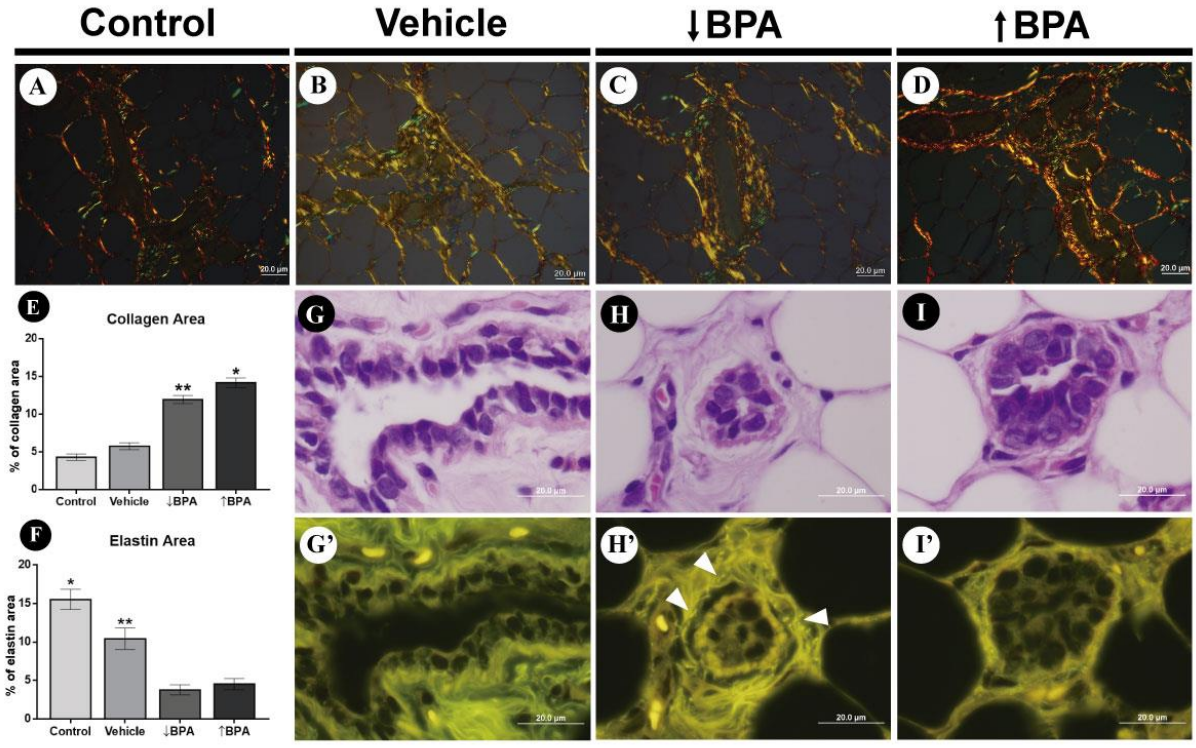


Figure 5. Stromal fibers in disrupted MG. (A-D') Collagen fibers were enhanced in BPA groups in surrounding stroma. Furthermore, ↑BPA presented the highest values in % of collagen area and differed from all groups (E). (F) Elastin showed a continuous feature and its area % increased in control and vehicle (G-G') groups. (H-H', I-I') In addition to a decreased area BPA groups presented rupture of elastin layer in MG stroma (arrowheads in H'). Staining: (A-D) Picrosirius red under polarized microscopy; (G-I) HE-Floxin under light and (G'-I') fluorescent microscopy. Asterisks indicate statistical

differences among groups, considering $p<0.05$ of significance (n=5, parametric data: One-Way ANOVA followed by Tukey's test (elastin area); non-parametric data: Kruskal-Wallis test followed by Dunn's test (collagen area). Scale Bars: 20 µm.

Metalloproteases expression and FAP-positive cells in stroma of normal and altered BPA exposed MG

The BPA exposed groups presented a higher incidence of cells expressing all MMPs compared to control and vehicle groups (Figure 6 A-C). MMP-2 positive cells were observed in the stromal compartment (Figure 6 D-E). Cells in carcinoma regions presented poor staining (Figure 6 E). MMP-3 positivity was also observed in fibroblasts among carcinoma cells (Figure 6 G), stromal cells, and the non-disrupted myoepithelial layer (Figure 6 H-I). MMP-3 cytoplasmic expression was predominant but several cells also presented nuclear positivity (Figure 6 H). MMP-9 was observed in stromal cells and poorly-diffused in ECM (Figure 6 J-L). Cytoplasmic staining of MMP-9 was observed in the disrupted epithelial compartment (Figure 6 K).

FAP expression (Figure 6 M-Q) in stromal cells was different between BPA exposed groups and both control and vehicle groups (Figure 6 N-O). FAP-positive fibroblasts (i.e., cancer-associated fibroblasts, CAFs) increased around 2- and 3-fold in MG of ↓BPA and ↑BPA groups in comparison to control (Figure 6 M). Furthermore, FAP-positive cells in BPA-exposed gerbil MGs were predominant in surrounding stroma and carcinoma (Figure 6 Q).

Discussion

Tumorigenic development was observed in MG of aged gerbils exposed to BPA

The female gerbils were exposed to BPA during two drastic remodeling periods for MG, pregnancy and lactation. We demonstrate the susceptibility of MG tissue to endocrine-chemical disruption and its potential to induce carcinogenesis during these exposure periods. Furthermore, aging is an aggravating factor to the onset of tumorigenic process (Fane & Weeraratna 2020). In the present study we observed the development of multifocal neoplastic structures in MG of aged gerbils exposed to BPA during pregnancy and lactation. These exposure windows deserve to be focus of discussion related to the development of neoplastic triggering after pregnancy (Schedin 2006; Hsiao et al. 2010; Allouch et al. 2020).

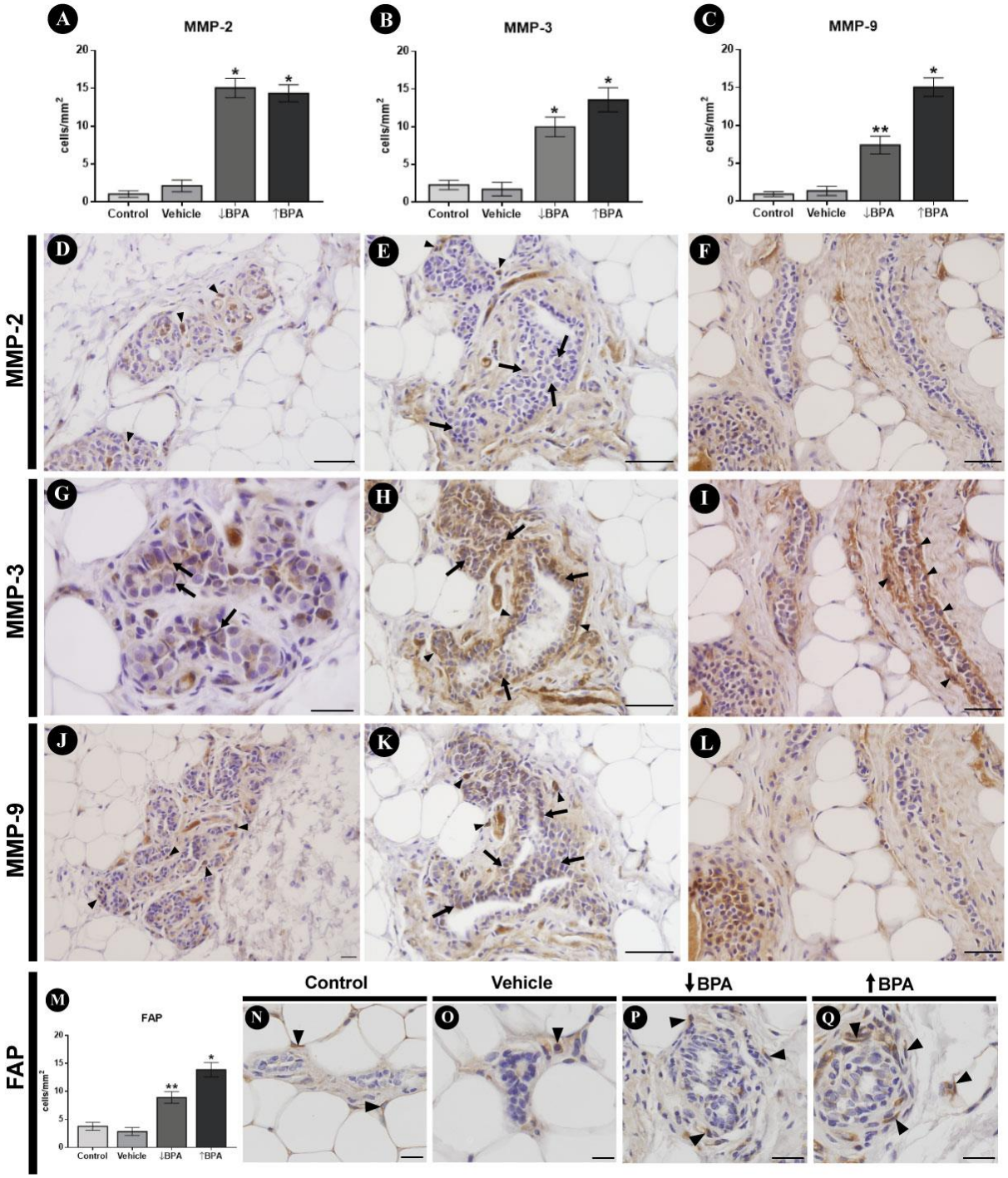


Figure 6. Metalloproteinases expression and FAP-positive cells BPA-exposed MG. (A), (B), and (C) indicate the incidence of positive cells per mm^2 for MMP-2, MMP-3, and MMP-9, respectively. (D-F) MMP-2. Cytoplasmic staining in stromal (arrowheads) and carcinoma cells (arrows) were observed. (G-I) MMP-3. (G) Fibroblasts (arrows) among disrupted epithelium expressing MMP-3 were found. (H-I) In normal alveoli and ducts, myoepithelial cells (arrowheads) express MMP-3. Arrows in both images demonstrate nuclear staining for MMP-3 in carcinomas. (J-L) MMP-9. Stromal (arrowheads) and carcinoma cells (arrows) present cytoplasmic staining for MMP-9. (M-Q) Incidence of FAP-positive cells. FAP-positive fibroblasts were observed only in peripheric stroma of control and vehicle groups (N-O, arrowheads), whereas in both BPA groups they were found among disrupted carcinoma (P-Q, arrowheads). Asterisks indicate statistical differences among groups, considering $p < 0.05$ of significance (n=5, parametric data: One-Way ANOVA followed by Tukey's test). Scale Bars: (D-L) 50 µm; (N-O) 20 µm; (P-Q) 30 µm.

In addition, pregnancy-lactational window must be recognized as a vulnerability period, which impacts a third window related to ageing. In fact, this study shows evidence that exposure to BPA (without the use of other carcinogenic inducers (Cheung et al. 2003)) is capable of promoting mammary cancer in a susceptible animal model. These negative impacts were demonstrated in MG of mothers directly exposed to BPA, a susceptible group not evaluated in the majority of published works, in which the main concern is the transgenerational effect (Yoshida et al. 2004; Wadia et al. 2007; Kunz et al. 2011; Lozada & Keri 2011; Gomez et al. 2017) resulted from disruption during the perinatal window of susceptibility.

The tumor development process was observed in the tissue, from stages for cancer establishment (nuclear atypia, phenotypic changes in epithelial cells, and hyperplasia) to alveolar remodeling and tumorigenic interaction of the epithelial and stromal compartments (Mallon et al. 2000). Hyperplasia was present in all groups, but enhanced in females exposed to BPA. Hyperplastic structures were characterized by discontinuity of the myoepithelial cell layer, which acts as a barrier between the secretory luminal epithelial cells and the stroma (Li et al. 2020). The myoepithelial cell layer also signals to the polarization of luminal epithelial cells (Gudjonsson et al. 2002). Thus, its loss leads to a disorder in the epithelial compartment where cells undergo proliferative disarray. Another marker considered for evaluating tumor progression to malignant and invasive phenotype was cytoplasmic expression of p63 (Hsiao et al. 2010), which increased in BPA exposed MG.

High neoplasia rates in BPA exposed aged female gerbils demonstrate the carcinogenic potential of this xenoestrogen (Seachrist et al. 2016). In vitro studies showed the ability of BPA to induce neoplastic development in breast epithelial cells (Fernandez & Russo 2010). Shafei and colleagues reviewed BPA molecular mechanisms of action and related mammary disorders to epigenetic changes and anti-apoptotic activity (Shafei et al. 2018a). In the present study, the proliferation rates in the BPA groups were high; contrarily, the number of cells in apoptotic process was reduced, establishing the tumorigenic process. MG from female Mongolian gerbil individuals at 18 months of age, when not exposed to the xenoestrogen BPA, presented similar characteristics of perimenopause involution phase (Oh et al. 2016). This is also an evidence that BPA alters cell machinery (Lee et al. 2013) to promote survival and transformation of cancer cells at late age.

Also, increase in proliferative activity was observed in MG of female gerbils from vehicle group, mostly associated to hyperplasia. Indeed, this finding can be related to the activity of phytoestrogens found in the vegetal oil (Lorand et al. 2010), which could promote hyperplasic features in gerbils MG, as described (Leonel et al. 2020), and a morphological feature often associated to aging (Attia 1996; Gomez et al. 2017).

BPA induces epithelial-mesenchymal transition in MG of aged gerbils

The epithelial-mesenchymal transition (EMT) is one of the initial processes in tumor progression and invasion of adjacent tissues (Lee et al. 2017). When exposed to BPA, breast cancer cells modulate key signal-proteins of endocrine response to promote EMT signaling, such as loss of cytokeratin and vimentin expression (Patel et al. 2015), similarly to that observed in our study. TGF-β1 is a cytokine with major responsibility for leading breast cancer cells to the EMT process, also contributing to the next invasiveness steps (Zhang et al. 2020). Its high expression in BPA groups, especially in ↑BPA, corroborates the EMT mechanism observed in MG of aging gerbils. Dong and colleagues associated TGF-β1 expression during mammary carcinoma development as a remarkable modulating signal for MMP-9 stromal overexpression in MG (Dong et al. 2019). The TGF-β1 increase in response to stromal modifications indicates the relevance of epithelial-stromal interactions. Thus, the interaction between compartments supports carcinoma development induced by BPA.

Even though stromal features influence the EMT process, epithelial protein expression also contributes to tumorigenic development. High epithelial expression of TGF-β1 in mammary cells of BPA exposed gerbils indicates that this marker participates in susceptibility to BPA-disruption (Betancourt et al. 2014). As TGF-β1 amplifies the EMT process (Goulet & Pouliot 2021) and cancer modulation in ECM (Vandenberg et al. 2012), its expression in normal epithelial cells and tissue of BPA groups indicates its role as a mediator in the tumor induction by this xenoestrogen in gerbil MG. Clearly, the tumorigenic process has several molecular influences, but TGF-β1 expression in disrupted tissue suggests its key role in BPA action.

Compared to non-exposed tissue of control and vehicle groups, in BPA groups a proliferative pathway was induced (Dairkee et al. 2013) by means of a cell surviving mechanism (Muraoka-Cook et al. 2006) in an environment supposed to show apoptotic and regressive patterns. This cell survival leads to the perpetuation of a tumoral architecture that allows a clonal and disordered growth (Zhang et al. 2017) through the

proliferative process, confirmed by an increase in PH-H3 positive cells in aged gerbil MG. BPA acts through estrogenic pathways to disrupt the cell cycle, such as in prostate (Huang et al. 2018). In addition, even though this EDC promotes mammary hyperplasia (Dumitrascu et al. 2020), in the present study we denote its tumor development potential in aging through the EMT mechanism, which could develop into an invasive and aggressive cancer.

The invasive profile can be monitored through p63 expression in carcinoma cells. Cytoplasmic and nuclear staining for this protein enables diagnosis of invasiveness and is present in early breast cancer (Sundqvist et al. 2020) and pregnancy-associated breast cancer (Hsiao et al. 2010). Here, after BPA exposure during pregnancy and lactation, gerbil MG tissue from aged females presented enhanced p63 expression mainly in the cytoplasm, indicating a poor prognosis. This increase is one of the mechanisms activated by the BPA disruption activity in mammary tissue, known as basal programming (Cheung et al. 2013), which contributes to the structural maintenance of carcinomas and leads to a collective stromal invasion. Furthermore, its expression in tissues prone to tumorigenesis can amplify the functions of TGF-β1 as an EMT promoter, leading to invasiveness (Sundqvist et al. 2020). The expression of p63 demonstrates regulation of the EMT process in tumor tissue, exhibiting a permissive character to the collective invasion of cells in transformation associated with the remodeling of ECM in pro-tumor conditions (Gatti et al. 2019).

Stromal remodeling promotes a tumor microenvironment in MG exposed to BPA

A favorable tumor microenvironment is provided by disrupted stroma in several breast cancer types (Bussard et al. 2016). Collagen and elastin constitute the major fibrillar components of ECM and its degradation and synthesis support tumor progression (Brassart-Pasco et al. 2020). In our study, this process occurred in stroma of MG from BPA exposed females with a strong correlation with MMP expression. Indeed, loss of fibrillar elastin in BPA leads to a decline in mechanical properties and fibrosis in the stromal compartment, that also affects the epithelial compartment (Brassart-Pasco et al. 2020). In contrast, the collagen area increased drastically in BPA groups and this ECM remodeling is a remarkable step, previously described for breast cancer promotion (Gehmert et al. 2020).

Collagen fibers were frequent around carcinoma and normal mammary structures in MG from aged females. TGF-β1 is known to increase the collagen

deposition in ductal areas (Silberstein et al. 1990) creating a fibrotic aspect of ECM, stimulating proliferative disorders in the epithelial compartment, and thus supporting tumor increase. Collagen and elastin dynamic in the MG of females exposed to BPA exhibits a tissue microenvironment that has undergone high remodeling. BPA promotes ECM stiffness in the MG of females exposed in utero through cellular machinery reprogramming for synthesis of extracellular elements (Wormsbaecher et al. 2020). Thus, we propose that this EDC promotes a tumor-associated collagen signature that rearranges the collagen matrix of the stroma (Lyons et al. 2011), supporting the development of carcinoma, in addition to being able to influence local invasiveness (Provenzano et al. 2006).

Tumor growth invasiveness is promoted by upregulation of ECM proteases. The reactive stroma concerns not only changes in the ECM fiber components, but also those in cellular assembly (Mao et al. 2013), either by differentiation or by cell recruitment. Increases in the expression of MMP-2, MMP-3, and MMP-9 were observed in the MG of aged females exposed to BPA. Although each one presents a specific action in the mammary tissue, in the present study these MMPs contributed to tumor growth and the installation of multifocal carcinoma in gerbil MG. MMP-2 and MMP-9, which showed an expressive increase in the BPA groups, were exclusively expressed in ECM and are related to stromal cells that promote its remodeling (Di Cara et al. 2018). Both are proteases that aid local invasion of tumor cells and tumor growth (Dong et al. 2019). However, interestingly, we detected MMP-3 expression in different cell types, which assisted in the understanding of remodeled tissue mechanisms. Cells potentially in the EMT process showed cytoplasmic MMP-3 staining, similarly to myoepithelial cells in the basal epithelium, when discontinuous or not.

In our study the expression profile of these MMPs suggests two tumorigenic process mechanisms induced by BPA: (I) EMT reprogramming is supported by MMP-3 expression in the epithelium and MMP-2 and MMP-9 expression in the stroma to develop a tumor feature; and (II) invasiveness and expansion of carcinoma occurred due to myoepithelial cell expression of MMP-3 and proteolytic activity in basement membrane. The first mechanism is related to the main role of MMPs described during breast cancer establishment (Radisky & Radisky 2015). These proteases decrease cell adhesion (Marcus et al. 2019) and regulate signals of stemness (Radisky et al. 2017), leading to the consequent EMT process. Specifically, MMP-9, which is also overexpressed in other cancer types after BPA disruption (Kim et al. 2015), promoted

the tumor signaling pathway of TGF-β1. MMP-9 intensifies TGF-β1 signaling during induction of the EMT process in breast tissue (Dong et al. 2019). This “cell adhesion” effect can also be induced by MMP-2 (Di Cara et al. 2018). In terms of basement membrane, the second mechanism correlates to the degradation activity of MMPs. MMP-3 expression in myoepithelial cells of BPA exposed MG promotes rupture of the last barrier between carcinoma and stroma (Deng et al. 2020). The surrounding stromal cells are recruited by BPA-induced carcinoma signaling pathways to express MMP-2 and MMP-9 for basement membrane discontinuity (Blavier et al. 2006). With basement membrane loss, disrupted epithelial cells are stimulated to a disordered proliferation and expansion towards stroma (Slepicka et al. 2019). BPA changes basement membrane deposition in MG, which impacts breast alveoli morphogenesis by epithelial cells (Marchese & Silva 2012). Thus, our results suggest that BPA disruption increases MMP-3 expression for tumor progression and collective invasion of the stroma, whereas MMP-2 and MMP-9 expression in stromal cells is increased to support invasion in MG from exposed aged females, mediating the epithelium-stroma interaction.

The epithelial-stroma interaction for tumor progression requires CAFs (Alexander & Cukierman 2020). Despite their multiple origins, CAFs are cellular elements that apparently arise from the establishment of reactive stroma in the breast (Elwakeel et al. 2019). According to Wormsbaecher and colleagues the mammary fibroblast phenotype is altered when an in-utero BPA exposure occurs, leading to subsequent ECM remodeling (Wormsbaecher et al. 2020). It is important to note that the recruitment of these cells increases dramatically in gerbil MG exposed to BPA, as observed by the staining of FAP-positive cells (Tao et al. 2017), suggesting that the endocrine disruption acts in some pathway for the establishment of this cell type. One of the pathways of fibroblast activation in cancer is through induction by TGF-β1 and FGF (Tao et al. 2017), both highly expressed in BPA-disrupted MG, which can be stated as a BPA endocrine disruption pathway for recruiting CAFs.

In summary, the gestational and lactational windows in MG represent an exposure period in which BPA imposes a delayed carcinogenic risk during aging. The analysis allowed us to identify cancer progression in gerbil MG associated with several elements that support tumoral invasiveness (Figure 7). TGF-β1 was highly expressed in normal epithelium of disrupted tissue and seems to be a remarkable feature in BPA disruption, not addressed in aged females so far. These BPA induced mechanisms

contribute to EMT reprogramming and stromal signaling for tumor microenvironment establishment. Furthermore, the epithelial-stromal interaction, by molecular pathways and remodeling MMPs, emphasizes the alterations provoked by the studied EDC and the repercussions which favor carcinogenesis in disrupted mammary tissue.

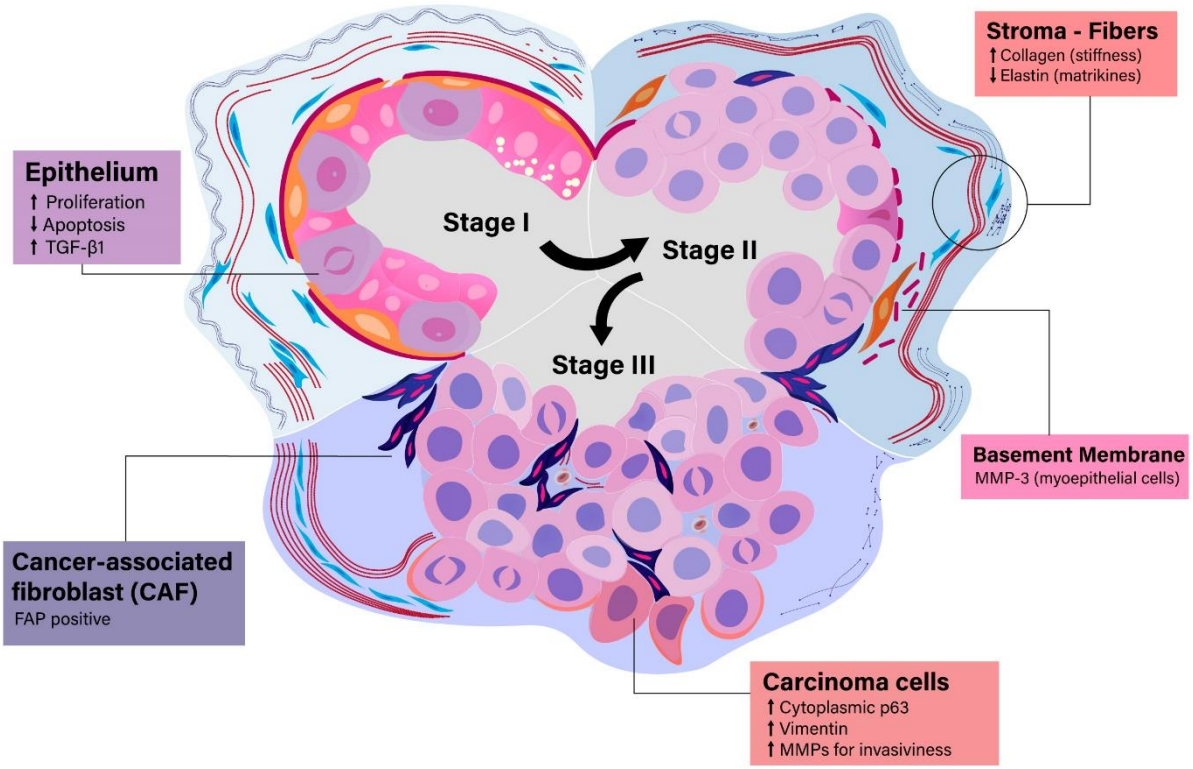


Figure 7. Graphical illustration of BPA-induced disruption in three stages of tumorigenesis in gerbil mammary gland.

References

Alexander J & Cukierman E 2020 Cancer associated fibroblast: Mediators of tumorigenesis. Matrix Biology 91–92 19–34. (doi:10.1016/j.matbio.2020.05.004)

Allouch S, Gupta I, Malik S, Al Farsi HF, Vranic S & Al Moustafa AE 2020 Breast Cancer During Pregnancy: A Marked Propensity to Triple-Negative Phenotype. Frontiers in Oncology 10 1–11. (doi:10.3389/fonc.2020.580345)

Alvarado-Cabrero I, Valencia-Cedillo R & Estevez-Castro R 2020 Preneoplasia of the Breast and Molecular Landscape. Archives of Medical Research 51 845–850. (doi:10.1016/j.arcmed.2020.09.011)

Atlas E & Dimitrova V 2019 Bisphenol S and Bisphenol A disrupt morphogenesis of MCF-12A human mammary epithelial cells. Scientific Reports 9 1–10. (doi:10.1038/s41598-019-52505-x)

Attia MA 1996 Neoplastic and non-neoplastic lesions in the mammary gland, endocrine and genital organs in aging male and female Sprague-Dawley rats. Archives of Toxicology 70 461–473. (doi:10.1007/s002040050300)

Betancourt A, Mobley JA, Wang J, Jenkins S, Chen D, Kojima K, Russo J & Lamartiniere CA 2014 Alterations in the rat serum proteome induced by prepubertal exposure to bisphenol A and genistein. Journal of Proteome Research 13 1502–1514. (doi:10.1021/pr401027q)

Biancardi MF, Perez APS, Caires CRS, Falleiros LR, Góes RM, Vilamaior PSL, Freitas DR, Santos FCA & Taboga SR 2017 Prenatal and pubertal testosterone exposure imprint permanent modifications in the prostate that predispose to the development of lesions in old Mongolian gerbils. Asian Journal of Andrology 19 160–167. (doi:10.4103/1008-682X.170436)

Blavier L, Lazaryev A, Dorey F, Shackleford GM & DeClerck YA 2006 Matrix metalloproteinases play an active role in Wnt1-induced mammary tumorigenesis. Cancer Research 66 2691–2699. (doi:10.1158/0008-5472.CAN-05-2919)

Brassart-Pasco S, Brézillon S, Brassart B, Ramont L, Oudart JB & Monboisse JC 2020 Tumor Microenvironment: Extracellular Matrix Alterations Influence Tumor Progression. Frontiers in Oncology 10 1–13. (doi:10.3389/fonc.2020.00397)

Bussard KM, Mutkus L, Stumpf K, Gomez-Manzano C & Marini FC 2016 Tumor-associated stromal cells as key contributors to the tumor microenvironment. Breast Cancer Research 18 1–11. (doi:10.1186/s13058-016-0740-2)

Campos SGP, Zanetoni C, Scarano WR, Vilamaior PSL & Taboga SR 2008 Age-related histopathological lesions in the Mongolian gerbil ventral prostate as a good model for studies of spontaneous hormone-related disorders. International Journal of Experimental Pathology 89 13–24. (doi:10.1111/j.1365-2613.2007.00550.x)

Di Cara G, Marabeti M, Musso R, Riili I, Cancemi P & Pucci Minafra I 2018 New Insights into the Occurrence of Matrix Metalloproteases -2 and -9 in a Cohort of Breast Cancer Patients and Proteomic Correlations. Cells 7 89. (doi:10.3390/cells7080089)

Cheung SY, Yuen MT, Choi HL, Cheng HK, Huang Y, Chen S & Chan FL 2003 An expression study of hormone receptors in spontaneously developed, carcinogen-induced and hormone-

induced mammary tumors in female Noble rats. International Journal of Oncology 22 1383–1395. (doi:10.3892/ijo.22.6.1383)

Cheung KJ, Gabrielson E, Werb Z & Ewald AJ 2013 Collective Invasion in Breast Cancer Requires a Conserved Basal Epithelial Program. Cell 155 1639–1651. (doi:10.1016/j.cell.2013.11.029)

Custodio AMG, Santos FCA, Campos SGP, Vilamaior PSL, Góes RM & Taboga SR 2008 Aging effects on the mongolian gerbil female prostate (Skene's paraurethral glands): Structural, ultrastructural, quantitative, and hormonal evaluations. Anatomical Record 291 463–474. (doi:10.1002/ar.20637)

Custodio AMG, Santos FCA, Campos SGP, Vilamaior PSL, Oliveira SM, Góes RM & Taboga SR 2010 Disorders related with ageing in the gerbil female prostate (Skene's paraurethral glands). International Journal of Experimental Pathology 91 132–143. (doi:10.1111/j.1365-2613.2009.00685.x)

Dairkee SH, Luciani-Torres MG, Moore DH & Goodson WH 2013 Bisphenol-A-induced inactivation of the p53 axis underlying deregulation of proliferation kinetics, and cell death in non-malignant human breast epithelial cells. Carcinogenesis 34 703–712. (doi:10.1093/carcin/bgs379)

Deng Y, Miki Y & Nakanishi A 2020 Estradiol/GPER affects the integrity of mammary duct-like structures in vitro. Scientific Reports 10 1–15. (doi:10.1038/s41598-020-57819-9)

Dhimolea E, Wadia PR, Murray TJ, Settles ML, Treitman JD, Sonnenschein C, Shioda T & Soto AM 2014 Prenatal exposure to BPA alters the epigenome of the rat mammary gland and increases the propensity to neoplastic development. PloS One 9 e99800–e99800. (doi:10.1371/journal.pone.0099800)

Dong H, Diao H, Zhao Y, Xu H, Pei S, Gao J, Wang J, Hussain T, Zhao D, Zhou X et al. 2019 Overexpression of matrix metalloproteinase-9 in breast cancer cell lines remarkably increases the cell malignancy largely via activation of transforming growth factor beta/SMAD signalling. Cell Proliferation 52 1–14. (doi:10.1111/cpr.12633)

Dumitrascu M, Mares C, Petca R-C, Sandru F, Popescu R-I, Mehedintu C & Petca A 2020 Carcinogenic effects of bisphenol A in breast and ovarian cancers (Review). Oncology Letters 20 1–1. (doi:10.3892/ol.2020.12145)

EFSA 2015 Scientific Opinion on the risks to public health related to the presence of bisphenol A (BPA) in foodstuffs. EFSA Journal 13. (doi:10.2903/j.efsa.2015.3978)

Elwakeel E, Brüggemann M, Fink AF, Schulz MH, Schmid T, Savai R, Brüne B, Zarnack K & Weigert A 2019 Phenotypic plasticity of fibroblasts during mammary carcinoma development. International Journal of Molecular Sciences 20. (doi:10.3390/ijms20184438)

Fane M & Weeraratna AT 2020 How the ageing microenvironment influences tumour progression. Nature Reviews Cancer 20 89–106. (doi:10.1038/s41568-019-0222-9)

Faupel-Badger JM, Arcaro KF, Balkam JJ, Heather Eliassen A, Hassiotou F, Lebrilla CB, Michels KB, Palmer JR, Schedin P, Stuebe AM et al. 2013 Postpartum remodeling, lactation, and breast cancer risk: Summary of a national cancer institute-sponsored workshop. Journal of the National Cancer Institute 105 166–174. (doi:10.1093/jnci/djs505)

Fernandez S V. & Russo J 2010 Estrogen and Xenoestrogens in breast cancer. Toxicologic Pathology 38 110–122. (doi:10.1177/0192623309354108)

Gatti V, Bongiorno-Borbone L, Fierro C, Annicchiarico-Petruzzelli M, Melino G & Peschiaroli A 2019 P63 At the Crossroads Between Stemness and Metastasis in Breast Cancer. International Journal of Molecular Sciences 20. (doi:10.3390/ijms20112683)

Gehmert S, Lehoczky G, Loibl M, Jung F, Prantl L & Gehmert S 2020 Interaction between extracellular cancer matrix and stromal breast cells. Clinical Hemorheology and Microcirculation 74 45–52. (doi:10.3233/CH-199234)

Giulivo M, Lopez de Alda M, Capri E & Barceló D 2016 Human exposure to endocrine disrupting compounds: Their role in reproductive systems, metabolic syndrome and breast cancer. A review. Environmental Research 151 251–264. (doi:10.1016/j.envres.2016.07.011)

Gomez AL, Delconte MB, Altamirano GA, Vigezzi L, Bosquiazzo VL, Barbisan LF, Ramos JG, Luque EH, Muñoz-de-Toro M & Kass L 2017 Perinatal Exposure to Bisphenol A or Diethylstilbestrol Increases the Susceptibility to Develop Mammary Gland Lesions After Estrogen Replacement Therapy in Middle-Aged Rats. Hormones and Cancer 8 78–89. (doi:10.1007/s12672-016-0282-1)

Goodson WH, Luciani MG, Sayeed SA, Jaffee IM, Moore DH & Dairkee SH 2011 Activation of the mTOR pathway by low levels of xenoestrogens in breast epithelial cells from high-risk women. Carcinogenesis 32 1724–1733. (doi:10.1093/carcin/bgr196)

Goulet CR & Pouliot F 2021 TGFβ Signaling in the Tumor Microenvironment. pp 89–105. (doi:10.1007/978-3-030-47189-7_6)

Gudjonsson T, Rønnov-jessen L, Villadsen R, Rank F, Mina J & Petersen OW 2002 Normal and tumor-derived myoepithelial cells differ in their ability to interact with luminal breast epithelial cells for polarity and basement membrane deposition. J Cell Sci. 115 39–50.

Hsiao YH, Su YA, Tsai H Der, Mason JT, Chou MC & Man YG 2010 Increased invasiveness and aggressiveness in breast epithelia with cytoplasmic p63 expression. International Journal of Biological Sciences 6 428–442. (doi:10.7150/ijbs.6.428)

Huang DY, Zheng CC, Pan Q, Wu SS, Su X, Li L, Wu JH & Sun ZY 2018 Oral exposure of low-dose bisphenol A promotes proliferation of dorsolateral prostate and induces epithelial-mesenchymal transition in aged rats. Scientific Reports 8 1–10. (doi:10.1038/s41598-017-18869-8)

Kim YS, Hwang KA, Hyun SH, Nam KH, Lee CK & Choi KC 2015 Bisphenol a and nonylphenol have the potential to stimulate the migration of ovarian cancer cells by inducing epithelial-mesenchymal transition via an estrogen receptor dependent pathway. Chemical Research in Toxicology 28 662–671. (doi:10.1021/tx500443p)

Kunz N, Camm EJ, Somm E, Lodygensky G, Darbre S, Aubert ML, Hüppi PS, Sizonenko SV & Gruetter R 2011 Developmental and metabolic brain alterations in rats exposed to bisphenol A during gestation and lactation. International Journal of Developmental Neuroscience 29 37–43. (doi:10.1016/j.ijdevneu.2010.09.009)

Lee SG, Kim JY, Chung JY, Kim YJ, Park JE, Oh S, Yoon YD, Yoo KS, Yoo YH & Kim JM 2013 Bisphenol a exposure during adulthood causes augmentation of follicular atresia and luteal regression by Decreasing 17β-estradiol synthesis via downregulation of aromatase in rat ovary. Environmental Health Perspectives 121 663–669. (doi:10.1289/ehp.1205823)

Lee HM, Hwang KA & Choi KC 2017 Diverse pathways of epithelial mesenchymal transition related with cancer progression and metastasis and potential effects of endocrine disrupting chemicals on epithelial mesenchymal transition process. Molecular and Cellular Endocrinology 457 103–113. (doi:10.1016/j.mce.2016.12.026)

Leonel ECR, Campos SGP, Bedolo CMB, Guerra LHA, Vilamaior PSL, Calmon MF, Rahal P, Amorim CA & Taboga SR 2020 Perinatal exposure to bisphenol A impacts in the mammary gland morphology of adult Mongolian gerbils. Experimental and Molecular Pathology 113 104374. (doi:10.1016/j.yexmp.2020.104374)

Leung YK, Biesiada J, Govindarajah V, Ying J, Kendler A, Medvedovic M & Ho SM 2020 Low-dose bisphenol a in a rat model of endometrial cancer: A clarity-bpa study. Environmental Health Perspectives 128 1–12. (doi:10.1289/EHP6875)

Li CMC, Shapiro H, Tsiobikas C, Selfors LM, Chen H, Rosenbluth J, Moore K, Gupta KP, Gray GK, Oren Y et al. 2020 Aging-Associated Alterations in Mammary Epithelia and Stroma Revealed by Single-Cell RNA Sequencing. Cell Reports 33 108566. (doi:10.1016/j.celrep.2020.108566)

Lorand T, Vigh E & Garai J 2010 Hormonal Action of Plant Derived and Anthropogenic Non-Steroidal Estrogenic Compounds: Phytoestrogens and Xenoestrogens. Current Medicinal Chemistry 17 3542–3574. (doi:10.2174/092986710792927813)

Lozada KW & Keri RA 2011 Bisphenol A increases mammary cancer risk in two distinct mouse models of breast cancer. Biology of Reproduction 85 490–497. (doi:10.1095/biolreprod.110.090431)

Lyons TR, O'Brien J, Borges VF, Conklin MW, Keely PJ, Eliceiri KW, Marusyk A, Tan AC & Schedin P 2011 Postpartum mammary gland involution drives progression of ductal carcinoma in situ through collagen and COX-2. Nature Medicine 17 1109–1116. (doi:10.1038/nm.2416)

Mallon E, Osin P, Nasiri N, Blain I, Howard B & Gusterson B 2000 The basic pathology of human breast cancer. Journal of Mammary Gland Biology and Neoplasia 5 139–163. (doi:10.1023/A:1026439204849)

Mandrup K, Boberg J, Isling LK, Christiansen S & Hass U 2016 Low-dose effects of bisphenol A on mammary gland development in rats. Andrology 4 673–683. (doi:10.1111/andr.12193)

Mao Y, Keller ET, Garfield DH, Shen K & Wang J 2013 Stromal cells in tumor microenvironment and breast cancer. Cancer and Metastasis Reviews 32 303–315. (doi:10.1007/s10555-012-9415-3)

Marchese S & Silva E 2012 Disruption of 3D MCF-12A Breast Cell Cultures by Estrogens - An In Vitro Model for ER-Mediated Changes Indicative of Hormonal Carcinogenesis. PLoS ONE 7 1–11. (doi:10.1371/journal.pone.0045767)

Marcus J, Bejerano-Sagie M, Patterson N, Bagchi S, Verkhusha V V., Connolly D, Goldberg GL, Golden A, Sharma VP, Condeelis J et al. 2019 Septin 9 isoforms promote tumorigenesis in mammary epithelial cells by increasing migration and ECM degradation through

metalloproteinase secretion at focal adhesions. Oncogene 38 5839–5859. (doi:10.1038/s41388-019-0844-0)

Mileva G, Baker SL, Konkle ATM & Bielajew C 2014 Bisphenol-A: epigenetic reprogramming and effects on reproduction and behavior. International Journal of Environmental Research and Public Health 11 7537–7561. (doi:10.3390/ijerph110707537)

Montévil M, Acevedo N, Schaeberle CM, Bharadwaj M, Fenton SE & Soto AM 2019 A combined morphometric and statistical approach to assess non-monotonicity in the developing mammary gland of rats in the CLARITY-BPA study. BioRxiv 2012 1–21. (doi:10.1101/783019)

Muraoka-Cook RS, Shin I, Yi JY, Easterly E, Barcellos-Hoff MH, Yingling JM, Zent R & Arteaga CL 2006 Activated type I TGFβ receptor kinase enhances the survival of mammary epithelial cells and accelerates tumor progression. Oncogene 25 3408–3423. (doi:10.1038/sj.onc.1208964)

Norris ML & Adams CE 1972 Incidence of cystic ovaries and reproductive performance in the mongolian gerbil, Meriones unguiculatus. Laboratory Animals 6 337–342. (doi:10.1258/002367772781006176)

Oh H, Bodelon C, Palakal M, Chatterjee N, Sherman ME, Linville L, Geller BM, Vacek PM, Weaver DL, Chicoine RE et al. 2016 Ages at menarche- and menopause-related genetic variants in relation to terminal duct lobular unit involution in normal breast tissue. Breast Cancer Research and Treatment 158 341–350. (doi:10.1007/s10549-016-3859-z)

Patel NA, Patel PS & Vora HH 2015 Role of PRL-3, Snail, Cytokeratin and Vimentin expression in epithelial mesenchymal transition in breast carcinoma. Breast Disease 35 113–127. (doi:10.3233/BD-140395)

Provenzano PP, Eliceiri KW, Campbell JM, Inman DR, White JG & Keely PJ 2006 Collagen reorganization at the tumor-stromal interface facilitates local invasion. BMC Medicine 4 1–15. (doi:10.1186/1741-7015-4-38)

Raafat A, Strizzi L, Lashin K, Ginsburg E, McCurdy D, Salomon D, Smith GH, Medina D & Callahan R 2012 Effects of Age and Parity on Mammary Gland Lesions and Progenitor Cells in the FVB/N-RC Mice. PLoS ONE 7 1–8. (doi:10.1371/journal.pone.0043624)

Radisky ES & Radisky DC 2015 Matrix metalloproteinases as breast cancer drivers and therapeutic targets. Frontiers in Bioscience - Landmark 20 1144–1163. (doi:10.2741/4364)

Radisky ES, Raeeszadeh-Sarmazdeh M & Radisky DC 2017 Therapeutic Potential of Matrix Metalloproteinase Inhibition in Breast Cancer. Journal of Cellular Biochemistry 118 3531–3548. (doi:10.1002/jcb.26185)

vom Saal FS & Vandenberg LN 2021 Update on the Health Effects of Bisphenol A: Overwhelming Evidence of Harm. Endocrinology 162 1–25. (doi:10.1210/endocr/bqaa171)

Schedin P 2006 Pregnancy-associated breast cancer and metastasis. Nature Reviews Cancer 6 281–291. (doi:10.1038/nrc1839)

Seachrist DD, Bonk KW, Ho SM, Prins GS, Soto AM & Keri RA 2016 A review of the carcinogenic potential of bisphenol A. Reproductive Toxicology 59 167–182. (doi:10.1016/j.reprotox.2015.09.006)

Shafei A, Ramzy MM, Hegazy AI, Husseny AK, EL-hadary UG, Taha MM & Mosa AA 2018a The molecular mechanisms of action of the endocrine disrupting chemical bisphenol A in the development of cancer. Gene 647 235–243. (doi:10.1016/j.gene.2018.01.016)

Shafei A, Matbouly M, Mostafa E, Al Sannat S, Abdelrahman M, Lewis B, Muhammad B, Mohamed S & Mostafa RM 2018b Stop eating plastic, molecular signaling of bisphenol A in breast cancer. Environmental Science and Pollution Research 25 23624–23630. (doi:10.1007/s11356-018-2540-y)

Silberstein GB, Strickland P, Coleman S & Daniel CW 1990 Epithelium-dependent extracellular matrix synthesis in transforming growth factor-β1-growth-inhibited mouse mammary gland. Journal of Cell Biology 110 2209–2219. (doi:10.1083/jcb.110.6.2209)

Slepicka PF, Cyrill SL & dos Santos CO 2019 Pregnancy and Breast Cancer: Pathways to Understand Risk and Prevention. Trends in Molecular Medicine 25 866–881. (doi:10.1016/j.molmed.2019.06.003)

Soriano S, Gil-Rivera M, Marroqui L, Alonso-Magdalena P, Fuentes E, Gustafsson JA, Nadal A & Martinez-Pinna J 2019 Bisphenol A Regulates Sodium Ramp Currents in Mouse Dorsal Root Ganglion Neurons and Increases Nociception. Scientific Reports 9 1–14. (doi:10.1038/s41598-019-46769-6)

Soto AM, Brisken C, Schaeberle C & Sonnenschein C 2013 Does cancer start in the womb? Altered mammary gland development and predisposition to breast cancer due to in utero exposure to endocrine disruptors. Journal of Mammary Gland Biology and Neoplasia 18 199–208. (doi:10.1007/s10911-013-9293-5)

Sprague BL, Trentham-Dietz A, Hedman CJ, Wang J, Hemming JDC, Hampton JM, Buist DSM, Aiello Bowles EJ, Sisney GS & Burnside ES 2013 Circulating serum xenoestrogens and mammographic breast density. Breast Cancer Research 15. (doi:10.1186/bcr3432)

Sundqvist A, Vasilaki E, Voytyuk O, Bai Y, Morikawa M, Moustakas A, Miyazono K, Heldin CH, ten Dijke P & van Dam H 2020 TGFβ and EGF signaling orchestrates the AP-1- and p63 transcriptional regulation of breast cancer invasiveness. Oncogene 39 4436–4449. (doi:10.1038/s41388-020-1299-z)

Tao L, Huang G, Song H, Chen Y & Chen L 2017 Cancer associated fibroblasts: An essential role in the tumor microenvironment (review). Oncology Letters 14 2611–2620. (doi:10.3892/ol.2017.6497)

Terry MB, Michels KB, Brody JG, Byrne C, Chen S, Jerry DJ, Malecki KMC, Martin MB, Miller RL, Neuhausen SL et al. 2019 Environmental exposures during windows of susceptibility for breast cancer: A framework for prevention research. Breast Cancer Research 21 1–16. (doi:10.1186/s13058-019-1168-2)

Vandenberg LN, Colborn T, Hayes TB, Heindel JJ, Jacobs DR, Lee DH, Shioda T, Soto AM, vom Saal FS, Welshons W V. et al. 2012 Epithelium-dependent extracellular matrix synthesis in transforming growth factor-β1-growth-inhibited mouse mammary gland. Cancer Prevention Research 13 370–378. (doi:10.1101/783019)

Vandenberg LN, Hunt PA & Gore AC 2019 Endocrine disruptors and the future of toxicology testing — lessons from CLARITY–BPA. Nature Reviews Endocrinology 15 366–374. (doi:10.1038/s41574-019-0173-y)

Vincent AL, Rodrick GE & Sodeman WA 1980 The mongolian gerbil in aging research. Experimental Aging Research 6 249–260. (doi:10.1080/03610738008258361)

Wadia PR, Vandenberg LN, Schaeberle CM, Rubin BS, Sonnenschein C & Soto AM 2007 Perinatal bisphenol A exposure increases estrogen sensitivity of the mammary gland in diverse mouse strains. Environmental Health Perspectives 115 592–598. (doi:10.1289/ehp.9640)

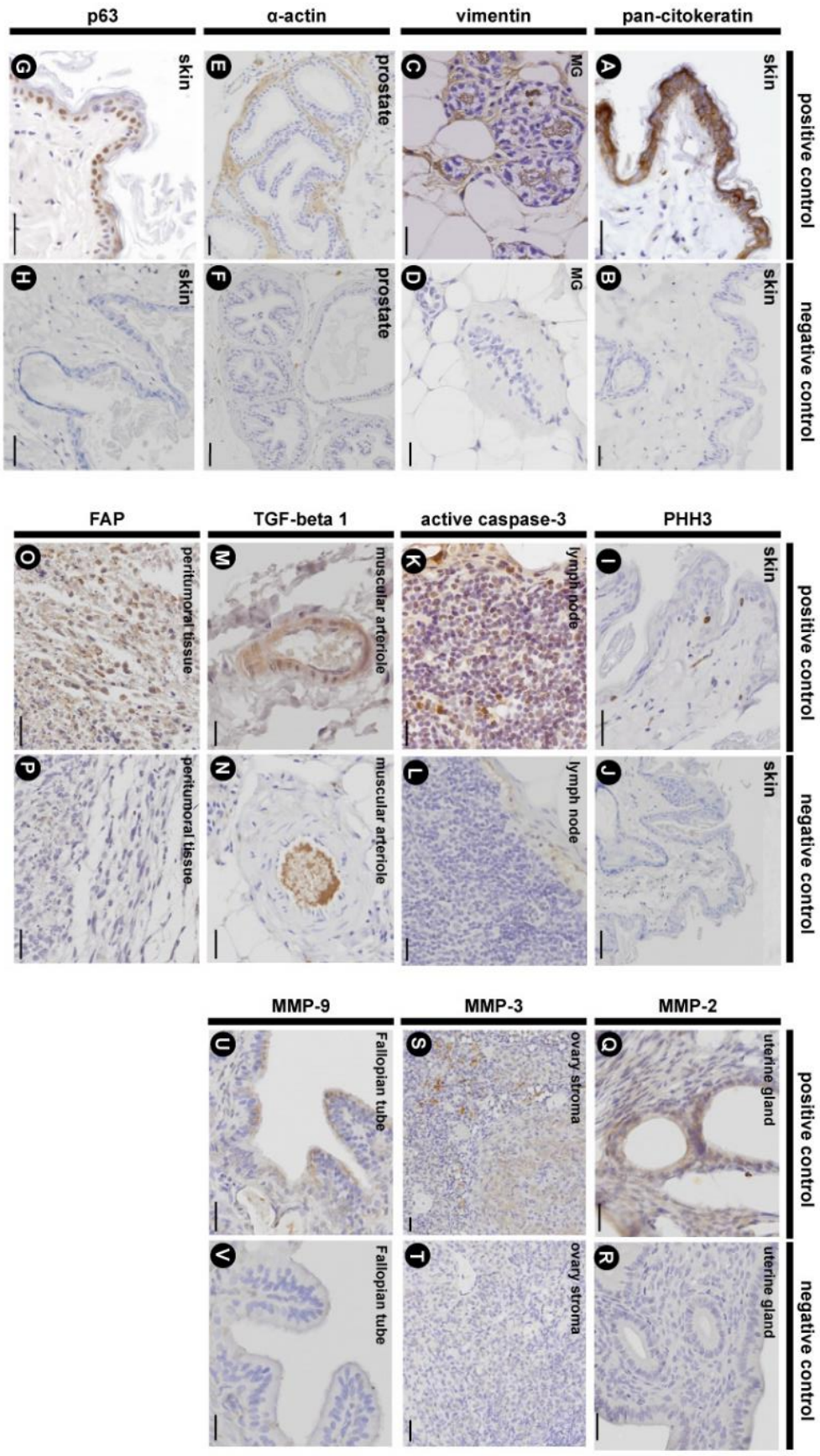
Wormsbaecher C, Hindman AR, Avendano A, Cortes-Medina M, Jones CE, Bushman A, Onua L, Kovalchin CE, Murphy AR, Helber HL et al. 2020 In utero estrogenic endocrine disruption alters the stroma to increase extracellular matrix density and mammary gland stiffness. Breast Cancer Research 22 1–12. (doi:10.1186/s13058-020-01275-w)

Yoshida M, Shimomoto T, Katashima S, Watanabe G, Taya K & Maekawa A 2004 Maternal exposure to low doses of bisphenol A has no effects on development of female reproductive tract and uterine carcinogenesis in Donryu rats. Journal of Reproduction and Development 50 349–360. (doi:10.1262/jrd.50.349)

Zhang M, Lee A V. & Rosen JM 2017 The cellular origin and evolution of breast cancer. Cold Spring Harbor Perspectives in Medicine 7 1–14. (doi:10.1101/cshperspect.a027128)

Zhang J, Thorikay M, van der Zon G, van Dinther M & Ten Dijke P 2020 Studying TGF-β Signaling and TGF-β-induced Epithelial-to-mesenchymal Transition in Breast Cancer and Normal Cells. Journal of Visualized Experiments : JoVE. (doi:10.3791/61830)

Material Suplementar 1



Supplementary Figure 1. Positive and negative controls for immunohistochemical reactions in Mongolian gerbils tissues. Pan-cytokeratin: (A, B) skin. Vimentin: (C, D) mammary gland. α-SMA: (E, F) prostate. p63: (G, H) skin. Phospho-histone H3: (I, J) skin. Active caspase-3: (K, L) lymph node. TGF-β1: (M, N) muscular arteriole. FAP: (O, P) peritumoral mammary tissue. MMP-2: (Q, R) uterine gland. MMP-3: (S, T) ovary stroma. MMP-9: (U, V) Fallopian tube. Scale bars: 20 µm. (*Mammary carcinoma in aged gerbil mothers after endocrine disruption in pregnancy and lactation*. Authors: TFR Ruiz, SJ Colleta, ECR Leonel, SR Taboga).

4.2. Capítulo 2: Hormone receptor expression in aging mammary tissue and carcinoma from a rodent model after xenoestrogen disruption

Revista *Life Sciences* (IF: 5.037)

Life Sciences 285 (2021) 120010

Contents lists available at ScienceDirect

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Hormone receptor expression in aging mammary tissue and carcinoma from a rodent model after xenoestrogen disruption

Thalles Fernando Rocha Ruiz [a,*], Simone Jacovaci Colleta [a], Débora Aparecida Pires de Campos Zuccari [b], Patrícia Simone Leite Vilamaior [a], Ellen Cristina Rivas Leonel [c], Sebastião Roberto Taboga [a,**]

[a] Department of Biology, Institute of Biosciences, Humanities and Exact Sciences, São Paulo State University (UNESP), Rua Cristóvão Colombo 2265, Jardim Nazareth, 15054-000 São José do Rio Preto, São Paulo, Brazil
[b] Laboratory of Molecular Investigation of Cancer (LIMC), Avenue Brigadeiro Faria Lima, 5416, Vila Sao Pedro, Sao Jose do Rio Preto, Sao Paulo 15090-000, Brazil
[c] Department of Histology, Embryology and Cell Biology, Institute of Biological Sciences (ICB III), Federal University of Goiás (UFG), Avenida Esperança, s/n, Campus Samambaia, 74001-970 Goiânia, Goiás, Brazil

Abstract

Aims: Hormone receptors are the main markers applied for prognosis of breast cancer subtypes. Among modulators, exogenous chemical agents known as endocrine disruptors interact with certain receptors, triggering molecular pathways or increasing their expression. Bisphenol A (BPA), a xenoestrogen, interacts with several hormone receptors. Thus, our aim was to characterize the hormone receptor status in the mammary gland (MG) of aged female Mongolian gerbils exposed to BPA in pregnancy and lactation.

Methods: We evaluated the expression of receptors for estrogens (ERα and ERβ), progesterone (PR), prolactin (PRL-R), HER2/ErbB2, and androgen (AR) in normal and hyperplastic mammary tissue and in carcinomas developed after BPA exposure.

Key findings: BPA-exposed MG presented increased ERα, whereas ERβ, PR, and PRL-R showed lower expression. AR and HER2/ErbB2 showed similar expression in normal and hyperplastic tissue from control, vehicle, and BPA groups. Both receptors were found in cytoplasm and nucleus in BPA-induced carcinoma. We demonstrate the presence of EZH2 expression, an epigenetic and epithelial-mesenchymal transition (EMT) marker, with a high H-score in BPA-exposed MG, which was associated with poor prognosis of cancer. Co-localization of ERα and EZH2 was present in normal and

carcinoma features, corroborating the installation of ERα-positive mammary cancer associated with the EMT process. Enhanced EZH2 in BPA-exposed mammary tissue could decrease ERβ expression and promote tumorigenesis progress through HER2/ErbB2.

Significance: The present study proposes the Mongolian gerbil as an experimental model for mammary carcinogenesis studies, based on BPA disruption that triggers a phenotype of increased ERα/HER2 positivity and depletion of ERβ/PR expression.

Keywords: Bisphenol A, Cancer marker, Mongolian gerbil, Morphology, Pathology

Introduction

The receptor-hormone binding signal is the base of hormone-responsive organs, such as the prostate and breast (Rodriguez-Gonzalez et al. 2008; Higa & Fell 2013; Levin & Hammes 2016). This signal alters molecular pathways that could accelerate pathological processes of cancer installation (Dhiman et al. 2018). The mammary gland is one of the most susceptible organs to hormonal regulation (Sternlicht et al. 2006; Brisken, Cathrin; O'Malley 2010; Brisken & Ataca 2015). Diagnosis of neoplastic lesions in this tissue is commonly based on the expression of key markers, such as estrogen receptors (ERα and ERβ), progesterone receptors (PR), and human epidermal growth factor receptor 2 (HER2/ErbB2) (Shah et al. 2014; Levin & Hammes 2016; Waks & Winer 2019). Variations in the expression of these receptors in mammary gland neoplastic lesions are related to impacts in tissue homeostasis through proliferative activity (Reis-Filho & Pusztai 2011) and changes in signaling for establishment of a tumor microenvironment t (Dhiman et al. 2018). Specifically, a decline or absence of these three receptors characterizes a triple negative (ER-/PR-/HER2-) tumor, which is one of the most aggressive types of breast cancers (Abramson et al. 2015). A new tumor subtype (quadruple negative) has been considered based on the absence of androgen receptor (AR) expression (Mina et al. 2017; Huang et al. 2020), which has a relevant role in healthy tissue (Wang et al. 2014a) and breast cancer (Caswell-Jin & Curtis 2021). Thus, the decreased expression of hormone receptors in neoplastic mammary tissue represents a poor prognosis for breast cancer, indicating low responsiveness to hormonal therapies (Aloisi et al. 2001; Thomas & Gustafsson 2015; Ma et al. 2017).

Recently, efforts have been made to describe an effective relationship between compounds known as endocrine disruptors (ED) and breast cancer (Seachrist et al.

2016; Terry et al. 2019; Vandenberg et al. 2019). EDs act by pathways that mimic or modulate the action of hormones and factors (Toppari 2008; Rodgers et al. 2018) to develop carcinogenesis. Among them, xenoestrogens have great relevance because they act mainly through ER binding (Viñas et al. 2012). However, bisphenol A (BPA), a ubiquitous xenoestrogen, acts by interacting with several other hormone receptors for disruption (Shafei et al. 2018). Furthermore, studies have suggested that the receptor-related action of BPA goes beyond direct binding interaction: it further promotes expression or silencing of genes related to these receptors (Zhang et al. 2021), and consequently impacts the expression of these proteins (Bhan et al. 2014). Indeed, epigenetic changes have been linked to BPA, as strong evidence of the endocrine disruption of this compound that leads to tumorigenesis (Doherty et al. 2010). Among these epigenetic alteration pathways (Monteiro et al. 2020), methylation is one of the main mechanisms of BPA disruption (Singh & Li 2012; Mileva et al. 2014). Enhancer of zeste homolog 2 (EZH2) is a methyltransferase of histone H3 (in lysine 27) from the polycomb protein group (Holm et al. 2012). It largely modulates the expression of genes that encode relevant proteins for tumor progression (Li et al. 2009; Bae & Hennighausen 2014) including some linked to estrogen-like compounds (Greathouse et al. 2012; Bhan et al. 2014). This modulation is particularly important when exposure to BPA occurs during the perinatal development susceptibility window (Doherty et al. 2010; Leonel et al. 2020a).

For better understanding of these proliferative lesion mechanisms the establishment of new rodent species as experimental models is relevant. The hypothesis that the Mongolian gerbil (Meriones unguiculatus) is an adequate model for these experiments is based on previous data and consistent evidence from our research group, with a focus on prostatic and mammary tissues (Campos et al. 2008; Custodio et al. 2010; Leonel et al. 2020a). Thus, we present analysis of the hormone receptor expression patterns in normal and hyperplastic mammary gland and in carcinoma induced by xenoestrogen BPA, allied with evaluation of the epigenetic marker EZH2. For this, the object of study was the late repercussions of BPA in females exposed during phases of major mammary morphological changes: pregnancy and lactation.

Materials and Methods

Experiments and ethics

The present study was conducted at the Animal Breeding Center of the Institute of Biosciences, Humanities and Exact Sciences (IBILCE, UNESP, São Paulo). Twenty female Mongolian gerbils, 3 months of age, were bred with fertile males. After birth the first litter was discarded in order to induce a second pregnancy in females. Eight days later, the females were divided into 4 experimental groups: control (gavage, water), vehicle (gavage, corn oil vehicle), ↓BPA (gavage, 50 µg/kg of BPA), and ↑BPA (gavage, 5000 µg/kg of BPA). The BPA dosage in the ↓BPA group reflects the daily safe exposure as considered by the US EPA (Soriano et al. 2019) and EFSA (EFSA 2015), while the ↑BPA group depicts overexposure to the compound. The pregnant females were subjected to gavage daily, from the 8th day of gestation until the end of lactation, comprising 39 days of exposure. After weaning the mothers were kept in polysulfone isolators with water and balanced food ad libitum until 18 months of age, characterizing an aging period. Euthanasia was performed in animals with the exclusive presence of cornified cells in the vaginal smear, performed to standardize the hormonal status among animals of the experiment, since this impacts on the mammary tissue morphology. The abdominal mammary glands were removed and fixed whole in 4% paraformaldehyde for 24 hours, and then embedded in paraffin in a Leica Semi Enclosed System (TP1020, Leica Biosystems).

The experiment followed the standards and protocols for animal experimentation of the National Council of Control and Animal Experimentation (CONCEA, Brazil) and was approved by the local Ethics Committee on the Use of Animals (CEUA, IBILCE, UNESP), number 217/2019.

Immunohistochemistry procedures and analysis

The fixed samples were sectioned at 4 µm thick and placed on silanized slides. They were then assigned to routine immunohistochemistry (IHC). Slides were deparaffinized and hydrated and before being subjected to antigen retrieval in 10 mM citrate buffer (97ºC) for 40 minutes. Subsequently, sections were destined to peroxidase blockage in H2O2 5% diluted in methanol for 20 minutes. Blocking of non-specific proteins was performed in 10% bovine serum albumin for 30 minutes. Antibody incubation was performed overnight with the following primary antibodies: estrogen receptors – anti-ERα (mouse monoclonal, sc-8005, 1:50, Santa Cruz Biotechnology) and anti-ERβ (rabbit polyclonal, PA1-310B, 1:50, Invitrogen, ThermoFisher), progesterone receptor – anti-PR (mouse monoclonal, 1:50, GTX22765, GeneTex),

anti-HER2/ErbB2 (mouse monoclonal, 3B5, ab16901, 1:75, Abcam), prolactin receptor – anti-PRL -R (rabbit monoclonal, D4A9, 1:100, Cell Signaling Technology), androgen receptor – anti-AR (mouse monoclonal, sc-7305, 1:75, Santa Cruz Biotechnology), and enhancer of zeste homolog 2 – anti-EZH2 (rabbit monoclonal, D2C9, 1:75, Cell Signaling Technology). The slides were then incubated with post-primary antibody and polymer (Novolink™ polymer detection system 1, Leica Biosystems Newcastle 118 Ltd., Newcastle, United Kingdom). Steps were interspersed with PBS or TBS wash buffers. Positive staining was detected by DAB chromogen (3-30'-diaminobenzidine tetrahydrochloride, NovolinkTM DAB, RE7270-CE, Leica Biosystems, Buffalo 121 Grove, USA) and counterstaining was performed with hematoxylin.

For analyses of immunohistochemical expression of ERα, ERβ, PR, and AR in the mammary gland, 15 microscopy fields were randomly selected for each animal (n = 5 per group). Incidence of the following structures with different morphological patterns was quantified: normal alveoli (1 or 2 layers of epithelial cells), hyperplastic foci (alveoli with more than 3 layers of epithelial cells), and carcinomas/neoplasia (regions with no lumen, showing non-polarized cells and with no basal delineation). Cells showing positive staining for each marker were counted, and the percentage of positive epithelial cells in relation to the total number of epithelial cells in the microscopic field was provided. These automated analyses were performed in QuPath software (Version 0.1.2, an open-source pathology software platform). In addition, the incidence of EZH2 expression was evaluated in entire tissue sections (cells/mm^2) and the H-score of EZH2 immunostaining was taken into account for quantifying the intensity of labeling in cells throughout the section, as described by Vougiouklakis et al. (2020). Both analyses were performed and standardized by QuPath software.

Immunofluorescence assay

Immunofluorescence was performed to detect localization of HER2/ErbB2 and AR, and co-localization of ERα and EZH2. Antigen retrieval and nonspecific protein blockage were performed as stated above for IHC. Primary antibody incubation was performed overnight (4ºC). Incubation was then carried out with the following specific fluorochrome conjugated secondary antibodies: anti-mouse FITC (sc-2010, 1:100, Santa Cruz Biotechnology, USA) and anti-rabbit Texas Red (sc-2780, 1:100, Santa Cruz Biotechnology, USA), for 1 hour at room temperature. The slides were stained and mounted with DAPI (Fluoroshield™ with DAPI, F6057, histology mounting

medium, Merck, Darmstadt, Germany) for nuclear fluorescence. The sections were analyzed with a Zeiss AX10 Fluorescence Microscope (Zeiss, Oberkochen, Germany) coupled to AxioVision (Zeiss) software.

Statistical analysis

The analysis of data normality was performed by the Kolmogorov-Smirnov test. Data of ERα, PR, and EZH2 (parametric data) were analyzed by one-way ANOVA followed by Tukey's test. For ERβ and AR (non-parametric data), the Kruskal-Wallis followed by Dunn's tests were applied. $P < 0.05$ value was considered for statistically significant differences between groups. The statistical analyses were performed in GraphPad Prism 5.00 software for Windows (GraphPad Software, San Diego, CA, USA, www.graphpad.com).

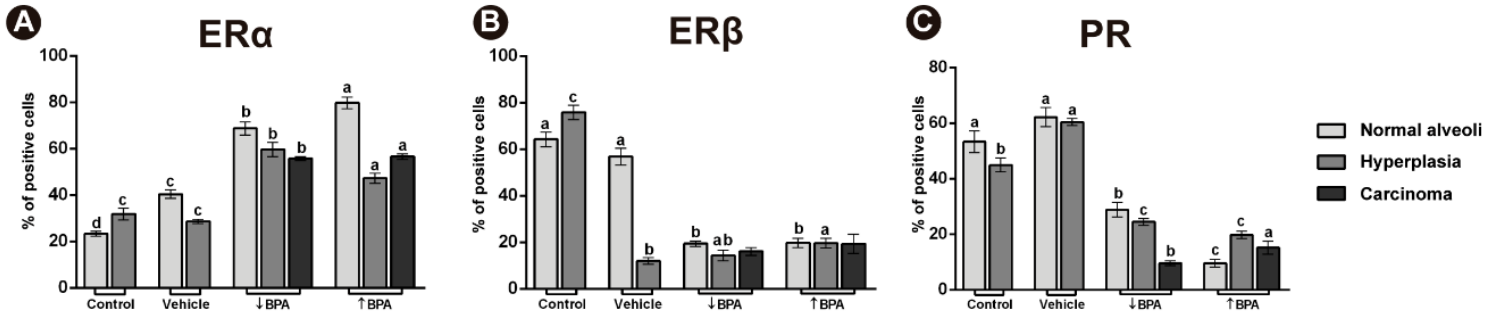

Figure 1. Incidence of ERα, ERβ, and PR expression (percentages) in normal and hyperplastic tissues and carcinoma regions of mammary gland exposed to BPA. (A) ERα; (B) ERβ; (C) PR. Carcinomas were absent in mammary glands from control and vehicle groups. Graphical values are expressed as mean ± SEM and different letters (a, b and c) indicate significant differences among treatment groups in each morphological pattern (normal, hyperplasic or carcinoma) (A, C: ANOVA test followed by Tukey's test; B: Kruskal-Wallis test followed by Dunn's test).

Results

ERα, ERβ and PR expression

The expression of ERα, ERβ, and PR was quantified in epithelial cells of normal and hyperplastic tissue, and in multifocal carcinoma features in mammary gland. The percentages of cells expressing these receptors over the total number of epithelial cells are shown in Fig. 1 (A) for ERα, Fig. 1 (B) for ERβ, and Fig. 1 (C) for PR, where different letters (a, b and c) indicate significant differences among treatment groups in normal, hyperplasic or carcinoma patterns. In addition, Fig. 2 presents the nuclear expression

of ERα in Fig. 2(A-E), ERβ in Fig. 2(F-J), and PR in Fig. 2 (K-O), in normal, hyperplastic, and carcinoma structures. Control and vehicle groups presented the lowest values of ERα-positive cells (Fig. 2(A-B)) in both normal and hyperplastic tissues; the ↓BPA group presented the highest percentage in hyperplasia (59.67±3.11) and ↑BPA showed the highest rates in normal alveoli (79.81±2.52) (Fig. 2(C-D), respectively). In BPA-induced carcinoma > 50% of cells were ERα-positive in both groups (Fig. 2(E)).

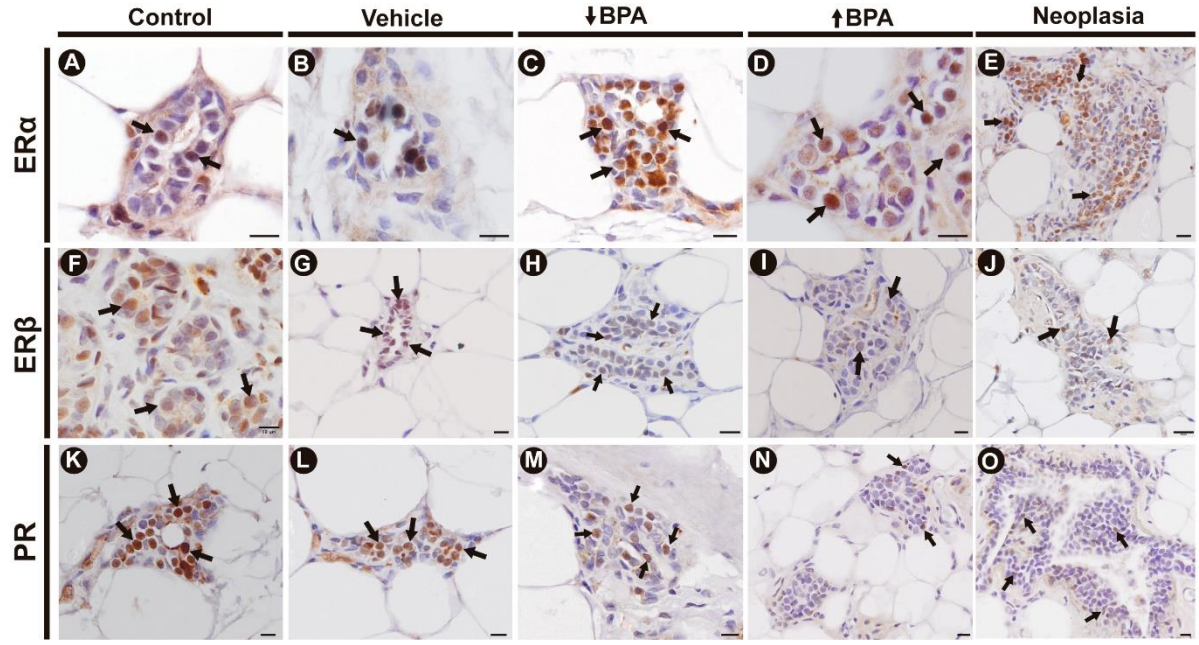

Figure 2. ERα, ERβ, and PR expression in mammary gland from different groups. (A-E) ERα. Normal epithelium in control and vehicle (A, B) presented low expression of ERα (arrows) in comparison to both BPA groups (C, D); this marker was also highly expressed in neoplastic structures disrupted by BPA (E). (F-J) ERβ. Nuclear expression (arrows) of ERβ was enhanced in normal alveoli of control and vehicle groups (F, G). However, its expression in ↓BPA (H) and ↑BPA (I) groups was rare. Also, neoplasia of BPA groups showed diminished ERβ expression (J). (K-O) PR. Expression of PR (arrows) was enhanced in control and vehicle (K, L), decreased in ↓BPA (M), and drastically reduced in ↑BPA (N) groups. Note sparse and reduced expression in neoplasia of BPA-exposed mammary gland. Scale Bars: (A, B, D, F) 10 µm; (C, G-O) 20 µm; (E) 30 µm.

ERβ and PR presented opposite patterns of expression in BPA groups in comparison to ERα. The percentages of ERβ and PR positive cells are shown in Fig. 1(B-C). ERβ was highly expressed in normal alveoli (Fig. 2(F)) and hyperplastic tissues from the control group (64.27±3.22 and 75.87±3.05, respectively), while its expression drastically decreased in tissues from BPA groups (Fig. 2(H-J)). Percentage of PR-positive cells also decreased in mammary gland of BPA groups (Fig. 1(C)). Normal

and hyperplasic structures in control and vehicle groups presented around 50% of epithelial cells expressing PR (Fig. 2(K-L)). However, in BPA groups (Fig. 2(M-N)), there was a lower rate of normal epithelium in ↑BPA compared to ↓BPA. Carcinomas from the ↓BPA group presented the lowest percentages of PR positive cells (Fig. 2(O)). These three receptors did not show significant expression in stromal cells.

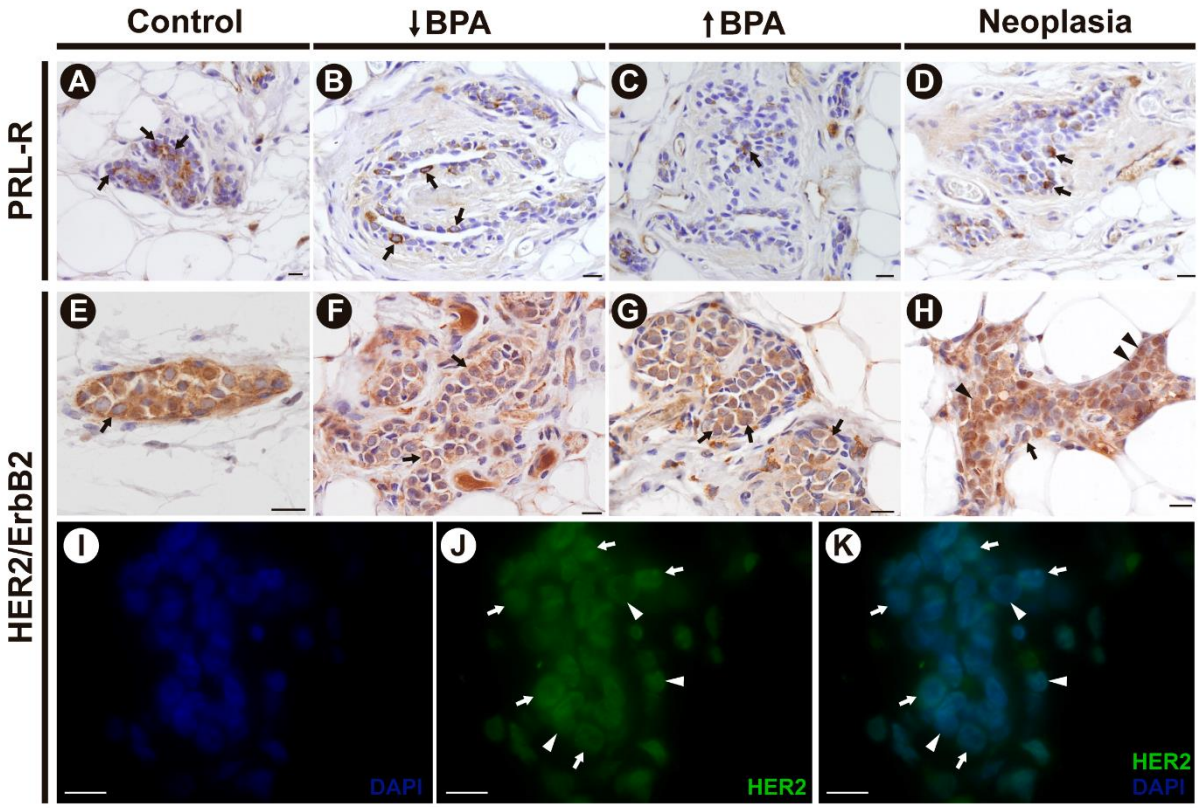

Figure 3. PRL-R and HER2/ErbB2 expression in BPA-disrupted mammary gland. (A-D) PRL-R. Note cytoplasmic expression (arrows) in control group in A, compared to scarce expression in normal alveoli of ↓BPA (B) and in hyperplasia of ↑BPA (C) groups, as in neoplastic regions (D). (E-K) HER2/ErbB2. Control group presented HER2/ErbB2 positivity in cytoplasm (arrows) of all epithelial compartment (A), as well as in ↑BPA and ↓BPA groups (F, G). However, in neoplastic structures of BPA group, nuclear (arrowheads) staining for HER2/ErbB2 was observed (H). Immunofluorescence assay enabled observation of the nuclear (arrowheads) and cytoplasmic (arrows) localization of HER2/ErbB2 that occurred in normal alveoli of BPA disrupted groups (I-K). Scale Bars: 20 µm.

PRL-R, HER2/ErbB2 and AR expression

The expression of PRL-R and HER2/ErbB2 showed a visible decrease in mammary tissue of BPA groups (Fig. 3). A diffuse cytoplasmic pattern of staining required the use of a particular qualitative method to quantify the expression of these receptors in the mammary tissue. Normal epithelium in the control group showed cytoplasmic localization of PRL-R (Fig. 3(A)). However, in normal (Fig. 3(B)) and

hyperplasic (Fig. 3(C)) alveoli from BPA groups, the PRL-R positive staining was scarce. A similar pattern was observed in neoplastic cells (Fig. 3(D)). HER2/ErbB2 expression was similar in normal and hyperplasic structures from all groups (Fig. 3(E-G)). However, in multifocal neoplasia from BPA groups, cytoplasmic and nuclear expression of HER2/ErbB2 were observed (Fig. 3(H)), which was an uncommon feature in normal epithelium from the control group (Fig. 3(E)), but often observed in disrupted and normal alveoli from BPA groups (Fig. 3(F-G)). These aspects were confirmed by immunofluorescence (Fig. 3(I-K)).

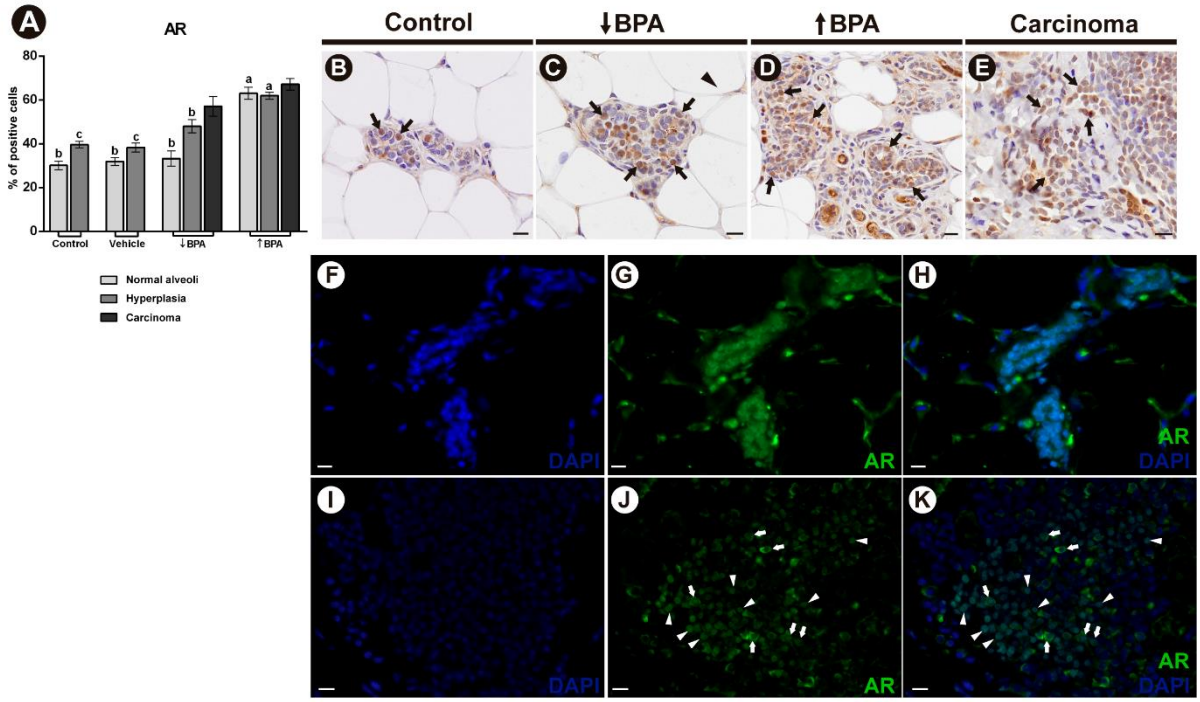

Figure 4. AR expression and localization in mammary gland. (A) Incidence (percentage) of AR positive cells. Graphical values are expressed as mean ± SEM and different letters (a, b and c) indicate significant differences among treatment groups in each morphological pattern (normal, hyperplasic or carcinoma) (ANOVA test followed by Tukey's test). (B-E) AR expression (arrows) in mammary gland tissue: lower in normal alveoli of control group (B), enhanced in hyperplasia of ↓BPA (C) and in normal tissue and hyperplasia of ↑BPA (D). Neoplasia/carcinoma regions presented high expression of AR (arrows). (F-K) Localization of AR in BPA groups. Normal alveoli of ↑BPA group presented high expression in nucleus (arrowheads) and cytoplasm (arrows) of epithelial cells (F-H), as well as in carcinoma of ↓BPA group (I-K). Scale Bars: 20 µm.

The percentages of AR-positive cells among groups quantified in epithelial compartments from normal and hyperplastic glands and carcinoma are shown in Fig. 4 (A, where a, b and c indicate significant differences among treatment groups in

normal, hyperplasic or carcinoma patterns). Control and vehicle groups presented a lower incidence of nuclear staining for AR (Fig. 4(B)). Hyperplastic structures presented more than 50% of AR-positivity staining in the ↓BPA group (Fig. 4(C)). The highest rates were observed in the ↑BPA group in both normal and hyperplasic epithelium (Fig. 4(D)), with statistically different percentages from other groups. Carcinomas presented the highest percentages of AR-positive cells in each BPA exposed group (Fig. 4(E)). AR expression was observed in stromal cells among normal and hyperplastic alveoli in BPA groups (Fig. 4(D)). Furthermore, in the immunofluorescence assay it was possible to observe the cytoplasmic and nuclear localization of AR in normal tissue (Fig. 4(F-H)) and carcinoma regions (Fig. 4(I-K)) from BPA groups.

EZH2 expression

The EZH2 H-score expression is shown in Fig. 5(A). In control and vehicle groups (Fig. 5(B-C)), mammary tissue presented a low incidence of EZH2 positive cells (1.56±0.18 and 1.80 ±0.23 cells/mm^2, respectively). Normal mammary tissue and carcinoma from BPA groups presented enhanced positivity for EZH2 (↓BPA: 41.12±3.29 and ↑BPA: 43.62±4.17 cells/ mm^2) (Fig. 5(D-F)). Immunoreactive intensity (Fig. 5(D, F)) was stronger in BPA groups, which presented elevated H-scores (↓BPA: 97.44±5.42 and ↑BPA: 103.7±7.51) in comparison with control and vehicle groups (control: 4.68±0.26 and vehicle: 6.41±0.62). In BPA groups, nuclear co-localization of ERα and EZH2 were observed in normal alveoli (Fig. 5(G-J)) and in carcinoma regions (Fig. 5(K-N)).

Discussion

The present study describes the hormone receptor standards in the tumorigenic process induced by BPA in the mammary gland of aged female gerbils. ERα expression was more frequent than PR and ERβ in carcinomas and in normal and hyperplastic structures from BPA exposed individuals. This demonstrates that the multifocal carcinoma developed in response to this xenoestrogen tends to be ERα-positive, similarly to what is most commonly described in breast cancer (Najim et al. 2019). We also emphasize the positivity of BPA-induced neoplastic regions for HER2/ErbB2, as these receptors are applied in the clinical diagnosis, for

understanding the malignant and metastatic status that may occur (Sharp & Harper-Wynne 2014; Makki 2015).

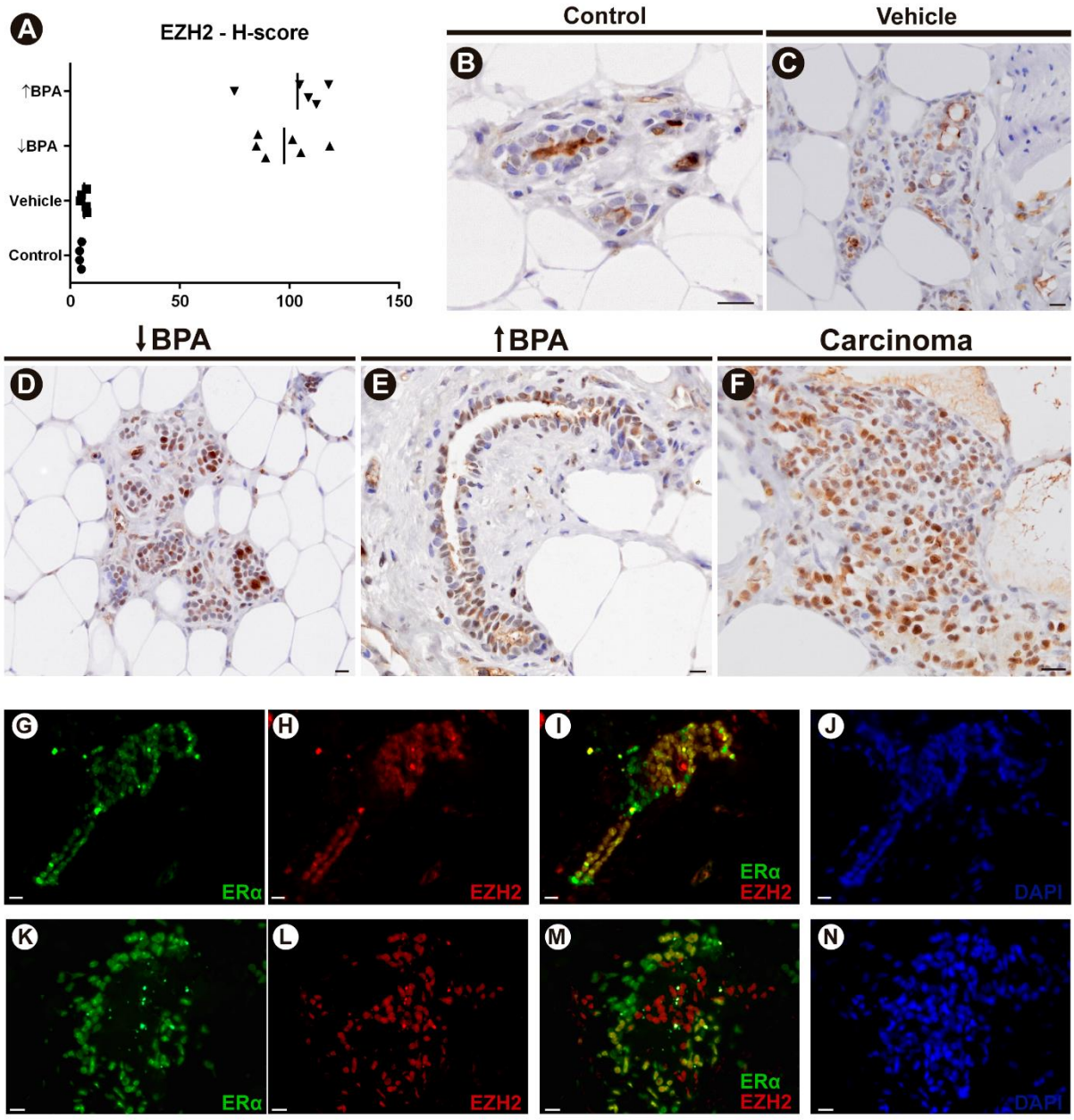

Figure 5. EZH2 expression. (A) H-score of EZH2. Both BPA groups presented high indices of H-score. (B-F) IHC for EZH2. (B) Control and (C) vehicle groups presented less EZH2 staining in mammary gland tissue. (D-E) Normal and hyperplasic alveoli presented heterogenous stain intensity for EZH2, reflecting in the H-score of these groups. (F) Carcinoma/neoplasia showed EZH2 positivity in almost every cell in different intensities. (G-N) Immunofluorescence assay for co-localization (yellow) of ERα (green) and EZH2 (red). (J and N) Shows the total nuclei in the same section with DAPI. Scale Bars: 20 µm.

According to previous descriptions, BPA presents molecular interaction with other hormone receptors, such as PR and AR (Ayyanan et al. 2011; Acconcia et al.

2015). Furthermore, an epigenetic action is assigned to BPA-disruptive processes that lead to tumor installation (Bromer et al. 2010; Doherty et al. 2010; Nair et al. 2020). In this aspect, BPA interacts with ERα-promoting regions through DNA methylation, increasing its expression in cells (La Rocca et al. 2014) and making it persistent (Doshi et al. 2011). It also increases ERα translocation to the nucleus (Weng et al. 2010). This epigenetic imprinting (Weng et al. 2010) was observed by the marking and incidence of EZH2, an epigenetic marker (Perrot-Applanat et al. 2018) in the mammary tissue. Furthermore, we suggest that this mechanism may have been a major candidate for the installation of ERα+ cancer in mothers exposed to both BPA dosages, shown in Fig. 6-1. Clinically, EZH2 overexpression is related to the increased incidence of hyperplasia and alveoli disarray (Li et al. 2009) in addition to chemotherapy resistance (Reid et al. 2021). H-score values were also increased in the BPA exposed animals, defining a pathological status of neoplasia (Vougiouklakis et al. 2020). The action of EZH2 impacts the protein machinery of normal cells during BPA endocrine disruption due to its methyltransferase activity, causing hypomethylation of histone H3 (Fig. 6-1) (Doherty et al. 2010; Singh & Li 2012). This EZH2 mechanism acts on cell signaling pathways linked to the estrogen receptor and its coregulatory elements (Greathouse et al. 2012; Bhan et al. 2014), being an attenuating factor for the progression of ERα positive carcinomas, as observed in the present study. This crosstalk between ERα and EZH2 may be related to the EMT phenotype observed in neoplastic cells disrupted by BPA (Fig. 6-2), since EZH2 is an inducer of this process (Tiwari et al. 2013; Tian & Schiemann 2017; Feng et al. 2019).

BPA action is based on the disruption of epigenetic relationships in mammary gland (dos Santos et al. 2015), leading to the hypothesis that these changes could be the reason for ER positivity in mammary carcinoma. BPA disruption is not only related to the onset, but also to the progression of mammary cancer, since this ED alters more than 170 breast cancer-related genes (Weng et al. 2010), some of them linked to HER2/ErbB2 signaling (Weng et al. 2010), and the main pathways are triggered by ERα (Jorgensen et al. 2016) (Fig. 6-2). In general, the alterations caused by BPA are selective to pathways related to genes under ERα transcriptional regulation (Jorgensen et al. 2016), associated with apoptosis inhibition (Sengupta et al. 2013) and EMT process induction (Atlas & Dimitrova 2019; Segovia-mendoza et al. 2020), as observed in the present study (Fig. 6-3).

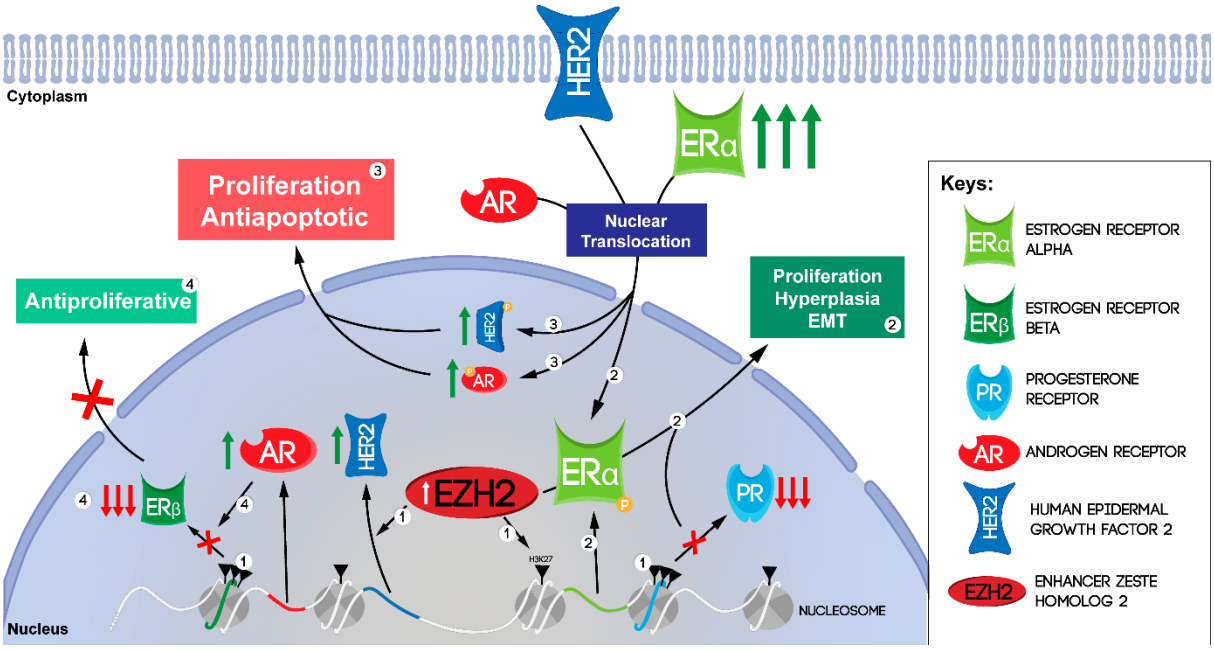

Figure 6. Role of hormonal receptor expression in aged gerbil mammary gland under BPA disruption. (1) EZH2 mediated BPA disruption: EZH2 overexpression indicates the enhanced methylation of histone H3 in (H3K27). This contributes to increasing (green arrows) or decreasing (red arrows) expression of hormonal receptors – modifying HER2/ErbB2 expression. (2) Hyperplasia/EMT process: in the BPA exposed mammary gland, EZH2 expression promotes increased ERα expression and its nuclear translocation. ERα overexpression increases molecular pathways of proliferation causing hyperplasia and the EMT process in disrupted cells. Also, low expression of PR contributes to proliferative disorders in mammary tissue. (3) Proliferative/Antiapoptotic action: BPA increases the translocation of AR and HER2/ErbB2 to the nucleus, which activates/inhibits cell proliferative/antiapoptotic pathways. (4) Deregulation in proliferative pathways: BPA reduces ERβ positive cells, impacting in the AR expression and, consequently, blocking the antiproliferative control for tissue homeostasis. Antiproliferative action: BPA promotes decreased expression and increases AR expression which contributes to the decrease in ERβ, and consequently the antiproliferative process.

Endocrine disruption by BPA led to the development of mammary carcinoma in aged female gerbils with PR, ERβ, and PRL-R loss of expression. This suggests that BPA modulates the response to certain hormones through depletion or exacerbation of hormonal-specific response, such as proliferation and expression of matrix remodeling proteins (Jorgensen et al. 2016). Under normal conditions, PR expression is mediated by ERα through the induction of estrogens (Feng et al. 2007), which was not observed in the present study. This demonstrates a pathway selectivity associated with BPA disruption in the expression of genes modulated by ERα (Aldad et al. 2011), as found in ER+ breast cancer stem-like cells (Lillo et al. 2017). Changes in PR expression modulate molecular pathways preferentially activated by the progesterone-

PR binding signal (Mohammed et al. 2015; Thomas & Gustafsson 2015) (Fig. 6-1). This promotes a molecular crosstalk with ERα, decreasing estrogenic responses in neoplastic cells (Kabos et al. 2012; Thomas & Gustafsson 2015). Among these signs, the main progesterone pathway in mammary gland is the establishment of a distinct alveolar structure and differentiation of the terminal end buds during estrous/menstrual cycles (Haslam & Shyamala 1979; Macias & Hinck 2012). The absence of PR in breast cancer is associated with the progression of an irreversible state determined by the EMT process (Fig. 6-4), associated with an invasive phenotype (Obr & Edwards 2012) and poor prognosis (Blows et al. 2010). Furthermore, in the present study the loss of PR enabled the classification of the developing cancer as luminal B (Cancello et al. 2013), which is positive for ER and HER2/ErbB2.

Regarding ERs, the present study demonstrated that even in regions with normal and hyperplastic alveoli, expressive depletion in ERβ in comparison to ERα was observed. As previously mentioned, BPA acts selectively through induction pathways linked to ERα, which are maintained through endocrine disruption. Differently, ERβ is associated with antiproliferative pathways (Jia et al. 2015). We observed a higher incidence of ERβ expression in the control group, probably due to perimenopause regression and aging (Dall et al. 2018), against a drastic decrease in the BPA groups. ERβ presents a protective function in normal hormone-responsive organs (Guo et al. 2016) and is less expressed in female mammary gland after perinatal exposure to BPA (Wadia et al. 2013; Leonel et al. 2020a). Loss of ERβ leads to diminished regulatory control of cell maintenance processes in normal breast epithelium and promotes premalignant growth (Rody et al. 2005) linked to invasiveness in breast cancer (Tan et al. 2016). Furthermore, ERβ is highly expressed under homeostatic conditions in mammary gland (Jia et al. 2015) while ERβ (ESR2) gene silencing occurs by hypermethylation events (Zhao et al. 2003) (Fig. 6-4). The therapeutic implications of ERβ expression in breast cancer treatment demonstrate resistance to endocrine therapy (Hopp et al. 2004; Ma et al. 2017).

ARs are relevant for the normal development of hormone-responsive organs (Wang et al. 2014b; Tarulli et al. 2019) and/or neoplastic lesions (Salvi et al. 2020). Although there is little investigation on breast cancer development, efforts in the last decade defined relevant and conflicting parameters for the prognosis of breast cancer related to this receptor (Peters et al. 2009; Vergneau-Grosset et al. 2021). As in the present study, more than 90% of mammary tumors present AR expression, this being

considered an interesting therapeutic target (Hickey et al. 2012). In addition, according to Farmer and collaborators (Farmer et al. 2005), AR and ER positivity define the development of luminal breast cancer, corroborating the characterization made from the expression of PR.

As suggested for prostate cancer cells (Hess-Wilson 2009), the increase in mammary gland androgen receptors induced by BPA dosages may present an interaction through molecular pathways that alter ERβ expression (Fig. 6-2). Furthermore, AR has the ability to antagonize ERα processes by crosstalk with PR (Liao et al. 1998; Peters et al. 2009); however, these pathways may be hampered, since PR showed low expression in BPA induced carcinomas. Oncogene activation mechanisms and cofactors for AR in prostate cancer are similar to those of ERα in the breast, and when expressed in the same context they can compete and increase the mitogenic activation capacity of cells (Risbridger et al. 2010). Thus, we suggest that proliferation in BPA-induced carcinoma may be triggered through AR and ERα proliferative pathways, both highly expressed in the present study.

Additionally, the expression and nuclear detection of AR and HER2/ErbB2 in carcinoma regions suggest a co-regulation (Venema et al. 2019), with the ability to indirectly modulate cell proliferation (Ni et al. 2011) (Fig. 6-3). Both receptors are commonly found in the cytoplasm in their non-phosphorylated/non-ligand-bound form (Golsteynl et al. 1990; Liao et al. 1998). When both events occur, translocation to the nucleus promotes effects in cells (Chua & Adams 2017; Wang 2017), such as proliferation and invasiveness, in addition to risk of cancer recurrence (Risbridger et al. 2010). This increased nuclear AR is associated with estrogenic effects (Liao et al. 1998). BPA could also present pathways for tumor progression by overexpression of HER2/ErbB2 through epigenetic reprogramming, since EZH2 participates in the tumorigenesis process mediated by this receptor (Smith et al. 2019). Indeed, some anticancer therapies aim to decrease nuclear translocation of AR and HER2/ErbB2, aiming to decrease the progression of effects through oncogenes (Mina et al. 2017; Bon et al. 2020). Thus, pathways activated by these receptors and mechanisms associated with tumor growth are affected by age, tumor size, and sentinel lymph node characteristics (Liu 2020). This correlates with the present study, since aging is the main factor for the development of neoplasia in the gerbil model (Rowe et al. 1974).

Several works identify features of mammary cancer progression in non-genetically modified experimental models from exposure to BPA (Wadia et al. 2013;

Dhimolea et al. 2014; Oral et al. 2016; Leonel et al. 2020a; Wormsbaecher et al. 2020). In most cases, there is evidence of neoplastic processes after induction with carcinogenic compounds, such as MNU (Durando et al. 2006; Zhang et al. 2021). Among these models, the most widely used in studies of BPA-induced neoplasia/preneoplasia is the Sprague-Dawley rat (Hussain et al. 2015; Varuzza et al. 2019). Recently, our research group has focused efforts on experiments related to mammary gland of the Mongolian gerbil and observed the effects BPA endocrine disruption in female offspring (Leonel et al. 2020b, a, 2021). This species is used as a model for prostate (Campos et al. 2008; Quintar et al. 2017) and gastric carcinogenesis (Noto et al. 2016), as females have developed prostate glands (Rochel-Maia et al. 2013). Furthermore, aging is a phase that greatly increases the incidence of neoplasia in hormone-responsive glands, such as the prostate (Custodio et al. 2008, 2010).

The female gerbil is prone to the development of neoplasia in old age (Vincent et al. 1980; Custodio et al. 2010), such as adrenal dysregulation and ovarian tumors (Vincent et al. 1979). In addition, due to hormone-responsive variations in several susceptibility windows (Lv & Shi 2011), the development of hormone-sensitive cancers in aging can be a useful application in this experimental model. Interestingly, in previous studies, the non-monotonicity of BPA effects at different concentrations found in Sprague-Dawley rats (Montévil et al. 2019; Prins et al. 2019), occurred in gerbil mammary gland (Leonel et al. 2020a, b), and similar effects are observed in both dosages for tumor development and progression.

Malignant pathological processes in gerbils are attributed to events related to hormone receptors in organs of the reproductive system (Gonçalves et al. 2013). Carcinogenic development should reach spontaneous relevance (Pollard & Luckert 1987) for observation of the progression and tumor establishment induced by hormonal pathways (Bosland et al. 1991; Gonçalves et al. 2013) or hormone-like pathways, such as by endocrine disruptors (Li et al. 2015). Thus, the effects observed in this study present the gerbil as an interesting model for the study of carcinogenesis in mammary tissue.

In summary, BPA-induced carcinoma with increased ERα positivity, loss of ERβ, PR, and PRL-R, and increasing nuclear incidence of HER2/ErbB2 and AR in mammary gland of aged female gerbils, defining the mechanisms of this ED in carcinogenesis (Fig. 6). Both BPA dosages triggered similar effects related to modulation of hormonal receptors, important to the progression of mammary cancer. In addition, the Mongolian

gerbil is proposed as an experimental model for carcinogenesis induced by BPA, and useful for the study of endocrine disruption mechanisms and effective hormonal therapies for mammary neoplasia.

References

Abramson VG, Lehmann BD, Ballinger TJ & Pietenpol JA 2015 Subtyping of triple-negative breast cancer: Implications for therapy. Cancer 121 8–16. (doi:10.1002/cncr.28914)

Acconcia F, Pallottini V & Marino M 2015 Molecular mechanisms of action of BPA. Dose-Response 13 1–9. (doi:10.1177/1559325815610582)

Aldad TS, Rahmani N, Leranth C & Taylor HS 2011 Bisphenol-A exposure alters endometrial progesterone receptor expression in the nonhuman primate. Fertility and Sterility 96 175–179. (doi:10.1016/j.fertnstert.2011.04.010)

Aloisi AM, Della Seta D, Ceccarelli I & Farabollini F 2001 Bisphenol-A differently affects estrogen receptors-α in estrous-cycling and lactating female rats. Neuroscience Letters 310 49–52. (doi:10.1016/S0304-3940(01)02092-4)

Atlas E & Dimitrova V 2019 Bisphenol S and Bisphenol A disrupt morphogenesis of MCF-12A human mammary epithelial cells. Scientific Reports 9 1–10. (doi:10.1038/s41598-019-52505-x)

Ayyanan A, Laribi O, Schuepbach-Mallepell S, Schrick C, Gutierrez M, Tanos T, Lefebvre G, Rougemont J, Yalcin-Ozuysal Ö & Brisken C 2011 Perinatal exposure to bisphenol A increases adult mammary gland progesterone response and cell number. Molecular Endocrinology 25 1915–1923. (doi:10.1210/me.2011-1129)

Bae WK & Hennighausen L 2014 Canonical and non-canonical roles of the histone methyltransferase EZH2 in mammary development and cancer. Molecular and Cellular Endocrinology 382 593–597. (doi:10.1016/j.mce.2013.05.002)

Bhan A, Hussain I, Ansari KI, Bobzean SAM, Perrotti LI & Mandal SS 2014 Histone methyltransferase EZH2 is transcriptionally induced by estradiol as well as estrogenic endocrine disruptors bisphenol-a and diethylstilbestrol. Journal of Molecular Biology 426 3426–3441. (doi:10.1016/j.jmb.2014.07.025)

Blows FM, Driver KE, Schmidt MK, Broeks A, van Leeuwen FE, Wesseling J, Cheang MC, Gelmon K, Nielsen TO, Blomqvist C et al. 2010 Subtyping of breast cancer by immunohistochemistry to investigate a relationship between subtype and short and long term survival: A collaborative analysis of data for 10,159 cases from 12 studies. PLoS Medicine 7. (doi:10.1371/journal.pmed.1000279)

Bon G, Pizzuti L, Laquintana V, Loria R, Porru M, Marchiò C, Krasniqi E, Barba M, Maugeri-Saccà M, Gamucci T et al. 2020 Loss of HER2 and decreased T-DM1 efficacy in HER2 positive

advanced breast cancer treated with dual HER2 blockade: the SePHER Study. Journal of Experimental and Clinical Cancer Research 39 1–14. (doi:10.1186/s13046-020-01797-3)

Bosland MC, Dreef-Van Der Meulen HC, Sukumar S, Ofner P, Leav I, Han X & Liehr JG 1991 Multistage prostate carcinogenesis: the role of hormones. Princess Takamatsu Symposia 22 109–123.

Brisken, Cathrin; O'Malley B 2010 Hormone Action in the Mammary Gland. Cold Spring Harbor Perspectives in Biology 4 1–16. (doi:10.1101/cshperspect.a013086)

Brisken C & Ataca D 2015 Endocrine hormones and local signals during the development of the mouse mammary gland. Wiley Interdisciplinary Reviews: Developmental Biology 4 181–195. (doi:10.1002/wdev.172)

Bromer JG, Zhou Y, Taylor MB, Doherty L & Taylor HS 2010 Bisphenol-A exposure in utero leads to epigenetic alterations in the developmental programming of uterine estrogen response. The FASEB Journal 24 2273–2280. (doi:10.1096/fj.09-140533)

Campos SGP, Zanetoni C, Scarano WR, Vilamaior PSL & Taboga SR 2008 Age-related histopathological lesions in the Mongolian gerbil ventral prostate as a good model for studies of spontaneous hormone-related disorders. International Journal of Experimental Pathology 89 13–24. (doi:10.1111/j.1365-2613.2007.00550.x)

Cancello G, Maisonneuve P, Rotmensz N, Viale G, Mastropasqua MG, Pruneri G, Montagna E, Iorfida I, Mazza M, Balduzzi A et al. 2013 Progesterone receptor loss identifies luminal B breast cancer subgroups at higher risk of relapse. Annals of Oncology 24 632–639. (doi:10.1093/annonc/mds430)

Caswell-Jin JL & Curtis C 2021 Androgen receptor agonists as breast cancer therapeutics. Nature Medicine 27 198–199. (doi:10.1038/s41591-021-01242-8)

Chua FY & Adams BD 2017 Androgen receptor and miR-206 regulation in prostate cancer. Transcription 8 313–327. (doi:10.1080/21541264.2017.1322668)

Custodio AMG, Santos FCA, Campos SGP, Vilamaior PSL, Góes RM & Taboga SR 2008 Aging effects on the mongolian gerbil female prostate (Skene's paraurethral glands): Structural, ultrastructural, quantitative, and hormonal evaluations. Anatomical Record 291 463–474. (doi:10.1002/ar.20637)

Custodio AMG, Santos FCA, Campos SGP, Vilamaior PSL, Oliveira SM, Góes RM & Taboga SR 2010 Disorders related with ageing in the gerbil female prostate (Skene's paraurethral glands). International Journal of Experimental Pathology 91 132–143. (doi:10.1111/j.1365-2613.2009.00685.x)

Dall G V., Hawthorne S, Seyed-Razavi Y, Vieusseux J, Wu W, Gustafsson JA, Byrne D, Murphy L, Risbridger GP & Britt KL 2018 Estrogen receptor subtypes dictate the proliferative nature of the mammary gland. Journal of Endocrinology 237 323–336. (doi:10.1530/JOE-17-0582)

Dhiman VK, Bolt MJ & White KP 2018 Nuclear receptors in cancer - Uncovering new and evolving roles through genomic analysis. Nature Reviews Genetics 19 160–174. (doi:10.1038/nrg.2017.102)

Dhimolea E, Wadia PR, Murray TJ, Settles ML, Treitman JD, Sonnenschein C, Shioda T & Soto AM 2014 Prenatal exposure to BPA alters the epigenome of the rat mammary gland and increases the propensity to neoplastic development. PloS One 9 e99800–e99800. (doi:10.1371/journal.pone.0099800)

Doherty LF, Bromer JG, Zhou Y, Aldad TS & Taylor HS 2010 In utero exposure to diethylstilbestrol (DES) or bisphenol-A (BPA) increases EZH2 expression in the mammary gland: An epigenetic mechanism linking endocrine disruptors to breast cancer. Hormones and Cancer 1 146–155. (doi:10.1007/s12672-010-0015-9)

Doshi T, Mehta SS, Dighe V, Balasinor N & Vanage G 2011 Hypermethylation of estrogen receptor promoter region in adult testis of rats exposed neonatally to bisphenol A. Toxicology 289 74–82. (doi:https://doi.org/10.1016/j.tox.2011.07.011)

Durando M, Kass L, Piva J, Sonnenschein C, Soto AM, Luque EH & Muñoz-De-Toro M 2006 Prenatal bisphenol a exposure induces preneoplastic lesions in the mammary gland in wistar rats. Environmental Health Perspectives 115 80–86. (doi:10.1289/ehp.9282)

EFSA 2015 Scientific Opinion on the risks to public health related to the presence of bisphenol A (BPA) in foodstuffs. EFSA Journal 13. (doi:10.2903/j.efsa.2015.3978)

Farmer P, Bonnefoi H, Becette V, Tubiana-Hulin M, Fumoleau P, Larsimont D, MacGrogan G, Bergh J, Cameron D, Goldstein D et al. 2005 Identification of molecular apocrine breast tumours by microarray analysis. Oncogene 24 4660–4671. (doi:10.1038/sj.onc.1208561)

Feng Y, Manka D, Wagner KU & Khan SA 2007 Estrogen receptor-α expression in the mammary epithelium is required for ductal and alveolar morphogenesis in mice. Proceedings of the National Academy of Sciences of the United States of America 104 14718–14723. (doi:10.1073/pnas.0706933104)

Feng X, Zhang M, Wang B, Zhou C, Mu Y, Li J, Liu X, Wang Y, Song Z & Liu P 2019 CRABP2 regulates invasion and metastasis of breast cancer through hippo pathway dependent on ER status. Journal of Experimental and Clinical Cancer Research 38 1–18. (doi:10.1186/s13046-019-1345-2)

Golsteynl EJ, Goren HJ, Lehoux J & Lefebvre YA 1990 PHORYLATION AND NUCLEAR PROCESSING OF THE ANDROGEN RECEPTOR. Biochemical and Biophysical Research Communications 171 336–341.

Gonçalves BF, De Campos SGP, Zanetoni C, Scarano WR, Falleiros LR, Amorim RL, Gões RM & Taboga SR 2013 A new proposed rodent model of chemically induced prostate carcinogenesis: Distinct time-course prostate cancer progression in the dorsolateral and ventral lobes. Prostate 73 1202–1213. (doi:10.1002/pros.22669)

Greathouse KL, Bredfeldt T, Everitt JI, Lin K, Berry T, Kannan K, Mittelstadt ML, Ho S & Walker CL 2012 Environmental Estrogens Differentially Engage the Histone Methyltransferase EZH2

to Increase Risk of Uterine Tumorigenesis. Molecular Cancer Research 10 546 LP – 557. (doi:10.1158/1541-7786.MCR-11-0605)

Guo L, Zhang YU, Yilamu D, Liu SHA & Guo C 2016 ER β overexpression results in endocrine therapy resistance and poor prognosis in postmenopausal ER α -positive breast cancer patients. Oncology Letters 11 1531–1536. (doi:10.3892/ol.2016.4095)

Haslam SZ & Shyamala G 1979 Progesterone receptors in normal mammary glands of mice: Characterization and relationship to development. Endocrinology 105 786–795. (doi:10.1210/endo-105-3-786)

Hess-Wilson JK 2009 Bisphenol A may reduce the efficacy of androgen deprivation therapy in prostate cancer. Cancer Causes and Control 20 1029–1037. (doi:10.1007/s10552-009-9337-8)

Hickey TE, Robinson JLL, Carroll JS & Tilley WD 2012 Minireview: The androgen receptor in breast tissues: Growth inhibitor, tumor suppressor, oncogene? Molecular Endocrinology 26 1252–1267. (doi:10.1210/me.2012-1107)

Higa GM & Fell RG 2013 Sex Hormone Receptor Repertoire in Breast Cancer. International Journal of Breast Cancer 2013 1–14. (doi:10.1155/2013/284036)

Holm K, Grabau D, Lövgren K, Aradottir S, Gruvberger-Saal S, Howlin J, Saal LH, Ethier SP, Bendahl PO, Stål O et al. 2012 Global H3K27 trimethylation and EZH2 abundance in breast tumor subtypes. Molecular Oncology 6 494–506. (doi:10.1016/j.molonc.2012.06.002)

Hopp TA, Weiss HL, Parra IS, Cui Y, Osborne CK & Fuqua SAW 2004 Low levels of estrogen receptor β protein predict resistance to tamoxifen therapy in breast cancer. Clinical Cancer Research 10 7490–7499. (doi:10.1158/1078-0432.CCR-04-1114)

Huang M, Wu J, Ling R & Li N 2020 Quadruple negative breast cancer. Breast Cancer 27 527–533. (doi:10.1007/s12282-020-01047-6)

Hussain I, Bhan A, Ansari KI, Deb P, Bobzean SAM, Perrotti LI & Mandal SS 2015 Bisphenol-A induces expression of HOXC6, an estrogen-regulated homeobox-containing gene associated with breast cancer. Biochimica et Biophysica Acta (BBA) - Gene Regulatory Mechanisms 1849 697–708. (doi:10.1016/j.bbagrm.2015.02.003)

Jia M, Dahlman-Wright K & Gustafsson JÅ 2015 Estrogen receptor alpha and beta in health and disease. Best Practice and Research: Clinical Endocrinology and Metabolism 29 557–568. (doi:10.1016/j.beem.2015.04.008)

Jorgensen EM, Alderman III MH & Taylor HS 2016 Preferential epigenetic programming of estrogen response after in utero xenoestrogen (bisphenol-A) exposure. The FASEB Journal 30 3194–3201.

Kabos P, Finlay-Schultz J, Li C, Kline E, Finlayson C, Wisell J, Manuel CA, Edgerton SM, Harrell JC, Elias A et al. 2012 Patient-derived luminal breast cancer xenografts retain hormone receptor heterogeneity and help define unique estrogen-dependent gene signatures. Breast Cancer Research and Treatment 135 415–432. (doi:10.1007/s10549-012-2164-8)

Leonel ECR, Campos SGP, Bedolo CMB, Guerra LHA, Vilamaior PSL, Calmon MF, Rahal P, Amorim CA & Taboga SR 2020a Perinatal exposure to bisphenol A impacts in the mammary gland morphology of adult Mongolian gerbils. Experimental and Molecular Pathology 113 104374. (doi:10.1016/j.yexmp.2020.104374)

Leonel ECR, Campos SGP, Guerra LHA, Bedolo CM, Vilamaior PSL, Calmon MF, Rahal P, Amorim CA & Taboga SR 2020b Impact of perinatal bisphenol A and 17β estradiol exposure: Comparing hormone receptor response. Ecotoxicology and Environmental Safety 188 109918. (doi:10.1016/j.ecoenv.2019.109918)

Leonel ECR, Ruiz TFR, Bedolo CM, Campos SGP & Taboga SR 2021 Inflammatory repercussions in female steroid responsive glands after perinatal exposure to bisphenol A and 17-β estradiol. Cell Biology International. (doi:10.1002/cbin.11665)

Levin ER & Hammes SR 2016 Nuclear receptors outside the nucleus : receptors. Nature Reviews Molecular Cell Biology 17 783–797. (doi:10.1038/nrm.2016.122.Nuclear)

Li X, Gonzalez ME, Toy K, Filzen T, Merajver SD & Kleer CG 2009 Targeted overexpression of EZH2 in the mammary gland disrupts ductal morphogenesis and causes epithelial hyperplasia. American Journal of Pathology 175 1246–1254. (doi:10.2353/ajpath.2009.090042)

Li L, Wang Q, Zhang Y, Niu Y, Yao X & Liu H 2015 The molecular mechanism of bisphenol A (BPA) as an endocrine disruptor by interacting with nuclear receptors: Insights from molecular dynamics (MD) simulations. PLoS ONE 10 1–18. (doi:10.1371/journal.pone.0120330)

Liao DZJ, Pantazis CG, Hou X & Li SA 1998 Promotion of estrogen-induced mammary gland carcinogenesis by androgen in the male Noble rat: Probable mediation by steroid receptors. Carcinogenesis 19 2173–2180. (doi:10.1093/carcin/19.12.2173)

Lillo MA, Nichols C, Seagroves TN, Miranda-Carboni GA & Krum SA 2017 Bisphenol A Induces Sox2 in ER+ Breast Cancer Stem-Like Cells. Hormones and Cancer 8 90–99. (doi:10.1007/s12672-017-0286-5)

Liu D 2020 AR pathway activity correlates with AR expression in a HER2-dependent manner and serves as a better prognostic factor in breast cancer. Cellular Oncology 43 321–333. (doi:10.1007/s13402-019-00492-6)

Lv XH & Shi DZ 2011 Variations in serum gonadotropin and prolactin levels during consecutive reproductive states in mongolian gerbils (Meriones unguiculatus). Experimental Animals 60 169–176. (doi:10.1538/expanim.60.169)

Ma R, Karthik GM, Lovrot J, Haglund F, Rosin G, Katchy A, Zhang X, Viberg L, Frisell J, Williams C et al. 2017 Estrogen Receptor B as a therapeutic target in breast cancer stem cells. Journal of the National Cancer Institute 109 1–14. (doi:10.1093/jnci/djw236)

Macias H & Hinck L 2012 Mammary gland development. Wiley Interdisciplinary Reviews: Developmental Biology 1 533–557. (doi:10.1002/wdev.35)

Makki J 2015 Diversity of breast carcinoma: Histological subtypes and clinical relevance. Clinical Medicine Insights: Pathology 8 23–31. (doi:10.4137/CPath.s31563)

Mileva G, Baker SL, Konkle ATM & Bielajew C 2014 Bisphenol-A: epigenetic reprogramming and effects on reproduction and behavior. International Journal of Environmental Research and Public Health 11 7537–7561. (doi:10.3390/ijerph110707537)

Mina A, Yoder R & Sharma P 2017 Targeting the androgen receptor in triple-negative breast cancer: Current perspectives. OncoTargets and Therapy 10 4675–4685. (doi:10.2147/OTT.S126051)

Mohammed H, Russell A, Stark R, Rueda O, Hickey T, Tarulli G, Serandour A, Birrell S, Bruna A, Saadi A et al. 2015 Progesterone receptor modulates estrogen receptor-α action in breast cancer. Nature 523 313–317. (doi:10.1038/nature14583)

Monteiro FL, Direito I & Helguero LA 2020 Hormone Signaling Pathways in the Postnatal Mammary Gland. In Tissue-Specific Cell Signaling, pp 279–315. Eds JV Silva, M Fardilha & MJ Freitas. Springer. (doi:10.1007/978-3-030-44436-5)

Montévil M, Acevedo N, Schaeberle CM, Bharadwaj M, Fenton SE & Soto AM 2019 A combined morphometric and statistical approach to assess non-monotonicity in the developing mammary gland of rats in the CLARITY-BPA study. BioRxiv 2012 1–21. (doi:10.1101/783019)

Nair VA, Valo S, Peltomäki P, Bajbouj K & Abdel-Rahman WM 2020 Oncogenic potential of bisphenol a and common environmental contaminants in human mammary epithelial cells. International Journal of Molecular Sciences 21. (doi:10.3390/ijms21103735)

Najim O, Seghers S, Sergoynne L, Van Gaver H, Papadimitriou K, Wouters K, Trinh XB, Huizing MT & Tjalma W 2019 The association between type of endocrine therapy and development of estrogen receptor-1 mutation(s) in patients with hormone-sensitive advanced breast cancer: A systematic review and meta-analysis of randomized and non-randomized trials. Biochimica et Biophysica Acta - Reviews on Cancer 1872. (doi:10.1016/j.bbcan.2019.188315)

Ni M, Chen Y, Lim E, Wimberly H, Bailey ST, Imai Y, Rimm DL, Shirley Liu X & Brown M 2011 Targeting Androgen Receptor in Estrogen Receptor-Negative Breast Cancer. Cancer Cell 20 119–131. (doi:10.1016/j.ccr.2011.05.026)

Noto JM, Romero-Gallo J, Piazuelo MB & Peek RM 2016 The Mongolian Gerbil: A Robust Model of Helicobacter pylori-Induced Gastric Inflammation and Cancer. In Physiology & Behavior, pp 263–280. (doi:10.1007/978-1-4939-3603-8_24)

Obr AE & Edwards DP 2012 The biology of progesterone receptor in the normal mammary gland and in breast cancer. Molecular and Cellular Endocrinology 357 4–17. (doi:10.1016/j.mce.2011.10.030)

Oral D, Erkekoglu P, Kocer-Gumusel B & Chao MW 2016 Epithelial-mesenchymal transition: A special focus on phthalates and bisphenol A. Journal of Environmental Pathology, Toxicology and Oncology 35 43–58. (doi:10.1615/JEnvironPatholToxicolOncol.2016014200)

Perrot-Applanat M, Kolf-Clauw M, Michel C & Beausoleil C 2018 Alteration of mammary gland development by bisphenol a and evidence of a mode of action mediated through endocrine disruption. Molecular and Cellular Endocrinology 475 29–53. (doi:10.1016/j.mce.2018.06.015)

Peters AA, Buchanan G, Ricciardelli C, Bianco-Miotto T, Centenera MM, Harris JM, Jindal S, Segara D, Jia L, Moore NL et al. 2009 Androgen receptor inhibits estrogen receptor-α activity and is prognostic in breast cancer. Cancer Research 69 6131–6140. (doi:10.1158/0008-5472.CAN-09-0452)

Pollard M & Luckert PH 1987 Autochthonous prostate adenocarcinomas in Lobund-Wistar rats: a model system. The Prostate 11 219–227. (doi:10.1002/pros.2990110303)

Prins GS, Patisaul HB, Belcher SM & Vandenberg LN 2019 CLARITY-BPA academic laboratory studies identify consistent low-dose Bisphenol A effects on multiple organ systems. Basic and Clinical Pharmacology and Toxicology 125 14–31. (doi:10.1111/bcpt.13125)

Quintar AA, Gonçalves BF, Taboga SR & Maldonado CA 2017 The mongolian gerbil (Meriones unguiculatus) as a model for inflammation-promoted prostate carcinogenesis. Cell Biology International 41 1234–1238. (doi:10.1002/cbin.10789)

Reid BM, Vyas S, Chen Z, Chen A, Kanetsky PA, Permuth JB, Sellers TA & Saglam O 2021 Morphologic and molecular correlates of EZH2 as a predictor of platinum resistance in high-grade ovarian serous carcinoma. BMC Cancer 21 1–14. (doi:10.1186/s12885-021-08413-3)

Reis-Filho JS & Pusztai L 2011 Gene expression profiling in breast cancer: Classification, prognostication, and prediction. The Lancet 378 1812–1823. (doi:10.1016/S0140-6736(11)61539-0)

Risbridger GP, Davis ID, Birrell SN & Tilley WD 2010 Breast and prostate cancer: More similar than different. Nature Reviews Cancer 10 205–212. (doi:10.1038/nrc2795)

La Rocca C, Tait S, Guerranti C, Busani L, Ciardo F, Bergamasco B, Stecca L, Perra G, Mancini FR, Marci R et al. 2014 Exposure to endocrine disrupters and nuclear receptor gene expression in infertile and fertile women from different italian areas. International Journal of Environmental Research and Public Health 11 10146–10164. (doi:10.3390/ijerph111010146)

Rochel-Maia SS, Santos FCA, Alonso-Magdalena P, Góes RM, Vilamaior PSL, Warner M, Gustafsson JÅ & Taboga SR 2013 Estrogen receptors alpha and beta in male and female gerbil prostates. Biology of Reproduction 88 1–7. (doi:10.1095/biolreprod.112.103614)

Rodgers KM, Udesky JO, Rudel RA & Brody JG 2018 Environmental chemicals and breast cancer: An updated review of epidemiological literature informed by biological mechanisms. Environmental Research 160 152–182. (doi:https://doi.org/10.1016/j.envres.2017.08.045)

Rodriguez-Gonzalez A, Cyrus K, Salcius M, Kim K, Crews CM, Deshaies RJ & Sakamoto KM 2008 Targeting steroid hormone receptors for ubiquitination and degradation in breast and prostate cancer. Oncogene 27 7201–7211. (doi:10.1038/onc.2008.320)

Rody A, Holtrich U, Solbach C, Kourtis K, Von Minckwitz G, Engels K, Kissler S, Gätje R, Karn T & Kaufmann M 2005 Methylation of estrogen receptor β promoter correlates with loss of ER-

β expression in mammary carcinoma and is an early indication marker in premalignant lesions. Endocrine-Related Cancer 12 903–916. (doi:10.1677/erc.1.01088)

Rowe S, Simmons J, Ringler D & Lay D 1974 Spontaneous neoplasms in aging gerbillinae - a summary of fourty-four neoplasms. Veterinary Pathology 11 38–51.

Salvi S, Bonafè M & Bravaccini S 2020 Androgen receptor in breast cancer: A wolf in sheep's clothing? A lesson from prostate cancer. Seminars in Cancer Biology 60 132–137. (doi:10.1016/j.semcancer.2019.04.002)

dos Santos CO, Dolzhenko E, Hodges E, Smith AD & Hannon GJ 2015 An Epigenetic Memory of Pregnancy in the Mouse Mammary Gland. Cell Reports 11 1102–1109. (doi:10.1016/j.celrep.2015.04.015)

Seachrist DD, Bonk KW, Ho SM, Prins GS, Soto AM & Keri RA 2016 A review of the carcinogenic potential of bisphenol A. Reproductive Toxicology 59 167–182. (doi:10.1016/j.reprotox.2015.09.006)

Segovia-mendoza M, Carmen TG, Ambrosio J, Nava-castro KE & Morales-montor J 2020 The chemical environmental pollutants BPA and BPS induce alterations of the proteomic profile of different phenotypes of human breast cancer cells : A proposed interactome. Environmental Research 191. (doi:10.1016/j.envres.2020.109960)

Sengupta S, Obiorah I, Maximov PY, Curpan R & Jordan VC 2013 Molecular mechanism of action of bisphenol and bisphenol A mediated by oestrogen receptor alpha in growth and apoptosis of breast cancer cells. Britsh Journal of Pharmacology 169 167–178. (doi:10.1111/bph.12122)

Shafei A, Ramzy MM, Hegazy AI, Husseny AK, EL-hadary UG, Taha MM & Mosa AA 2018 The molecular mechanisms of action of the endocrine disrupting chemical bisphenol A in the development of cancer. Gene 647 235–243. (doi:10.1016/j.gene.2018.01.016)

Shah R, Rosso K & David Nathanson S 2014 Pathogenesis, prevention, diagnosis and treatment of breast cancer. World Journal of Clinical Oncology 5 283–298. (doi:10.5306/wjco.v5.i3.283)

Sharp A & Harper-Wynne C 2014 Treatment of Advanced Breast Cancer (ABC): The Expanding Landscape of Targeted Therapies. Journal of Cancer Biology & Research 2 106.

Singh S & Li SSL 2012 Epigenetic effects of environmental chemicals bisphenol A and phthalates. International Journal of Molecular Sciences 13 10143–10153. (doi:10.3390/ijms130810143)

Smith HW, Hirukawa A, Sanguin-Gendreau V, Nandi I, Dufour CR, Zuo D, Tandoc K, Leibovitch M, Singh S, Rennhack JP et al. 2019 An ErbB2/c-Src axis links bioenergetics with PRC2 translation to drive epigenetic reprogramming and mammary tumorigenesis. Nature Communications 10. (doi:10.1038/s41467-019-10681-4)

Soriano S, Gil-Rivera M, Marroqui L, Alonso-Magdalena P, Fuentes E, Gustafsson JA, Nadal A & Martinez-Pinna J 2019 Bisphenol A Regulates Sodium Ramp Currents in Mouse Dorsal

Root Ganglion Neurons and Increases Nociception. Scientific Reports 9 1–14. (doi:10.1038/s41598-019-46769-6)

Sternlicht MD, Kouros-Mehr H, Lu P & Werb Z 2006 Hormonal and local control of mammary branching morphogenesis. Differentiation 74 365–381. (doi:10.1111/j.1432-0436.2006.00105.x)

Tan W, Li Q, Chen K, Su F, Song E & Gong C 2016 Estrogen receptor beta as a prognostic factor in breast cancer patients: A systematic review and meta-analysis. Oncotarget 7 10373–10385. (doi:10.18632/oncotarget.7219)

Tarulli GA, Laven-Law G, Shehata M, Walters KA, Denis IM, Rahman MM, Handelsman DJ, Dean NR, Tilley WD & Hickey TE 2019 Androgen Receptor Signalling Promotes a Luminal Phenotype in Mammary Epithelial Cells. Journal of Mammary Gland Biology and Neoplasia 24 99–108. (doi:10.1007/s10911-018-9406-2)

Terry MB, Michels KB, Brody JG, Byrne C, Chen S, Jerry DJ, Malecki KMC, Martin MB, Miller RL, Neuhausen SL et al. 2019 Environmental exposures during windows of susceptibility for breast cancer: A framework for prevention research. Breast Cancer Research 21 1–16. (doi:10.1186/s13058-019-1168-2)

Thomas C & Gustafsson JÅ 2015 Progesterone receptor-estrogen receptor crosstalk: A novel insight. Trends in Endocrinology and Metabolism 26 453–454. (doi:10.1016/j.tem.2015.08.002)

Tian M & Schiemann WP 2017 TGF-β Stimulation of EMT Programs Elicits Non-genomic ER-α Activity and Anti-estrogen Resistance in Breast Cancer Cells. Journal of Cancer Metastasis and Treatment 3 150–160. (doi:10.20517/2394-4722.2017.38)

Tiwari N, Tiwari VK, Waldmeier L, Balwierz PJ, Arnold P, Pachkov M, Meyer-Schaller N, Schübeler D, vanNimwegen E & Christofori G 2013 Sox4 Is a Master Regulator of Epithelial-Mesenchymal Transition by Controlling Ezh2 Expression and Epigenetic Reprogramming. Cancer Cell 23 768–783. (doi:10.1016/j.ccr.2013.04.020)

Toppari J 2008 Environmental endocrine disrupters. Sexual Development 2 260–267. (doi:10.1159/000152042)

Vandenberg LN, Hunt PA & Gore AC 2019 Endocrine disruptors and the future of toxicology testing — lessons from CLARITY–BPA. Nature Reviews Endocrinology 15 366–374. (doi:10.1038/s41574-019-0173-y)

Varuzza MB, Zapaterini JR, Colombelli KT, Barquilha CN, Justulin LA, Muñoz-de-Toro M, Kass L & Barbisan LF 2019 Impact of gestational low protein diet and postnatal bisphenol A exposure on chemically induced mammary carcinogenesis in female offspring rats. Environmental Toxicology 34 1263–1272. (doi:10.1002/tox.22827)

Venema CM, Bense RD, Steenbruggen TG, Nienhuis HH, Qiu SQ, van Kruchten M, Brown M, Tamimi RM, Hospers GAP, Schröder CP et al. 2019 Consideration of breast cancer subtype in targeting the androgen receptor. Pharmacology and Therapeutics 200 135–147. (doi:10.1016/j.pharmthera.2019.05.005)

Vergneau-Grosset C, Cluzel C, Beauchamp G, Hubbard NE, Paul-Murphy J & Paquet M 2021 Altered prolactin and androgen receptors expression in companion rat benign mammary tumours. Veterinary and Comparative Oncology 19 213–221. (doi:10.1111/vco.12664)

Viñas R, Jeng YJ & Watson CS 2012 Non-genomic effects of xenoestrogen mixtures. International Journal of Environmental Research and Public Health 9 2694–2714. (doi:10.3390/ijerph9082694)

Vincent AL, Rodrick GE & Sodeman WAJ 1979 The pathology of the Mongolian Gerbil (Meriones unguiculatus): a review. Laboratory Animal Science 29 645–651.

Vincent AL, Rodrick GE & Sodeman WA 1980 The Mongolian gerbil in aging research. Experimental Aging Research 6 249–260. (doi:10.1080/03610738008258361)

Vougiouklakis T, Belovarac BJ, Lytle A, Chiriboga L & Ozerdem U 2020 The diagnostic utility of EZH2 H-score and Ki-67 index in non-invasive breast apocrine lesions. Pathology Research and Practice 216 153041. (doi:10.1016/j.prp.2020.153041)

Wadia PR, Cabaton NJ, Borrero MD, Rubin BS, Sonnenschein C, Shioda T & Soto AM 2013 Low-Dose BPA Exposure Alters the Mesenchymal and Epithelial Transcriptomes of the Mouse Fetal Mammary Gland. PLoS ONE 8. (doi:10.1371/journal.pone.0063902)

Waks AG & Winer EP 2019 Breast Cancer Treatment: A Review. JAMA - Journal of the American Medical Association 321 288–300. (doi:10.1001/jama.2018.19323)

Wang Z 2017 ErbB Receptors and Cancer. (doi:10.1007/978-1-4939-7219-7_1)

Wang X, Yarid N, McMahon L, Yang Q & Hicks DG 2014a Expression of androgen receptor and its association with estrogen receptor and androgen receptor downstream proteins in normal/benign breast luminal epithelium. Applied Immunohistochemistry and Molecular Morphology 22 498–504. (doi:10.1097/PAI.0b013e3182a20933)

Wang J, Jenkins S & Lamartiniere CA 2014b Cell proliferation and apoptosis in rat mammary glands following combinational exposure to bisphenol A and genistein. BMC Cancer 14 1–12. (doi:10.1186/1471-2407-14-379)

Weng Y, Hsu P, Liyanarachchi S, Liu J, Deatherage DE, Huang Y, Zuo T, Rodriguez B, Lin C, Cheng A et al. 2010 Epigenetic in fl uences of low-dose bisphenol A in primary human breast epithelial cells. Toxicology and Applied Pharmacology 248 111–121. (doi:10.1016/j.taap.2010.07.014)

Wormsbaecher C, Hindman AR, Avendano A, Cortes-Medina M, Jones CE, Bushman A, Onua L, Kovalchin CE, Murphy AR, Helber HL et al. 2020 In utero estrogenic endocrine disruption alters the stroma to increase extracellular matrix density and mammary gland stiffness. Breast Cancer Research 22 1–12. (doi:10.1186/s13058-020-01275-w)

Zhang X, Cheng C, Zhang G, Xiao M, Li L, Wu S & Lu X 2021 Co-exposure to BPA and DEHP enhances susceptibility of mammary tumors via up-regulating Esr1/HDAC6 pathway in female rats. Ecotoxicology and Environmental Safety 221 112453. (doi:10.1016/j.ecoenv.2021.112453)

Zhao C, Lam EWF, Sunters A, Enmark E, De Bella MT, Coombes RC, Gustafsson JÅ & Dahlman-Wright K 2003 Expression of estrogen receptor β isoforms in normal breast epithelial cells and breast cancer: Regulation by methylation. Oncogene 22 7600–7606. (doi:10.1038/sj.onc.1207100)

4.3. Capítulo 3: Mammary tumor microenvironment after xenoestrogen disruption: inflammatory response and phenotypic cell polarization

Revista *International Journal of Cancer (ISSN: 1097-0215)* – (IF: 7.3)

Thalles Fernando Rocha Ruiz[1]; Simone Jacovaci Colleta[1]; Diego Dias dos Santos[2]; Nayara Fernanda da Costa Castro[1]; Ágata Silva Cabral[4]; Marilia Freitas Calmon[4]; Paula Rahal[4]; Cristiane Damas Gil[3]; Ana Paula Girol[1,4]; Patricia Simone Leite Vilamaior[1]; Ellen Cristina Rivas Leonel[5]; Sebastião Roberto Taboga[1].

[1] Department of Biological Sciences, Institute of Biosciences, Humanities and Exact Sciences, São Paulo State University (UNESP), Rua Cristóvão Colombo 2265, Jardim Nazareth, 15054-000 São José do Rio Preto, São Paulo, Brazil

[2] Department of Morphology and Genetics, Paulista School of Medicine, Federal University of São Paulo (UNIFESP), São Paulo, SP 04023-900, Brazil

[3] Laboratory of Genomic Studies, São Paulo State University, São José do Rio Preto, São Paulo 15054-000, Brazil

[4] University Center Padre Albino (UNIFIPA), Catanduva, SP, Brazil (Department of Basics Sciences)

[5] Department of Histology, Embryology and Cell Biology, Institute of Biological Sciences (ICB III), Federal University of Goiás (UFG), Avenida Esperança, s/n, Campus Samambaia, 74690-900 Goiânia, Goiás, Brazil

Short title: Tumor microenvironment in mammary neoplasia under endocrine disruption

Corrensponding Author: Prof. Dr. Sebastião Roberto Taboga (sebastiao.taboga@unesp.br). São Paulo State University (Unesp), Department of Biological Sciences, IBILCE, Cristóvão Colombo St., 2255, Postal Code 15054-000, Jardim Nazareth, São José do Rio Preto, São Paulo, Brazil.
Correspondence may also be addressed to Thalles F. R. Ruiz (thalles.ruiz@unesp.br).

Novelty and Impact:

Breast cancer development during aging is associated with exposure to endocrine disruptors that modulate the mammary microenvironment. Here, we demonstrate that BPA is able to promote macrophage and mast cell polarization to malignant phenotypes, supporting a tumor microenvironment. Furthermore, BPA increases the expression of inflammatory mediators for epithelial-mesenchymal transition and tumor progression in the mammary gland. Our analyses provide potential pathways for invasiveness and metastasis triggered by exposure to BPA during windows of susceptibility.

Abstract

Friend or foe, inflammation in the established tumor microenvironment (TME) is often associated with a poor prognosis of breast cancer. Bisphenol A (BPA) is an endocrine-disrupting chemical that acts as inflammatory promoter and tumoral facilitator in mammary tissue. An aged mammary gland (MG) environment from females exposed to BPA during pregnancy and lactation revealed indications of epithelial-mesenchymal transition (EMT) and TME development. Contrarily to non-exposed MG, BPA induced carcinogenic development mediated by COX-2 and p-STAT3 expression. BPA was also able to promote macrophage and mast cell polarization in tumoral phenotype, evidenced by pathways for recruitment and activation of these inflammatory cells and tissue invasiveness triggered by TNF-α and TGF-β1. Increase of tumor-associated macrophages, M1 (CD68+iNOS+) and M2 (CD163+) expressing pro-tumoral mediators and metalloproteases was observed; this aspect greatly contributed to stromal remodeling and invasion of neoplastic cells. In addition, the mast cell population drastically increased in BPA-exposed MG. Tryptase-positive mast cells increased in disrupted MG and expressed TGF-β1, contributing to EMT process during carcinogenesis mediated by BPA. Thus, BPA exposure interfered in inflammatory response by releasing and enhancing the expression of mediators that contribute to tumor growth and recruitment of inflammatory cells that promote a malignant profile.

Introduction

Tumor microenvironment (TME) is the main target studied in cancer development and therapeutic approaches. TME promotes the neoplastic cross-talk required for tumor progression (Bussard, Mutkus, Stumpf, Gomez-Manzano, & Marini, 2016). One of the main elements for TME promotion is the establishment of inflammatory process, by production of cytokines and inflammatory-tumor mediators (Polyak & Kalluri, 2010). Breast cancer is one type of adenocarcinoma that presents a great imbalance between inflammation and malignancy (Liubomirski et al., 2019). These phenomena are associated to cancer progression through inflammatory mediators that stimulate the micrometastasis and recruitment of tumor-associated stromal cells (Fouad, Kogawa, Reuben, & Ueno, 2014).

Exposure to environmental chemicals and pollutants increase the risk of inflammation and breast cancer (Koual et al., 2020). Several compounds act as disruptors of stromal-epithelial interaction, impairing tissue homeostasis and developing neoplasms (Koual et al., 2020). Bisphenol A (BPA) is considered a multitarget disruptor of the female reproductive system (Pivonello et al., 2020) and is among the compounds recognized as tumor promoters in the mammary gland (MG). In addition to being considered a promoter of breast cancer (Nair, Valo, Peltomäki, Bajbouj, & Abdel-Rahman, 2020), BPA has been linked as an promoter of inflammatory process (Murata & Kang, 2018). This is due to the fact that exposure to BPA upregulates the expression of inflammatory cytokines recognized as attractant chemokines for inflammatory cells (Kanaya et al., 2019).

We recently demonstrated the carcinogenic potential of BPA in MG of elderly females after previous exposure during pregnancy and lactation (Ruiz, Colleta, Leonel, & Taboga, 2021). In these phases an intense remodeling occurs in epithelial and stromal compartments (Terry et al., 2019) and exposure to BPA triggers a late tumor development onset at aging (Ruiz, Colleta, Leonel, et al., 2021). Since TME at aging predicts a worse prognosis (Fane & Weeraratna, 2020) and BPA is one of the most environmental-spread endocrine disruptors (Vandenberg, Hauser, Marcus, Olea, & Welshons, 2007), the crosstalk between neoplastic development and BPA-exposed TME are matter of concern and deserve to be studied. Here, we investigate the expression of inflammatory cytokines in epithelial and stromal compartments and describe the phenotype of recruited inflammatory cells, mast cells (MC) and

macrophages, in TME during BPA-induced carcinogenesis of aged females exposed in gestational/lactational window.

Materials and Methods

Experimental Design and Animals

Female Mongolian gerbils (Meriones unguiculatus) were allocated with fertile males (N=20) in reproductive age (4 months old) for allowing copulation. On the 8th gestational day, the pregnant females were sorted into four experimental groups (n=5): control (subjected to water gavage/daily); vehicle (subjected to 0.1 ml corn oil gavage/daily); ↓BPA (subjected to 50 µg/kg of BPA/daily – safety dose according to EFSA(EFSA, 2015) and FDA(Soriano et al., 2019)); and ↑BPA (subjected to 5000 µg/kg of BPA/daily – acute exposure).Gavage was performed during 39 days – 18 days of pregnancy and 21 days of lactation. After, females were kept with no other treatment until 18 months of age when they were anesthetized and euthanized. Euthanasia was performed in the estrous phase of cycle, confirmed by vaginal smear. Left MG was collected and fixed in paraformaldehyde 4%, processed in histological routine and serially sectioned (4.5 µm) for immunohistochemistry analysis. Right MG was frozen at -80 ºC for western blotting analysis.

The animals were kept in Animal Breeding Center of the Institute of Biosciences, Humanities and Exact Sciences (IBILCE, UNESP, São Paulo). They were allocated in polysulfone isolators with balanced food and water ad libitum. Ethical standards and protocols for animal experimentation determined by the National Council of Control and Animal Experimentation (CONCEA, Brazil) were followed and experiment was approved by the local Ethics Committee on the Use of Animals (CEUA, IBILCE, UNESP, n. 217/2019).

Immunohistochemistry and Histochemical analysis

Histological sections were destined to immunohistochemistry technique for inflammatory cells and markers detection. The sections were deparaffinized and hydrated. Antigen retrieval was performed with 10 mM citrate buffer (95ºC) or Tris+EDTA (90ºC) for 45 minutes. H2O2 5% diluted in methanol was used for peroxidase blockage. Blocking of non-specific proteins was performed in 10% skimmed milk. After, sections were incubated overnight with primary antibodies for p-STAT3 (rabbit monoclonal, D3A7, #9145; Cell Signaling, Danvers, MA, USA), COX-2

(rabbit monoclonal, D5H5, #12282; Cell Signaling), TNF-α (rabbit monoclonal, D2D4, #11948; Cell Signaling), F4/80 (rabbit monoclonal, IgG, D2S9R, #70076; Cell Signaling), and IL-6 (mouse monoclonal, E-4, sc-28343, Santa Cruz Biotechnology, Dallas, TX, USA). These steps were interspersed with buffer washes in PBS or TBS. Slides were incubated with post-primary antibody and after with polymer (Novolink™, Leica Biosystems Newcastle Ltd.). Positive staining detection of immunohistochemistry reaction was performed with 3-30 diaminobenzidine tetrahydrochloride (DAB) (Novolink™, Leica Biosystems) and counterstaining with hematoxylin. Also, Toluidine blue (pH 4.0) staining was performed in histological sections for total mast cells detection.

Double immunofluorescence assay

Double immunofluorescence assay was performed to determine co-localization or specific cell markers. The steps were similar to immunohistochemistry techniques, except for the peroxidase blockage that was not carried. Incubation of the following primary antibodies was performed with a (1:50) dilution : CD68 (goat polyclonal, M-20, Santa Cruz Biotechnology), CD163 (rabbit polyclonal, M-96, Santa Cruz Biotechnology; or mouse monoclonal, ED2, sc-58965, Santa Cruz Biotechnology), NOS2/iNOS (mouse monoclonal, C-11, sc-7271, Santa Cruz Biotechnology), mast cell tryptase (mouse monoclonal, MAB1222, Sigma-Aldrich; or, rabbit polyclonal, ab196772, Abcam), mast cell chymase (mouse monoclonal, ab2377, Abcam), COX-2 (rabbit monoclonal, D5H5, #12282; Cell Signaling), MMP2 (mouse monoclonal, sc-13595, Santa Cruz Biotechnology), MMP9 (mouse monoclonal, sc-21733, Santa Cruz Biotechnology), TGF-β1 (rabbit polyclonal, sc-146), and TNF-α (rabbit monoclonal, D2D4, #11948; Cell Signaling), and IL-6 (mouse monoclonal, E-4, sc-28343, Santa Cruz Biotechnology). After, the incubation with specific fluorochrome conjugated secondary antibodies was performed, following the host of primary antibodies: anti-mouse FITC (sc-2010, Santa Cruz Biotechnology); anti-rabbit FITC (sc-2359, Santa Cruz Biotechnology); anti-mouse Rhodamine (sc-2092, , Santa Cruz Biotechnology); anti-goat Texas Red (sc-2783, Santa Cruz Biotechnology) and/or anti-rabbit Texas Red (sc-2780, Santa Cruz Biotechnology). DAPI (Fluoroshield™ with DAPI, F6057, histology mounting medium, Merck, Darmstadt, Germany) was used for nuclear fluorescence and mounting.

Western blotting

MG was stored in -80ºC for Western blotting analysis. Protein extraction was performed with an extraction buffer containing protease inhibitor (Protease Inhibitor Cocktail, P8340, Sigma Aldrich, St. Louis, MO, USA) in smashed samples, which were then centrifuged (20 min; 14,000 rpm; 4ºC). Protein concentration was quantified by absorbance using BCA Protein Assay Kit (Pierce BCA Protein Assay Kit, 23,227, Thermo Fischer Scientific, Rockford, IL, USA) in microplate reader SPECTROstar Omega (BMG Labtech, Ortenberg, Germany). Electrophoresis in SDS page gel (15 ug of sample; 105V; 90 min) and electrophoretic transfer to a nitrocellulose membrane (Amersham Protram, 10,600,003, GE Healthcare, Darmstadt, Germany) were performed. TNF-α expression amount was evaluated by Western blotting assay. Blots were blocked with skimmed milk 3% in TBS + Tween (0.1%). After, they were incubated with primary antibody anti-TNF-α (rabbit monoclonal, D2D4, #11948; Cell Signaling) and anti-GAPDH (rabbit monoclonal, 14C10, #2118, Cell Signaling, used as endogenous positive control) overnight. Secondary antibody incubation was performed with horseradish peroxidase conjugated (anti-rabbit IgG, HRP-linked Antibody, #7074). All steps were interposed with TBSt washes. Antibody detection was made with Chemiluminescent substrates – Luminol Enhancer and Peroxide solution reagents (Westar Antares, Cyanagen, XLS142,0250) and revealed in ChemiDoc Image System (BioRad, Hercules, CA, USA). Image J software (version 1.52a, USA) was used for densitometry analysis and protein quantification.

Quantification of positive cells and Statistical analysis

For the quantitative analyses, the number of positive cells per mammary tissue area (cell/mm^2) was considered. For IHC (pSTAT3, TNF-α, CD163+, COX-2, F4/80+), analyzes were performed in QuPath software (version 0.1.2, an open-source pathology software platform). Specific cell detection was evaluated by double immunofluorescence (CD68+iNOS+, Tryptase+Chymase+ or Tryptase+Chymase-). For these analyses, eight random fields were captured (100x) per sample on the AX10 Fluorescence Microscope (Zeiss, Oberkochen, Germany) coupled with AxioVision (Zeiss) software. The number of positive cells was counted after merge fluorescence channels – (total positive cells)/analyzed rea (mm^2).

Statistical analyses were performed in GraphPad Prism 6.00 for Windows (GraphPad Software). Kolmogorov–Smirnov test was used to check data normality distribution.

One-way ANOVA followed by Tukey's test was applied in parametric data. Kruskal–Wallis test followed by Dunn's test was applied in non-parametric data (TNF-α).

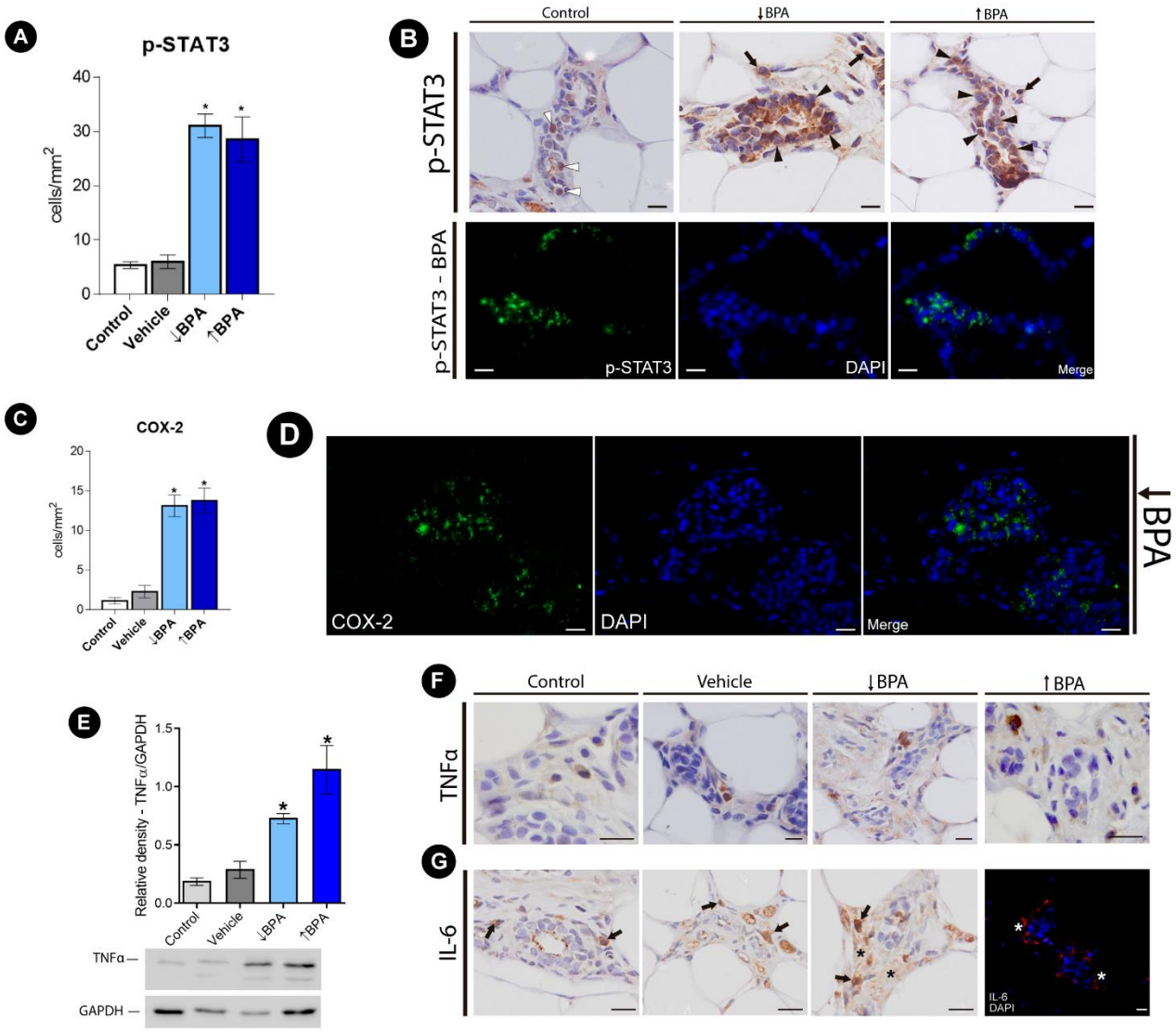

Figure 1. Inflammatory markers in the mammary gland exposed to BPA. (A-B) p-STAT3. Both BPA groups presented high expression of p-STAT3. Control group presented nuclear expression (white arrowheads) in normal epithelium, whereas p-STAT3 expression was observed in cytoplasm of stromal (arrows) and epithelial cells (black arrowheads) from BPA groups. (C-D) COX-2. High expression of COX-2 was observed in BPA groups. (D) Representative illustration of carcinoma cells expressing cytoplasmic COX-2 in BPA groups. (E-F) TNF-α. As shown by immunohistochemistry, TNF-α positive cells did not differ among groups. (E) Western blotting analysis, however, demonstrated an increased expression of TNF-α in BPA groups in comparison to controls. (F) Expression of TNF-α in MG stromal cells. (G) IL-6. The expression of IL-6 was restricted to cytoplasm of stromal cells (arrow) in control and vehicle groups, whereas in BPA groups a diffuse staining (asterisks) was observed. IL-6 positivity was confirmed by immunofluorescence assay in BPA groups (representative image of ↑BPA group). Mean ± SEM was expressed in graphical figures and asterisks indicate significant differences among experimental groups. Scale Bars: (B) 20 µm; (D) 30 µm; (F) 20 µm; (G) 30 µm.

Results

Expression of inflammatory markers in mammary tissue disrupted by BPA

Inflammatory mediators in mammary tissue were evaluated by immunohistochemistry (IHC) in aged females, exposed to a safe dosage (50 µg/kg) and acute exposure (5,000 µg/kg) of BPA during pregnancy and lactation. Expression of p-STAT3 showed higher rates in MG exposed to BPA, than in control groups (Figure 1A). P-STAT3 expression enhanced in vehicle group compared to control, in which positive cells presented nuclear staining (Figure 1 B). In both BPA-exposed groups, contrarily, p-STAT3 was observed in cytoplasm of stromal and epithelial cells (Figure 1B).

Control and vehicle groups presented the lowest values of COX-2 expression (Figure 1 C), observed scarcely in stromal cells. Compared to control groups, expression of COX-2 increased almost 4-folds in BPA exposed animals. In BPA groups, COX-2 was expressed in the cytoplasm of normal epithelium, carcinoma cells, and surrounding stroma (Figure 1 D). In IHC analysis, TNF-α-positive cells did not present statistical differences among groups (Figure 1 E-F). However, Western blotting analysis detected an increase of this protein expression (concentration) in BPA groups (Figure 1 E). IL-6 showed less cytoplasmic positivity of stromal cells in control and vehicle groups, whereas in both BPA groups IL-6-positive cells were more abundant (Figure 1 G). Also, diffuse staining in stroma of BPA groups for IL-6 was confirmed by immunofluorescence (Figure 1 G).

Macrophages in tumor microenvironment promoted by BPA in aged females

Total macrophages in mammary tissue were evaluated by F4/80 IHC (Figure 2 A-B). BPA groups presented high rates of macrophages in comparison to control and vehicle groups, which also differed between them. Clusters of macrophages were observed near to disrupted tissue; intraepithelial macrophages were also observed in BPA groups (Figure 2 B). Furthermore, M1 (CD68+iNOS+) and M2 (CD163+) macrophages were quantified and compared among groups (Figure 2 C). BPA groups presented an increase in M1 macrophages compared to control and vehicle groups (Figure 2 A). Also, M2 macrophages were 2-fold increased in ↓BPA and 3-fold increased in ↑BPA compared to M1 macrophages in the same group. Macrophages CD68+ expressed TNF-α and COX-2 in peritumoral tissue and non-disrupted structures of BPA groups (Figure 2 D). In addition, CD68 and TGF-β1 co-expression

was rare in these groups (Figure 2 E). CD163+ macrophages presented TGF-β1 positivity in BPA groups (Figure 2 E). MMP9 expression was found in CD68+ and CD163+ macrophages (Figure 2 F).

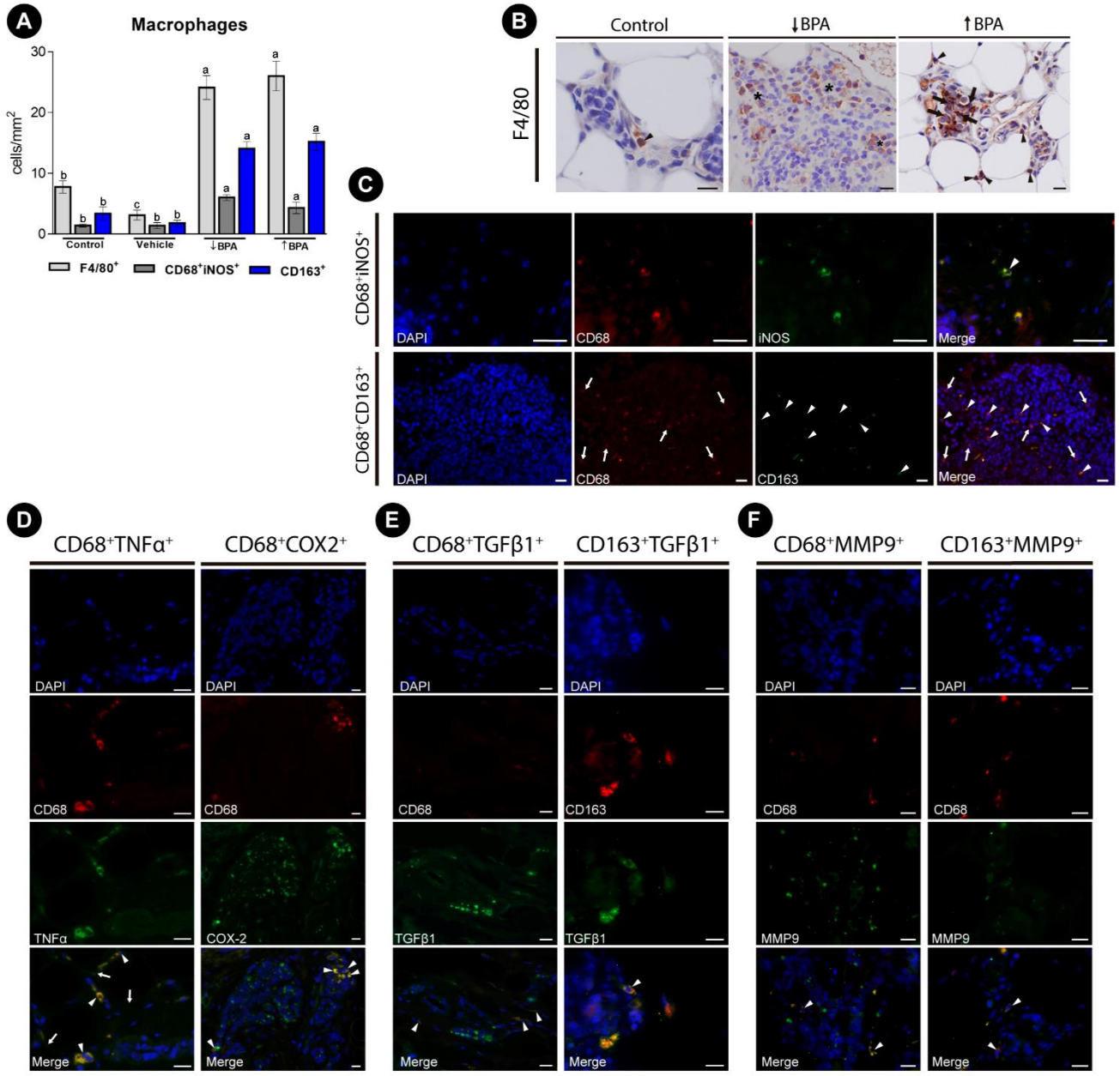

Figure 2. Macrophages in MG of aged females exposed to BPA during pregnancy/lactation window. (A) Macrophage population in mammary tissues. Total macrophages (F4/80+) enhanced in BPA groups compared to control group and vehicle, which presented lowest values. M2 macrophages (CD163+) were increased in BPA exposed tissues, as well as M1 macrophages (CD68+iNOS+) in the same groups. Mean ± SEM is expressed in graphical figures and different letters (a, b, c) indicate significant differences among experimental groups in each parameter (F4/80+, CD68+iNOS+, CD163+). (B) Macrophages (arrowhead) were observed in stromal compartment of control group. In carcinoma of BPA exposed MG, clusters of

macrophages (↓BPA group - asterisks) were observed. Intraepithelial macrophages (↑BPA group - arrows) were also observed in these experimental groups. (C) Tumor-associated macrophages. M1 macrophages were verified by co-expression of CD68 and iNOS. M2 macrophages were verified by expression of CD163 and CD68. Carcinoma features in BPA groups presented CD68+CD163+ (arrowheads) and CD68-CD163+ (arrows), M1 and M2 macrophages, respectively. (D) CD68+ cells (arrowheads) as well as adipocytes (arrows) express TNF-α. CD68+ cells also express COX-2 (arrowheads). (E) TGF-β1 (arrowheads) was expressed in CD68+ and CD163+ cells, as well as (F) MMP9 (arrowheads). Groups: (C-E) ↓BPA; (F) ↑BPA.Scale Bars: (B) 20 µm; (C) 40 µm; (D-F) 20 µm.

Mast cells in tumor microenvironment promoted by BPA in aged females

Total MC were evaluated by toluidine blue staining in mammary tissue (Figure 3 A-B). MCs were increased in BPA groups, mainly in ↑BPA. Analysis of tryptase-/chymase+ and tryptase+/chymase+ were performed to detect different mature MC populations (Figure 3 A, C). Tryptase+ MC (MCT - tryptase+/chymase-) were enhanced in BPA groups, presenting higher rates in ↓BPA. Also, chymase+ MC (MCCT - tryptase+/chymase+) presented no statistical differences among control, vehicle and ↓BPA groups, whereas ↑BPA presented high rate of MCCT. MCT expressed TFG-β1 and MMP2 in stromal compartment of BPA groups (Figure 3 D), while MCCT presented positivity for TGF-β1 (Figure 3 D) and rare expression of MMP2.

Discussion

We demonstrate in the present study the patterns of expression of inflammatory markers and cells in the TME of BPA-induced carcinogenesis. Epithelial and stromal inflammatory responses indicate cancer prognosis of MG tissue and a possible target for therapeutics (Baumgarten & Frasor, 2012). Nonetheless, inflammation is a remarkable feature disturbed by endocrine disruptors, which unbalance the communication between compartments (Murata & Kang, 2018). We demonstrated that increased expression of COX-2 and p-STAT3 in mammary neoplastic cells after BPA exposure, in addition to TGF-β1, are presented as pathways of interaction between the established neoplastic process and the surrounding stroma. Also, our analyses allowed us to present molecular pathways, such as TGF-β1 and TNF-α, preferentially triggered by macrophages and mast cells with pro-tumor phenotypes to promote the initiation of neoplastic development in MG from BPA-exposed females.

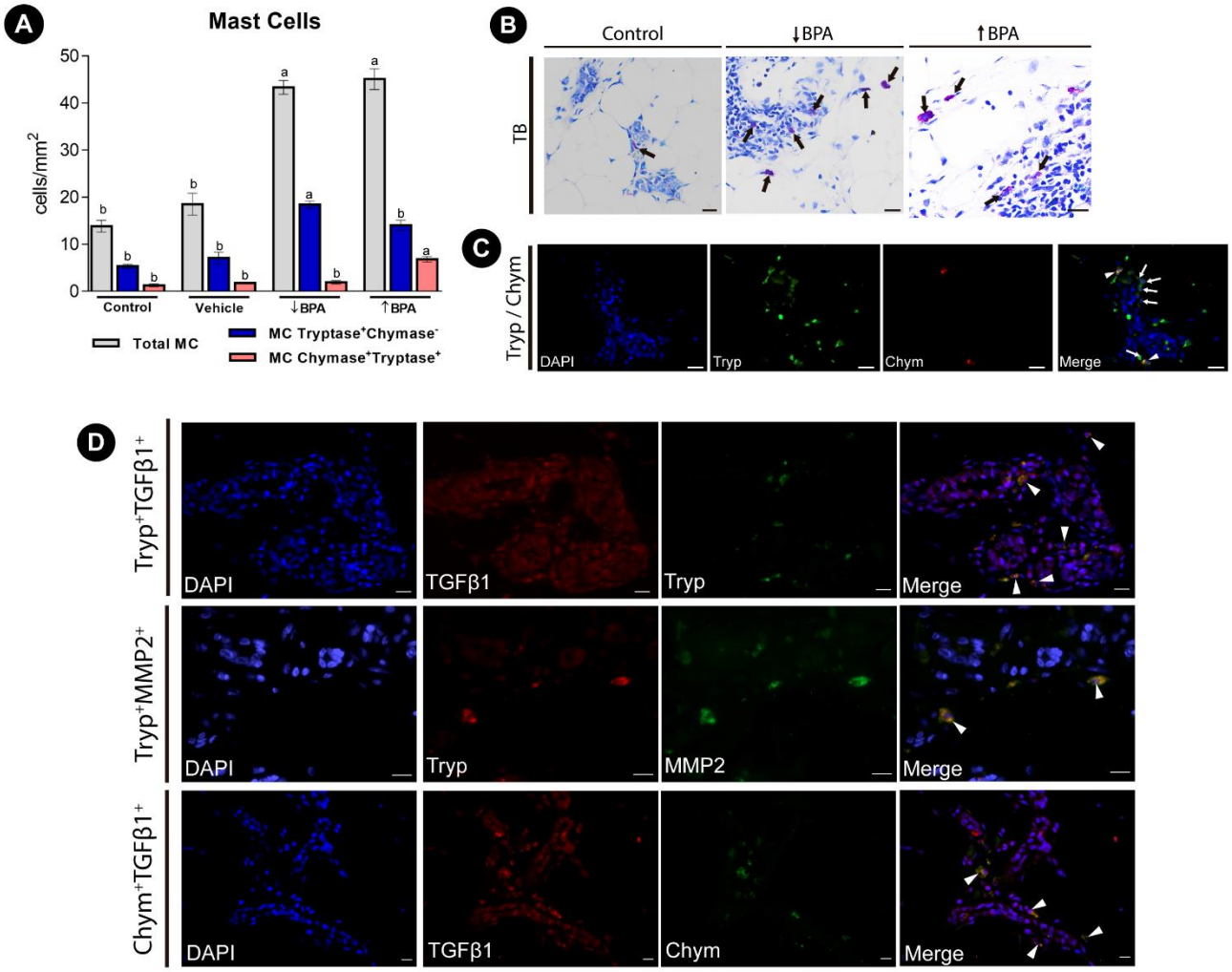

Figure 3. MC in MG of aged females exposed to BPA during pregnancy/lactation window. (A) MC population in mammary tissues. MC were increased in BPA groups compared to control and vehicle groups. Tryptase+Chymase- cells were enhanced in BPA groups, whereas only ↑BPA presented an increase in Tryptase+Chymase+ cells. Mean ± SEM was expressed in graphical figures and different letters (a, b, c) indicate significant differences among experimental groups in each parameter (total MC toluidine blue (TB), Tryptase+Chymase-, Tryptase+Chymase+). (B) Toluidine blue (pH 4) presented a metachromatic reaction (purple) with MC cytoplasm/granules (arrows). Few MC were observed in the control group compared to the abundant MC population surrounding carcinoma features in ↓BPA and ↑BPA groups. (C) MC population. MCT (Tryptase+Chymase-, arrows) and MCCT (Tryptase+Chymase+, arrowhead) were observed in the stromal compartment. (D) Tryptase+ cells express TGF-β1 in neoplasia and MMP2 in stroma of BPA exposed MG. Chymase+ cells also express TGF-β1 (arrowhead). Groups: (C), (D – Tryp+TGF+) ↓BPA; (D – Tryp+MMP2+; Chym+TGF+) ↑BPA. Scale Bars: 20 µm.

P-STAT3 is a marker of tumoral inflammation (Kang et al., 2017). Our results suggest that BPA modulated STAT3 expression (Dumitrascu et al., 2020) , enhancing

cell proliferation in breast cancer through this mechanism (Nair et al., 2020). We analyzed the expression of the phosphorylated form of STAT3. This form occurs in the cytoplasm and after its dimerization it goes through nuclear translocation (Schust, Sperl, Hollis, Mayer, & Berg, 2006). We found that control and vehicle groups showed a nuclear STAT3 expression, while in the BPA groups it was mostly identified in the cytoplasm. Our hypothesis is that BPA interferes in the p-STAT3 translocation machinery, either in dimerization or in the translocation process itself. Future studies could be explored to detect interferences in these events during BPA-induced carcinogenesis.

Tumor-associated macrophages are mainly activated and recruited via TGF-β1 signaling (Gratchev, 2017). In addition to the expression of CD68+, M1 macrophages are also positive for iNOS and TNF-α (Mantovani, Sozzani, Locati, Allavena, & Sica, 2002). The presence of M1 phenotype is associated to an increasing tissue inflammation in TME (Murray et al., 2014). TGF-β1 signaling can be amplified by COX-2 expression (Gan et al., 2016; Hugo, Saunders, Ramsay, & Thompson, 2015) in addition to p-STAT3 (Clarkson et al., 2006). Since BPA increases the expression of these proteins in epithelial cells and macrophages, the increase in M1 is probably linked to this pro-inflammatory cascade (COX-2/p-STAT3/TGF-β1) in neoplastic mammary tissue. Thus, disruption of BPA in the MG of aged females is linked to an intrinsic communication between inflammatory markers expressed in the epithelium and stromal cells.

Also, the marker CD163 was used in the present work as a marker for M2 macrophages (Kim et al., 2020). Even though an anti-inflammatory function is signaled for this macrophage phenotype (He et al., 2014), studies suggest a stimulatory effect on tumor progression (Kim et al., 2020; Najafi et al., 2019). The proportion of M2 is almost 2-fold that of M1 in the MG of aged females exposed to BPA. Thus, M2 macrophage polarization was favored during BPA endocrine disruption. In a tumorigenic environment, M2 macrophages orchestrated by epithelial-derived cancer cells influence extracellular matrix (ECM) remodeling, mainly by MMP9 (Sousa et al., 2015). Also, M2 enhance is related to a poor prognosis due to its association to an invasive environment (Jeong, Hwang, Kang, Shin, & Kwon, 2019). Our results confirm that BPA-promoted polarization occurs by means of estrogenic pathways (Teixeira et al., 2016) that are favored in the MG of aged females, as previously shown(Ruiz, Colleta, Zuccari, et al., 2021).

MC are responsible for important events in tumor signaling and ECM remodeling during carcinogenesis (Komi & Redegeld, 2020). We demonstrated that exposure to BPA during pregnancy and lactation increases MC recruitment to mammary tissue in the onset of neoplastic development in aging. MCT was the increased phenotype in the stroma of both BPA exposure doses, whereas MCCT was more recruited in MG exposed to high dose BPA, probably as a long-term response to an acute exposure. MCT initiates differentiation and proliferation of fibroblasts and stromal remodeling by releasing degradative proteases, such as MMPs, in the TME (Mangia et al., 2011). MMP9 is expressed by macrophages (Zollo et al., 2014) and MC (Xu, Cai, Yang, & Chen, 2017) in response to active estrogenic pathways (Inman, Robertson, Mott, & Bissell, 2015). Also, MCT is involved with collagen remodeling in the ECM (Westbury et al., 2014). Expression of MMPs was observed in differentiated MC, such as MCT and MCCT, after endocrine disruption by BPA. Both MMP-2 and MMP-9 expressed in active MC phenotypes hardly contribute to neoplastic-associated tissue remodeling (Di Cara et al., 2018). Thus, BPA recruits remodeling MC in the onset of carcinogenesis, favoring invasiveness in the MG of aged females.

In addition, MCT increases angiogenesis and micrometastasis in the early stages of carcinogenesis (Ranieri et al., 2009). Mainly in breast carcinoma, an increase of MCT (Kankkunen, Harvima, & Naukkarinen, 1997) is associated with invasion and migration (Liu et al., 2011). In this condition, MCT are found peri- and intra-tumor and are also associated with the communication process between disrupted/cancerous cells and stromal compartments (Khan et al., 2013). TGF-β1 is a chemotactic protein (Ramírez-Valadez, Vázquez-Victorio, Macías-Silva, & González-Espinosa, 2017) enhanced in disrupted epithelial cells and MCT in MG from aged females exposed to BPA. Also, MC communicates with breast cancer cells via TGF-β1, promoting their proliferation and stromal migration (Zollo et al., 2012). As we described previously (Ruiz, Colleta, Leonel, et al., 2021), TGF-β1 is a potential candidate for mediating the EMT process in BPA disrupted tissues. Also, EMT influences this process through inflammatory cytokines that enable tumor cell communication, and prepares invasion fields for cancerous cells (Aponte-López, Fuentes-Pananá, Cortes-Muñoz, & Muñoz-Cruz, 2018).

BPA induced an increase in mature MC, contrarily to what was observed in control and vehicle groups, which had low number of total MC. This demonstrates the recruiting and maturation effect of this cell type in MG exposed to BPA, as well as an

unexplored molecular mechanism of cell-mediated TME (Leoh, Daniels-Wells, & Penichet, 2015). Also, the increase in TNF-α and TGF-β1 by BPA in other cell types may be associated to the increase of mature MC in these groups and the development of a TME (Fleming et al., 2012). In addition to TNF-α, IL-6 mediates inflammation via MC, promoting macrophage activation (Dahdah et al., 2014) and MC own activation (Komi, Wöhrl, & Bielory, 2020). Thus, BPA modulates MC self-activation and co-activation of inflammatory cells during MG carcinogenesis in aged females (Figure 4).

In summary, BPA modulates the progress of TME in MG of aged females during the onset of cancer installation. Neoplastic cells exposed to BPA express COX-2 and p-STAT3 that contribute to EMT process and inflammatory signaling to stroma. Macrophages and MC, especially malignant phenotypes, were hardly recruited in BPA-disrupted tissue. M1 and M2 macrophages phenotypes promote the TME establishment and were responsible for ECM remodeling. MC enhances inflammatory-chemokine signaling and communication for EMT progress of BPA-disrupted epithelial cells by TGF-β and TNF-α. Thus, BPA-induced TME favors neoplastic development in MG of aged females by inflammatory responses and signaling, which also contributes to tumor growth and invasiveness.

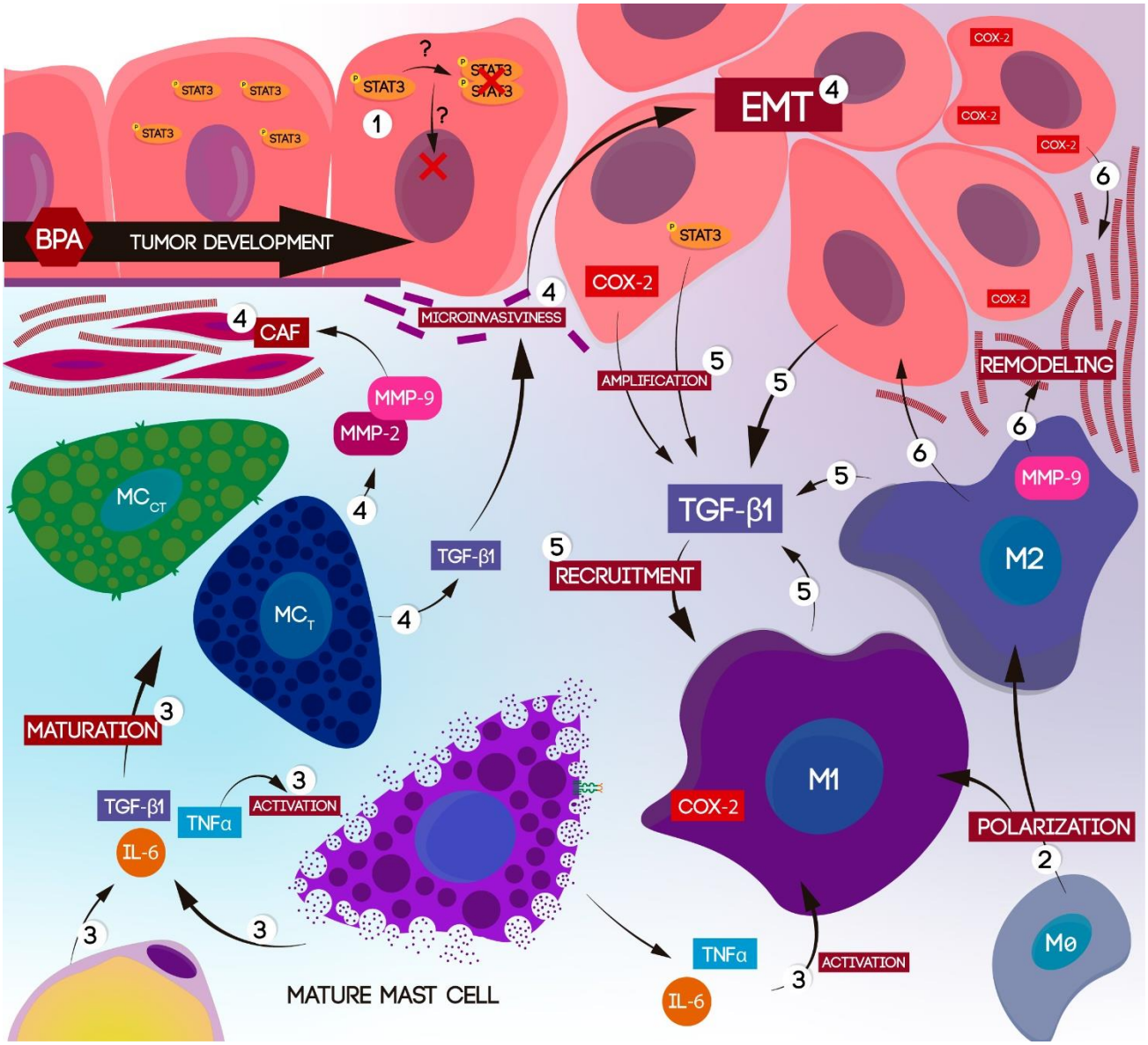

Figure 4. Tumor microenvironment in MG from aged females exposed to BPA. (1) p-STAT3: cytoplasmic expression in epithelial normal and neoplastic cells in disrupted tissue. The hypothesis of non-dimerization and impaired nuclear translocation after BPA exposure remains unclear. (2) Macrophage polarization: BPA interfered in the polarization of resident and recruited macrophages. Induction of M2 pro-tumoral phenotype and M1 pro-inflammatory phenotype was stimulated by BPA. (3) Activation and maturation of M1 macrophages and MC: IL-6 and TNF-α expressed and released by mature MC act as activation-key chemokines for M1 and MC. With TGF-β1, these chemokines act in maturation of MCT (Tryptase+Chymase-) and MCCT (Tryptase+Chymase+). (4) Role of MCT: expression of MMP2 and MMP9 are associated with remodeling of stromal compartment. These proteases alter fibrillar elements of ECM and promote the activation of fibroblasts, influencing the recruitment of cancer-associated fibroblasts (CAF). Also, MCT participates in microinvasive profile and epithelial-mesenchymal transition of neoplastic cells through TGF-β1 pathway. (5) Role of M1 macrophages: this tumor-associated macrophage phenotype acts expressing COX-2 and TGF-β1. COX-2 and p-STAT3 expressed by carcinoma cells amplified TGF-β1 signaling pathway to recruit inflammatory cells, particularly macrophages. TGF-β1 expressed by carcinoma cells, M1 and M2 also enhances the TGF-β1

synthesis and activity in these cells. (6) Role of M2: M2 macrophages express MMP9 that exerts a remodeling activity in ECM collagen. COX-2 expressed by carcinoma cells also contributes to matrix remodeling, promoting a metastatic environment.

References

Aponte-López, A., Fuentes-Pananá, E. M., Cortes-Muñoz, D., & Muñoz-Cruz, S. (2018). Mast Cell, the Neglected Member of the Tumor Microenvironment: Role in Breast Cancer. Journal of Immunology Research, 2018. https://doi.org/10.1155/2018/2584243

Baumgarten, S. C., & Frasor, J. (2012). Minireview: Inflammation: An instigator of more aggressive estrogen receptor (ER) positive breast cancers. Molecular Endocrinology, 26(3), 360–371. https://doi.org/10.1210/me.2011-1302

Bussard, K. M., Mutkus, L., Stumpf, K., Gomez-Manzano, C., & Marini, F. C. (2016). Tumor-associated stromal cells as key contributors to the tumor microenvironment. Breast Cancer Research, 18(1), 1–11. https://doi.org/10.1186/s13058-016-0740-2

Clarkson, R. W. E., Boland, M. P., Kritikou, E. A., Lee, J. M., Freeman, T. C., Tiffen, P. G., & Watson, C. J. (2006). The genes induced by signal transducer and activators of transcription (STAT)3 and STAT5 in mammary epithelial cells define the roles of these STATs in mammary development. Molecular Endocrinology, 20(3), 675–685. https://doi.org/10.1210/me.2005-0392

Dahdah, A., Gautier, G., Attout, T., Fiore, F., Lebourdais, E., Msallam, R., … Launay, P. (2014). Mast cells aggravate sepsis by inhibiting peritoneal macrophage phagocytosis. Journal of Clinical Investigation, 124(10), 4577–4589. https://doi.org/10.1172/JCI75212

Di Cara, G., Marabeti, M., Musso, R., Riili, I., Cancemi, P., & Pucci Minafra, I. (2018). New Insights into the Occurrence of Matrix Metalloproteases -2 and -9 in a Cohort of Breast Cancer Patients and Proteomic Correlations. Cells, 7(8), 89. https://doi.org/10.3390/cells7080089

Dumitrascu, M., Mares, C., Petca, R.-C., Sandru, F., Popescu, R.-I., Mehedintu, C., & Petca, A. (2020). Carcinogenic effects of bisphenol A in breast and ovarian cancers (Review). Oncology Letters, 20(6), 1–1. https://doi.org/10.3892/ol.2020.12145

EFSA. (2015). Scientific Opinion on the risks to public health related to the presence of bisphenol A (BPA) in foodstuffs. EFSA Journal, 13(1). https://doi.org/10.2903/j.efsa.2015.3978

Fane, M., & Weeraratna, A. T. (2020). How the ageing microenvironment influences tumour progression. Nature Reviews Cancer, 20(2), 89–106. https://doi.org/10.1038/s41568-019-0222-9

Fleming, J. M., Miller, T. C., Kidacki, M., Ginsburg, E., Stuelten, C. H., Stewart, D. A., … Vonderhaar, B. K. (2012). Paracrine interactions between primary human macrophages and human fibroblasts enhance murine mammary gland humanization in vivo. Breast Cancer Research, 14(3), 1–18. https://doi.org/10.1186/bcr3215

Fouad, T. M., Kogawa, T., Reuben, J. M., & Ueno, N. T. (2014). The Role of Inflammation in Inflammatory Breast Cancer. In International Journal of Radiation Biology (Vol. 44, pp. 53–73). https://doi.org/10.1007/978-3-0348-0837-8_3

Gan, L., Qiu, Z., Huang, J., Li, Y., Huang, H., Xiang, T., … Ren, G. (2016). Cyclooxygenase-2 in tumor-associated macrophages promotes metastatic potential of breast cancer cells through akt pathway. International Journal of Biological Sciences, 12(12), 1533–1543. https://doi.org/10.7150/ijbs.15943

Gratchev, A. (2017). TGF-β signalling in tumour associated macrophages. Immunobiology, 222(1), 75–81. https://doi.org/10.1016/j.imbio.2015.11.016

He, K. F., Zhang, L., Huang, C. F., Ma, S. R., Wang, Y. F., Wang, W. M., … Sun, Z. J. (2014). CD163+ tumor-associated macrophages correlated with poor prognosis and cancer stem cells in oral squamous cell carcinoma. BioMed Research International, 2014. https://doi.org/10.1155/2014/838632

Hugo, H. J., Saunders, C., Ramsay, R. G., & Thompson, E. W. (2015). New Insights on COX-2 in Chronic Inflammation Driving Breast Cancer Growth and Metastasis. Journal of Mammary Gland Biology and Neoplasia, 20(3–4), 109–119. https://doi.org/10.1007/s10911-015-9333-4

Inman, J. L., Robertson, C., Mott, J. D., & Bissell, M. J. (2015). Mammary gland development: Cell fate specification, stem cells and the microenvironment. Development (Cambridge), 142(6), 1028–1042. https://doi.org/10.1242/dev.087643

Jeong, H., Hwang, I., Kang, S. H., Shin, H. C., & Kwon, S. Y. (2019). Tumor-associated macrophages as potential prognostic biomarkers of invasive breast cancer. Journal of Breast Cancer, 22(1), 38–51. https://doi.org/10.4048/jbc.2019.22.e5

Kanaya, N., Chang, G., Wu, X., Saeki, K., Bernal, L., Shim, H.-J., … Chen, S. (2019). Single-cell RNA-sequencing analysis of estrogen- and endocrine-disrupting chemical-induced

reorganization of mouse mammary gland. Communications Biology, 2, 406. https://doi.org/10.1038/s42003-019-0618-9

Kang, H. J., Yi, Y. W., Hou, S. J., Kim, H. J., Kong, Y., Bae, I., & Brown, M. L. (2017). Disruption of STAT3-DNMT1 interaction by SH-I-14 induces reexpression of tumor suppressor genes and inhibits growth of triple-negative breast tumor. Oncotarget, 8(48), 83457–83468. https://doi.org/10.18632/oncotarget.4054

Kankkunen, J. P., Harvima, I. T., & Naukkarinen, A. (1997). Quantitative analysis of tryptase and chymase containing mast cells in benign and malignant breast lesions. International Journal of Cancer, 72(3), 385–388. https://doi.org/10.1002/(SICI)1097-0215(19970729)72:3<385::AID-IJC1>3.0.CO;2-L

Khan, M. W., Keshavarzian, A., Gounaris, E., Melson, J. E., Cheon, E. C., Blatner, N. R., … Khazaie, K. (2013). PI3K/AKT Signaling Is Essential for Communication between Tissue-Infiltrating Mast Cells, Macrophages, and Epithelial Cells in Colitis-Induced Cancer. Clinical Cancer Research, 19(9), 2342–2354. https://doi.org/10.1158/1078-0432.CCR-12-2623

Kim, M. G., Lim, K., Lee, Y. J., Yang, J., Oh, S. W., Cho, W. Y., & Jo, S. K. (2020). M2 macrophages predict worse long-term outcomes in human acute tubular necrosis. Scientific Reports, 10(1), 1–7. https://doi.org/10.1038/s41598-020-58725-w

Komi, D. E. A., & Redegeld, F. A. (2020). Role of Mast Cells in Shaping the Tumor Microenvironment. Clinical Reviews in Allergy & Immunology, 58(3), 313–325. https://doi.org/10.1007/s12016-019-08753-w

Komi, D. E. A., Wöhrl, S., & Bielory, L. (2020). Mast Cell Biology at Molecular Level: a Comprehensive Review. Clinical Reviews in Allergy and Immunology, 58(3), 342–365. https://doi.org/10.1007/s12016-019-08769-2

Koual, M., Tomkiewicz, C., Cano-Sancho, G., Antignac, J. P., Bats, A. S., & Coumoul, X. (2020). Environmental chemicals, breast cancer progression and drug resistance. Environmental Health: A Global Access Science Source, 19(1), 1–25. https://doi.org/10.1186/s12940-020-00670-2

Leoh, L. S., Daniels-Wells, T. R., & Penichet, M. L. (2015). Ige immunotherapy against cancer. Current Topics in Microbiology and Immunology (Vol. 388). https://doi.org/10.1007/978-3-319-13725-4_6

Liu, J., Zhang, Y., Zhao, J., Yang, Z., Li, D., Katirai, F., & Huang, B. (2011). Mast cell: Insight into remodeling a tumor microenvironment. Cancer and Metastasis Reviews, 30(2), 177–184. https://doi.org/10.1007/s10555-011-9276-1

Liubomirski, Y., Lerrer, S., Meshel, T., Rubinstein-Achiasaf, L., Morein, D., Wiemann, S., … Ben-Baruch, A. (2019). Tumor-stroma-inflammation networks promote pro-metastatic chemokines and aggressiveness characteristics in triple-negative breast cancer. Frontiers in Immunology, 10(APR), 1–24. https://doi.org/10.3389/fimmu.2019.00757

Mangia, A., Malfettone, A., Rossi, R., Paradiso, A., Ranieri, G., Simone, G., & Resta, L. (2011). Tissue remodelling in breast cancer: Human mast cell tryptase as an initiator of myofibroblast differentiation. Histopathology, 58(7), 1096–1106. https://doi.org/10.1111/j.1365-2559.2011.03842.x

Mantovani, A., Sozzani, S., Locati, M., Allavena, P., & Sica, A. (2002). Macrophage polarization: Tumor-associated macrophages as a paradigm for polarized M2 mononuclear phagocytes. Trends in Immunology, 23(11), 549–555. https://doi.org/10.1016/S1471-4906(02)02302-5

Murata, M., & Kang, J.-H. (2018). Bisphenol A (BPA) and cell signaling pathways. Biotechnology Advances, 36(1), 311–327. https://doi.org/10.1016/j.biotechadv.2017.12.002

Murray, P. J., Allen, J. E., Biswas, S. K., Fisher, E. A., Gilroy, D. W., Goerdt, S., … Wynn, T. A. (2014). Macrophage Activation and Polarization: Nomenclature and Experimental Guidelines. Immunity, 41(1), 14–20. https://doi.org/10.1016/j.immuni.2014.06.008

Nair, V. A., Valo, S., Peltomäki, P., Bajbouj, K., & Abdel-Rahman, W. M. (2020). Oncogenic potential of bisphenol a and common environmental contaminants in human mammary epithelial cells. International Journal of Molecular Sciences, 21(10). https://doi.org/10.3390/ijms21103735

Najafi, M., Hashemi Goradel, N., Farhood, B., Salehi, E., Nashtaei, M. S., Khanlarkhani, N., … Mortezaee, K. (2019). Macrophage polarity in cancer: A review. Journal of Cellular Biochemistry, 120(3), 2756–2765. https://doi.org/10.1002/jcb.27646

Pivonello, C., Muscogiuri, G., Nardone, A., Garifalos, F., Provvisiero, D. P., Verde, N., … Pivonello, R. (2020). Bisphenol A: An emerging threat to female fertility. Reproductive Biology and Endocrinology, 18(1). https://doi.org/10.1186/s12958-019-0558-8

Polyak, K., & Kalluri, R. (2010). The Role of the Microenvironment in Mammary Gland Development and Cancer. Cold Spring Harbor Perspectives in Biology, 2(11), a003244–a003244. https://doi.org/10.1101/cshperspect.a003244

Ramírez-Valadez, K. A., Vázquez-Victorio, G., Macías-Silva, M., & González-Espinosa, C. (2017). Fyn kinase mediates cortical actin ring depolymerization required for mast cell migration in response to TGF-β in mice. European Journal of Immunology, 47(8), 1305–1316. https://doi.org/10.1002/eji.201646876

Ranieri, G., Ammendola, M., Patruno, R., Celano, G., Zito, F. A., Montemurro, S., … Ribatti, D. (2009). Tryptase-positive mast cells correlate with angiogenesis in early breast cancer patients. International Journal of Oncology, 35(01), 547–557. https://doi.org/10.3892/ijo_00000319

Ruiz, T. F. R., Colleta, S. J., Leonel, E. C. R., & Taboga, S. R. (2021). Mammary carcinoma in aged gerbil mothers after endocrine disruption in pregnancy and lactation. Endocrine-Related Cancer. https://doi.org/10.1530/ERC-21-0198

Ruiz, T. F. R., Colleta, S. J., Zuccari, D. A. P. de C., Vilamaior, P. S. L., Leonel, E. C. R., & Taboga, S. R. (2021). Hormone receptor expression in aging mammary tissue and carcinoma from a rodent model after xenoestrogen disruption. Life Sciences, 285(October), 120010. https://doi.org/10.1016/j.lfs.2021.120010

Schust, J., Sperl, B., Hollis, A., Mayer, T. U., & Berg, T. (2006). Stattic: A Small-Molecule Inhibitor of STAT3 Activation and Dimerization. Chemistry and Biology, 13(11), 1235–1242. https://doi.org/10.1016/j.chembiol.2006.09.018

Soriano, S., Gil-Rivera, M., Marroqui, L., Alonso-Magdalena, P., Fuentes, E., Gustafsson, J. A., … Martinez-Pinna, J. (2019). Bisphenol A Regulates Sodium Ramp Currents in Mouse Dorsal Root Ganglion Neurons and Increases Nociception. Scientific Reports, 9(1), 1–14. https://doi.org/10.1038/s41598-019-46769-6

Sousa, S., Brion, R., Lintunen, M., Kronqvist, P., Sandholm, J., Mönkkönen, J., … Määttä, J. A. (2015). Human breast cancer cells educate macrophages toward the M2 activation status. Breast Cancer Research, 17(1), 1–14. https://doi.org/10.1186/s13058-015-0621-0

Teixeira, D., Marques, C., Pestana, D., Faria, A., Norberto, S., Calhau, C., & Monteiro, R. (2016). Effects of xenoestrogens in human M1 and M2 macrophage migration, cytokine release, and estrogen-related signaling pathways. Environmental Toxicology, 31(11), 1496–1509. https://doi.org/10.1002/tox.22154

Terry, M. B., Michels, K. B., Brody, J. G., Byrne, C., Chen, S., Jerry, D. J., … Fisher, C. (2019). Environmental exposures during windows of susceptibility for breast cancer: A framework for prevention research. Breast Cancer Research, 21(1), 1–16. https://doi.org/10.1186/s13058-019-1168-2

Vandenberg, L. N., Hauser, R., Marcus, M., Olea, N., & Welshons, W. V. (2007). Human exposure to bisphenol A (BPA). Reproductive Toxicology, 24(2), 139–177. https://doi.org/10.1016/j.reprotox.2007.07.010

Westbury, C. B., Freeman, A., Rashid, M., Pearson, A., Yarnold, J. R., & Short, S. C. (2014). Changes in mast cell number and stem cell factor expression in human skin after radiotherapy for breast cancer. Radiotherapy and Oncology, 111(2), 206–211. https://doi.org/10.1016/j.radonc.2014.02.020

Xu, L., Cai, Z., Yang, F., & Chen, M. (2017). Activation-induced upregulation of MMP9 in mast cells is a positive feedback mediator for mast cell activation. Molecular Medicine Reports, 15(4), 1759–1764. https://doi.org/10.3892/mmr.2017.6215

Zollo, M., Di Dato, V., Spano, D., De Martino, D., Liguori, L., Marino, N., … Guglielmotti, A. (2012). Targeting monocyte chemotactic protein-1 synthesis with bindarit induces tumor regression in prostate and breast cancer animal models. Clinical and Experimental Metastasis, 29(6), 585–601. https://doi.org/10.1007/s10585-012-9473-5

Zollo, M., Di Dato, V., Spano, D., De Martino, D., Liguori, L., Marino, N., … Guglielmotti, A. (2014). Tumor-induced immune dysfunction: The macrophage connection. Journal of Biological Chemistry, 66(3), 1777–1783. https://doi.org/10.1007/s10585-012-9473-5

5. CONSIDERAÇÕES FINAIS E CONCLUSÕES

A partir do presente estudo considera-se que:

- O BPA atua como agente carcinogênico na glândula mamária durante o envelhecimento, corroborando com estudos anteriores que demonstraram seu potencial, principalmente quando ocorre a exposição durante as janelas de susceptibilidade gestacional/lactacional;
- Ambas as doses de exposição ao BPA apresentaram efeitos histopatológicos semelhantes na glândula mamária, demonstrando um risco à exposição a doses consideradas seguras e que os impactos do BPA não são dose dependentes;
- O desenvolvimento tumoral inicial induzido pelo BPA modula mediadores celulares e teciduais que contribuem com o processo de transição epitélio-mesenquimal, característico do câncer de mama;
- O carcinoma multifocal nas glândulas mamárias de fêmeas expostas ao BPA apresentam um aumento na expressão de ERα e uma perda da expressão dos receptores de ERβ, PR e PRL-R, além de atuar na translocação nuclear dos receptores AR e HER2/ErbB2;
- A alta expressão de ER no tecido mamário exposto à disrupção endócrina está associada à expressão do marcador de alterações epigenéticas EZH2, o que confere um fenótipo pró-invasivo e maligno às neoplasias;
- Houve o estabelecimento de um microambiente tumoral que corrobora com o desenvolvimento neoplásico a partir de uma resposta inflamatória das células do carcinoma e elementos estromais;
- A polarização de macrófagos e mastócitos para fenótipos pró-tumorais e pró-inflamatórios foi uma marcante característica nas glândulas mamárias no início da carcinogênese induzida pelo BPA.

Assim, conclui-se que a exposição ao BPA nas janelas gestacional e lactacional apresenta um risco para o desenvolvimento neoplásico tardiamente na fase senil. As repercussões histopatológicas demonstraram o início do processo carcinogênico com características marcantes como a transição epitélio-mesenquimal, um estroma reativo e inflamação que favorece um microambiente tumoral.

6. REFERÊNCIAS

ACCONCIA, F.; PALLOTTINI, V.; MARINO, M. Molecular mechanisms of action of BPA. **Dose-Response**, v. 13, n. 4, p. 1–9, 2015.

ACEVEDO, C.; AMAYA, C.; LÓPEZ-GUERRA, J. L. Rare breast tumors: Review of the literature. **Reports of Practical Oncology and Radiotherapy**, v. 19, n. 4, p. 267–274, 2014.

AKERS, R. M. **Lactation and the Mammary Gland**. [s.l: s.n.].

ALOISI, A. M. et al. Bisphenol-A differently affects estrogen receptors-α in estrous-cycling and lactating female rats. **Neuroscience Letters**, v. 310, n. 1, p. 49–52, 2001.

ALONSO-MAGDALENA, P. et al. Bisphenol a exposure during pregnancy disrupts glucose homeostasis in mothers and adult male offspring. **Environmental Health Perspectives**, v. 118, n. 9, p. 1243–1250, 2010.

ALONSO-MAGDALENA, P. et al. Bisphenol-A treatment during pregnancy in mice: A new window of susceptibility for the development of diabetes in mothers later in life. **Endocrinology**, v. 156, n. 5, p. 1659–1670, 2015.

ALONSO-MAGDALENA, P. et al. Effects of gestational exposure to bisphenol-A on the development of diabetes in mothers. **Toxicology Letters**, v. 259, n. 2016, p. S31–S32, 2016.

BASAK, S.; DAS, M. K.; DUTTAROY, A. K. Plastics derived endocrine-disrupting compounds and their effects on early development. **Birth Defects Research**, v. n/a, n. n/a, jun. 2020.

BIGSBY, R. et al. Evaluating the effects of endocrine disrupters on endocrine function during development. **Environmental Health Perspectives**, v. 107, n. SUPPL. 4, p. 613–618, 1999.

BOUCHER, J. G. et al. In Vitro Effects of Bisphenol A β-D-Glucuronide (BPA-G) on Adipogenesis in Human and Murine Preadipocytes. **Environmental health perspectives**, v. 123, n. 12, p. 1287–1293, 2015.

BROMER, J. G. et al. Bisphenol-A exposure in utero leads to epigenetic alterations in the developmental programming of uterine estrogen response. **The FASEB Journal**, v. 24, n. 7, p. 2273–2280, fev. 2010.

CUNHA, G. R.; COOKE, P. S.; KURITA, T. Role of stromal-epithelial interactions in hormonal responses. **Archives of Histology and Cytology**, v. 67, n. 5, p. 417–434, 2004.

CUSTODIO, A. M. G. et al. Disorders related with ageing in the gerbil female prostate (Skene's paraurethral glands). **International Journal of Experimental Pathology**, v. 91, n. 2, p. 132–143, 2010.

DAWSON, C. A.; VISVADER, J. E. The Cellular Organization of the Mammary Gland: Insights From Microscopy. **Journal of Mammary Gland Biology and Neoplasia**, n. 0123456789, 2021.

DZIĘGELEWSKA, Ż.; GAJEWSKA, M. Stromal-Epithelial Interactions during Mammary Gland Development. **Stromal Cells - Structure, Function, and**

Therapeutic Implications, 2019.

ENGLUND, J. I. et al. Laminin alpha 5 is Necessary for Mammary Epithelial Growth and Function by Maintaining Luminal Epithelial Cell Identity. **bioRxiv**, p. 2019.12.27.889451, 2019.

FEINBERG, T. Y. et al. Functional roles of mmp14 and mmp15 in early postnatal mammary gland development. **Development (Cambridge)**, v. 143, n. 21, p. 3956–3968, 2016.

FERNANDEZ, S. V.; RUSSO, J. Estrogen and Xenoestrogens in breast cancer. **Toxicologic Pathology**, v. 38, n. 1, p. 110–122, 2010.

GASSMAN, N. R. Induction of Oxidative Stress by Bisphenol A and Its Pleiotropic Effects. **Environmental and Molecular Mutagenesis**, v. 58, n. 2, p. 60–71, 2017.

GOLOUBKOVA, T.; SPRITZER, P. M. Xenoestrogênios: o exemplo do bisfenol-A. **Arquivos Brasileiros de Endocrinologia & Metabologia**, v. 44, n. 4, p. 323–330, 2000.

GREEN, K. A.; STREULI, C. H. Apoptosis regulation in the mammary gland. **Cellular and Molecular Life Sciences**, v. 61, n. 15, p. 1867–1883, 2004.

HASS, U. et al. Low-dose effect of developmental bisphenol A exposure on sperm count and behaviour in rats. **Andrology**, v. 4, n. 4, p. 594–607, 2016.

HYTTEL, P.; SINOWATZ, F.; VEJLSTED, M. **Embriologia veterinária**. [s.l.] Elsevier Brasil, 2012.

INMAN, J. L. et al. Mammary gland development: Cell fate specification, stem cells and the microenvironment. **Development (Cambridge)**, v. 142, n. 6, p. 1028–1042, 2015.

LABRIE, F. et al. Is dehydroepiandrosterone a hormone? **Journal of Endocrinology**, v. 187, n. 2, p. 169–196, 2005.

LEONEL, E. C. R. et al. Perinatal exposure to bisphenol A impacts in the mammary gland morphology of adult Mongolian gerbils. **Experimental and Molecular Pathology**, v. 113, n. January, p. 104374, 2020.

LEONEL, E. C. R. R. et al. Histological and immunohistochemical characterization of the Mongolian gerbil's mammary gland during gestation, lactation and involution. **Acta Histochemica**, v. 119, n. 3, p. 273–283, 2017.

MACIAS, H.; HINCK, L. Mammary gland development. **Wiley Interdisciplinary Reviews: Developmental Biology**, v. 1, n. 4, p. 533–557, abr. 2012.

MAFFINI, M. V. et al. Endocrine disruptors and reproductive health: The case of bisphenol-A. **Molecular and Cellular Endocrinology**, v. 254–255, n. August, p. 179–186, 2006.

MALLER, O.; MARTINSON, H.; SCHEDIN, P. Extracellular matrix composition reveals complex and dynamic stromal-epithelial interactions in the mammary gland. **Journal of Mammary Gland Biology and Neoplasia**, v. 15, n. 3, p. 301–318, 2010.

MAN, Y. G.; SANG, Q. X. A. The significance of focal myoepithelial cell layer disruptions in human breast tumor invasion: A paradigm shift from the "protease-centered" hypothesis. **Experimental Cell Research**, v. 301, n. 2, p. 103–118, 2004.

MASSO-WELCH, P. A. et al. A developmental atlas of rat mammary gland histology.

Journal of Mammary Gland Biology and Neoplasia, v. 5, n. 2, p. 165–185, 2000.

MEANEY, M. J.; SZYF, M.; SECKL, J. R. Epigenetic mechanisms of perinatal programming of hypothalamic-pituitary-adrenal function and health. **Trends in Molecular Medicine**, v. 13, n. 7, p. 269–277, 2007.

MELI, R. et al. Oxidative stress and BPA toxicity: An antioxidant approach for male and female reproductive dysfunction. **Antioxidants**, v. 9, n. 5, 2020.

MORI, H. et al. Transmembrane/cytoplasmic, rather than catalytic, domains of Mmp14 signal to MAPK activation and mammary branching morphogenesis via binding to integrin β1. **Development (Cambridge)**, v. 140, n. 2, p. 343–352, 2013.

NEVILLE, M. C. et al. The Mammary Fat Pad. **Journal of Mammary Gland Biology and Neoplasia**, v. 3, n. 2, p. 109–116, 1998.

NIETO, C. M.; RIDER, L. C.; CRAMER, S. D. Influence of stromal-epithelial interactions on androgen action. **Endocrine-Related Cancer**, v. 21, n. 4, 2014.

NISHINO, N.; TOTSUKAWA, K. **Study on the Estrous Cycle in the Mongolian gerbil (Meriones unguiculatus)Experimental Animals**, 1996.

NORRIS, M. L.; ADAMS, C. E. Incidence of cystic ovaries and reproductive performance in the mongolian gerbil, Meriones unguiculatus. **Laboratory Animals**, v. 6, n. 3, p. 337–342, 1972.

PAMARTHY, S. et al. The V-atpase a2 isoform controls mammary gland development through notch and TGF-β signaling. **Cell Death and Disease**, v. 7, n. 11, p. 1–13, 2016.

PAWLINA, W.; ROSS, M. **Histologia: texto e atlas**. [s.l: s.n.].

RODGERS, K. M. et al. Environmental chemicals and breast cancer: An updated review of epidemiological literature informed by biological mechanisms. **Environmental Research**, v. 160, p. 152–182, 2018.

RUBIN, B. S. Bisphenol A: An endocrine disruptor with widespread exposure and multiple effects. **Journal of Steroid Biochemistry and Molecular Biology**, v. 127, n. 1–2, p. 27–34, 2011.

RUIZ, T. F. R.; TABOGA, S. R.; LEONEL, E. C. R. Molecular mechanisms of mammary gland remodeling: A review of the homeostatic versus bisphenol a disrupted microenvironment. **Reproductive Toxicology**, v. 105, n. May, p. 1–16, out. 2021.

RUSSO, I. H.; RUSSO, J. Mammary gland neoplasia in long-term rodent studies. **Environmental Health Perspectives**, v. 104, n. 9, p. 938–967, 1996.

RUSSO, J. et al. Pattern of distribution of cells positive for estrogen receptor a and progesterone receptor in relation to proliferating cells in the mammary gland. **Breast Cancer Research and Treatment**, v. 53, n. 3, p. 217–227, 1999.

SETA, D. DELLA et al. Bisphenol-A exposure during pregnancy and lactation affects maternal behavior in rats. **Brain Research Bulletin**, v. 65, n. 3, p. 255–260, 2005.

SHAFEI, A. et al. Stop eating plastic, molecular signaling of bisphenol A in breast cancer. **Environmental Science and Pollution Research**, v. 25, n. 24, p. 23624–23630, 2018.

SILVA, J. P. A. et al. Bisphenol-S promotes endocrine-disrupting effects similar to

those promoted by bisphenol-A in the prostate of adult gerbils. **Reproductive Toxicology**, v. 85, n. September 2018, p. 83–92, 2019.

STAPLES, C. A. et al. A review of the environmental fate, effects, and exposures of bisphenol A. **Chemosphere**, v. 36, n. 10, p. 2149–2173, 1998.

TERRY, M. B. et al. Environmental exposures during windows of susceptibility for breast cancer: A framework for prevention research. **Breast Cancer Research**, v. 21, n. 1, p. 1–16, 2019.

TOPPARI, J. Environmental endocrine disrupters. **Sexual Development**, v. 2, n. 4–5, p. 260–267, 2008.

TOPPER, Y. J.; FREEMAN, C. S. Multiple hormone interactions in the developmental biology of the mammary gland. **Physiological reviews**, v. 60, n. 4, p. 1049–1106, 1980.

TRISMEGISTRO, H. **O Caibalion**: Estudo da filosofia hermética do antigo Egito e da Grécia. 2ª (Padrão). Pensamento, 1908.

UNSWORTH, A.; ANDERSON, R.; BRITT, K. Stromal fibroblasts and the immune microenvironment: Partners in mammary gland biology and pathology? **Journal of Mammary Gland Biology and Neoplasia**, v. 19, n. 2, p. 169–182, 2014.

VIÑAS, R.; JENG, Y. J.; WATSON, C. S. Non-genomic effects of xenoestrogen mixtures. **International Journal of Environmental Research and Public Health**, v. 9, n. 8, p. 2694–2714, 2012.

VISVADER, J. E.; STINGL, J. Mammary stem cells and the differentiation hierarchy: Current status and perspectives. **Genes and Development**, v. 28, n. 11, p. 1143–1158, 2014.

WANG, Q. A.; SCHERER, P. E. Remodeling of Murine Mammary Adipose Tissue during Pregnancy, Lactation, and Involution. **Journal of Mammary Gland Biology and Neoplasia**, v. 24, n. 3, p. 207–212, 2019.

WANG, X. et al. A complex 3D human tissue culture system based on mammary stromal cells and silk scaffolds for modeling breast morphogenesis and function. **Biomaterials**, v. 31, n. 14, p. 3920–3929, 2010.

WISEMAN, B. S.; WERB, Z. Development: Stromal effects on mammary gland development and breast cancer. **Science**, v. 296, n. 5570, p. 1046–1049, 2002.

WISNIEWSKI, P. et al. Adult exposure to bisphenol A (BPA) in Wistar rats reduces sperm quality with disruption of the hypothalamic-pituitary-testicular axis. **Toxicology**, v. 329, p. 1–9, 2015.

YANG, Y. J. et al. Bisphenol A exposure is associated with oxidative stress and inflammation in postmenopausal women. **Environmental Research**, v. 109, n. 6, p. 797–801, 2009.

ANEXO I – Protocolo CEUA 217/2019

unesp UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"
Câmpus de São José do Rio Preto

COMISSÃO DE ÉTICA NO USO DE ANIMAIS – IBILCE/UNESP-CSJRP

CERTIFICADO

Certificamos que a proposta intitulada "Efeitos tardios da exposição ao bisfenol A na mama de fêmeas de gerbilos durante o período gestacional e lactacional", registrada com o nº. 217/2019 - CEUA, sob a responsabilidade do Professor Doutor Sebastião Roberto Taboga, que envolve a produção, manutenção ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto humanos), para fins de pesquisa científica (ou de ensino), encontra-se de acordo com os Preceitos da Lei nº 11.794, de 08 de outubro de 2008, do Decreto nº 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi aprovada pela COMISSÃO DE ÉTICA NO USO DE ANIMAIS (CEUA), do IBILCE/UNESP, em 30 de julho de 2020.

Finalidade	( ) Ensino (X) Pesquisa Científica
Vigência da autorização	01/08/2020 a 01/08/2022
Espécie/linhagem/Raça	Meriones unguiculatus (gerbilo da Mongólia)
Nº de animais	40
Peso/Idade	70 g/03 meses
Sexo	Macho e fêmea
Origem	Biotério do Departamento de Biologia do Instituto de Biociências, Letras e Ciências Exatas – UNESP/Campus São José do Rio Preto - SP.

São José do Rio Preto, 30 de julho de 2020.

Profa. Dra. Eliane Gonçalves de Freitas
Presidente da CEUA

Observações:

1) O Relatório Final deverá ser encaminhado em Formulário próprio à CEUA no prazo de até 30 (trinta) dias após o término da pesquisa;
2) Qualquer alteração na pesquisa deverá ser encaminhada à CEUA para apreciação.

ANEXO II – Ruiz et al., 2021 (Reproductive Toxicology)

Reproductive Toxicology 105 (2021) 1–16

Contents lists available at ScienceDirect

Reproductive Toxicology

journal homepage: www.elsevier.com/locate/reprotox

Molecular mechanisms of mammary gland remodeling: A review of the homeostatic versus bisphenol a disrupted microenvironment

Thalles Fernando Rocha Ruiz [a], Sebastião Roberto Taboga [a], Ellen Cristina Rivas Leonel [a,b,*]

[a] São Paulo State University (Unesp), Department of Biology, Institute of Biosciences, Humanities and Exact Sciences, São José Do Rio Preto, Brazil
[b] Federal University of Goiás (UFG), Department of Histology, Embryology and Cell Biology, Institute of Biological Sciences, Goiânia, Brazil

ARTICLE INFO

Handling Editor: Dr. Anna Bal-Price

Keywords:
Endocrine disruptor
Development
Breast
Hormone
Pathway
Susceptibility

ABSTRACT

Mammary gland (MG) undergoes critical points of structural changes throughout a woman's life. During the perinatal and pubertal stages, MG develops through growth and differentiation to establish a pre-mature feature. If pregnancy and lactation occur, the epithelial compartment branches and differentiates to create a specialized structure for milk secretion and nurturing of the newborn. However, the ultimate MG modification consists of a regression process aiming to reestablish the smaller and less energy demanding structure until another production cycle happens. The unraveling of these fascinating physiologic cycles has helped the scientific community elucidate aspects of molecular regulation of proliferative and apoptotic events and remodeling of the stromal compartment. However, greater understanding of the hormonal pathways involved in MG developmental stages led to concern that endocrine disruptors such as bisphenol A (BPA), may influence these specific development/involution stages, called "windows of susceptibility". Since it is used in the manufacture of polycarbonate plastics and epoxy resins, BPA is a ubiquitous chemical present in human everyday life, exerting an estrogenic effect. Thus, descriptions of its deleterious effects on the MG, especially in terms of serum hormone concentrations, hormonal receptor expression, molecular pathways, and epigenetic alterations, have been widely published. Therefore, allied to a didactic description of the main physiological mechanisms involved in different critical points of MG development, the current review provides a summary of key mechanisms by which the endocrine disruptor BPA impacts MG homeostasis at different windows of susceptibility, causing short- and long-term effects.

1. Introduction

The mammary gland (MG) is one of the most dynamic organs in organisms of mammalian species. During life, the MG goes through several repeated cycles of growth and involution to allow the preparation of the tissue for milk production and nourishment. Although slight morphological alterations take place during the estrous/menstrual cycle as a response to ovarian hormones [1], profound modifications occur during gestation and lactation to allow full mammary development and its regression after weaning [2].

Indeed, an estrogenic environment dictates mammary fate to a well-developed gland in females [3]. This is the reason why so much interest has been given to the effects of endocrine-disrupting chemicals' on MG mechanisms of development and regression [4]. Endocrine disruptors (ED) are a class of chemicals that modify the normal functionality of the endocrine system [5]. Several synthetic chemicals (including plasticizers, pesticides, and synthetic compounds used in drugs) and naturally occurring compounds (such as phytoestrogens) are classified as EDs. They disrupt the orchestrated action of endogenous hormones by altering their synthesis, transportation, metabolism, and excretion, or even by competing for hormone receptor binding in the target tissue [6]. Xenoestrogens are EDs that impact pivotal estrogenic pathways [7] and consequently disrupt the reproductive system in humans and other mammals [8]. The harmful effects are not limited to the exposed individual and may also extend to the progeny, causing changes in their postnatal endocrine function [9].

Research groups have focused on the context of xenoestrogens exposure during developmental "windows of susceptibility" in different tissues [10]. These windows are stages of development which involve cell proliferation, differentiation, and apoptosis, as well as a

* Corresponding author at: Federal University of Goiás (UFG), Department of Histology, Embryology and Cell Biology, Institute of Biological Sciences, Avenida Esperança, s/n Câmpus Samambaia, Goiânia, 74001970, Brazil.
E-mail addresses: thalles.ruiz@unesp.br (T.F.R. Ruiz), sebastiao.taboga@unesp.br (S.R. Taboga), ellenleonel@ufg.br (E.C.R. Leonel).

https://doi.org/10.1016/j.reprotox.2021.07.011
Received 6 May 2021; Received in revised form 26 July 2021; Accepted 29 July 2021
Available online 31 July 2021

well-defined stroma/epithelium crosstalk process controlled by hormones [2,11]. Thus, in the MG not only fetal or infant exposure is cause for concern; adult exposure may also impact developmental windows of susceptibility and have adverse consequences [10]. In this aspect, since MG has the ability to grow and involute several times during the reproductive age in a homeostatic environment, xenoestrogen exposure and disruption during these tissue remodeling phases comprise a risk [7,12]. Currently, the most commonly studied MG windows of susceptibility are the in utero and postnatal developmental phases, however, in addition to these, the periods of puberty, gestation/lactation, and involution during post-weaning and menopause are matters of concern, since the gland is undergoing deep tissue morphological and physiological remodeling [13].

This review brings together aspects of morphology, physiology, and molecular signaling of the mammary structure – which will be designated as breast, when talking about the humans' structure and MG when related to other mammals – differentiation phases considered windows of susceptibility for endocrine disruption, aiming to discuss in parallel the pathological alterations caused by endocrine disruptor bisphenol A (BPA) exposure (Table 1). The "Normal mammary development" topic addresses homeostatic physiological effectors and molecular pathways that present relevant alterations when disrupted by BPA.

2. Normal mammary development

During fetal development, the origin of the MG is epidermal cells, which invaginate to form the ductal tree. From birth to puberty, the MG shows allometric growth and no significant differentiation takes place [2]. Once puberty arrives, the mature hypothalamic-pituitary-gonadal axis promotes the cyclic secretion of gonadotropins and sexual hormones, contributing to dramatic modifications in the gland (Fig. 1) [14].

The MG is under constant hormonal regulation, modifications in gene expression, cell metabolism, and plasticity [15]. These changes may impact physiological and metabolic homeostasis and make the individual prone to future disorders due to chemical exposure [16]. Three main windows of susceptibility were described and are responsive to these regulating factors, which orchestrate the following changes:

(1) Postnatal and pubertal developmental phases: a massive architectural change takes place in response to estrogenic regulation, which mainly influences stromal-epithelial interactions [17] and induces intense epithelial cell proliferation [2]. During this window, xenoestrogens may increase the length of the period of ductal growth and alveologenesis throughout the menstrual cycle, by acting simultaneously to endogenous E2 [10].
(2) Pregnancy and lactation (Figs. 2 and 3): the gland development is influenced by progesterone and prolactin receptor binding [18]. Impacts of gestational/lactational exposure to xenoestrogens have been described in the post-weaning gland and are related to changes in the expression of estrogen and progesterone receptors [19].
(3) Post-weaning and menopause involution (Fig. 4): the remodeling MG mimics a tumorigenic malignant microenvironment in terms of proteinase expression [20] and inflammatory process [21]. In this aspect, not only epithelial cells but also stromal fibroblasts are targets of concern due to their role in the extracellular matrix (ECM) synthesis [22]. When exposed to disruptors during menopause, MG may be prone to an increase in ductal cell proliferation, with higher chances of DNA mutations and, consequently, breast cancer susceptibility [23].

Molecular signaling and physiological control act in the gland morphogenesis by different intra- and extracellular pathways. In the estrous/menstrual cycle, the MG undergoes a proliferation process followed by apoptosis of its ductal structures (Fig. 1B and F). In pregnancy, as detailed in Fig. 2, rapid proliferation of the duct and secretory epithelium (Fig. 1C and G) occurs, stimulated by estrogen, and progesterone leads to the differentiation of specific epithelial cells for synthesis and secretion of milk (Fig. 2) [24]. This epithelial structure, called alveolus, presents luminal epithelial cells that synthesize milk components by prolactin stimulus to be released during lactation (Fig. 3) [11]. Bipotent mammary stem cells (MSC) proliferate and differentiate into luminal and myoepithelial progenitor cells [25]. These latter cells are located around the ductal and alveoli system and, when stimulated by oxytocin, contract to expel milk from the luminal cells to the nipples [11]. At weaning, the lack of suckling stimulus decreases the production of oxytocin and, later, lactating hormones, leading to the process of MG involution, with a drastic rearrangement of cell compartments mediated by apoptotic events (Fig. 4) [26].

Hormones, growth factors, cytokines, and several metabolites interact among complex pathways to trigger proliferation, differentiation, and/or apoptosis in epithelial and stromal cells and ECM. The role of physiological and molecular key elements to understand the mechanisms involved in MG processes, has been little studied [27–32]. Thus, the role of the following elements will be discussed in this review: hormones; growth factors; adipokines; epigenetic regulation; and microRNAs (miRNA).

2.1. Hormones

The steroid hormones estrogen and progesterone play a central role during the menstrual/estrous cycle and coordinate structural changes in mammary tissue. Post-natal activity of MG correlates to ovarian onset secretion of these hormones and their systemic release [33]. In MG, estrogen and progesterone interact with other physiological regulators through a paracrine mechanism in the epithelial compartment, and through paracrine/autocrine pathways in the stroma, mainly to promote proliferation during development, as well as secretion (lactation) and apoptosis (involution) [34,35].

Prolactin contributes with progesterone to stimulate development and establishment of the lobuloalveolar tree in late pregnancy [36–38]. Its highest levels are present with the post-parturition decrease in estrogen and progesterone (milk secretion-inhibiting hormones) due to removal of the placenta [39]. Suckling is the mechanical stimulation that promotes prolactin and oxytocin release during lactation [40]. Oxytocin is a hypothalamic hormone released in response to sucking stimulation by the newborn, transmitted through the neuronal system [41]. Its targets are myoepithelial cells acting to expel milk toward the nipple region [42]. Recently it was demonstrated that myoepithelial cell contraction is crucial not only for the ejection of milk secreted by luminal cells in alveoli but also for the extrusion of lipid droplets of these cells [43].

2.1.1. Estrogen

In puberty, estrogen is required for MG development each estrous/menstrual cycle [44–46]. Serum estrogens act on target cells, crossing the cell membrane and binding to estrogen receptors (ER). Targets are mainly epithelial cells, which develop a relevant proliferative signaling. Two major ER receptors have been described: ER-alpha (ERα) and ER-beta (ERβ), which present different expression rates during pubertal, pregnancy, and lactation stages [47]. ERα and ERβ may be co-expressed in cells and have been described during lactation, although estrogen levels are decreased for the action of lactating hormones, such as prolactin [45,48]. In rodents, both are expressed in mammary epithelial and stromal cells and develop an essential and independent role to coordinate the branching process of the ductal system [35,49]. ERα is fundamentally expressed in mammary duct epithelial cells, promoting its elongation during the developmental stage [35]. When expressed during pregnancy, ERα triggers proliferative and differentiation activity in epithelial cells [45,50]. For ERβ, an antiproliferative role is assigned [49].

ER activation triggers the activity of many transcription factors and

Table 1
Aspects impacting mammary gland during different windows of susceptibility: normal versus BPA disruption.

	Normal physiology	BPA disruption*
Systemic changes	HPG axis regulates in puberty the estrous/menstrual cycles, promoting a follicular phase with estrogenic induction of MG growth and luteal phase with progesterone stimuli for establishment of structures for a possible pregnancy [1,2]	*Perinatal* - disruption of the hypothalamic-pituitary-gonadal axis [266]; - increase in estrous cycles length [181]; - reduced serum levels of E2 and progesterone during pregnancy [180]; *Puberty* - insulin and leptin resistance [183]; - alteration in adiponectin levels [267]; *Gestational* - gestational diabetes [268]; - damage in pancreatic cell cycle [185];
Morphological and Molecular changes	Mammary gland develops during embryonic period from epidermal cells originating rudimentary bud structures [2]. Each estrous/menstrual cycle modifies the MG through hormonal signal and stimuli of many growth factors [29,30,31,32]. When pregnancy occurs, there is induction of proliferation through progesterone regulation for structural changes of secretory acini [66,68,69,70,71,72]. Luminal cells accumulate milk and secrete it after parturition, when lactation takes place [18]. Prolactin regulates the subcellular production of milk [36,37,38,39] and the mechanical-dependent hormone oxytocin controls the contraction of myoepithelial cells for milk ejection [11]. In post-weaning, apoptotic pathways are activated and immune cells are recruited for MG involution of epithelium and stroma [26].	*Perinatal* - acceleration of epithelial morphogenesis [181]; - increase in estrogen sensitivity [208]; - changes in expression of pathways and growth factors that regulates proliferation (AKT/ERK and TGFβ) [216]; - MMP expression in stroma induced by ERR-γ activity – stromal organization [217]; - alteration in sensitivity to progesterone – triggers Wnt-4 and Nf-kB [219]; - increases RANKL expression, enhancing PR signaling [219,220]; - inflammation and autophagic activity induced by NF-kB and mTOR signaling [221]; - stromal changes by collagen fibers synthesis enhanced and in epithelial producing hyperplasia [209,255,269]; - tissue inflammation [256,257]; *Puberty* - adipocytes differentiation [270]; - increase in caspase-3 cascade for apoptosis [209]; - changes in expression of proliferative proteins – Ki-67, p16 and cyclin E [209,210,211] *Gestational* - overexpression of Areg [215]] *Perimenopausal* - increase in production of inflammatory and oxidative markers [237]; - increase in mammographic density [197]
ER-related effects	At puberty, ER signal acts in MG development at each estrous/menstrual cycle [44,45,46]. Co-activators and co-repressors modulate the gene expression in MG [38,53,55]. ER-estrogen binding signal modulates activity of mitogens [61], regulates the stromal response [35,56], and differentiation activity in epithelial cells during pregnancy [45,49,50].	*General* - high affinity and boosted effect in ERβ+ signal; - act as agonist or antagonist in interactions with ERα; - long-lasting epigenetic alterations that impact gene expression [200];
Epigenetics and microRNA	Epithelial-luminal differentiation is regulated by several DNAm during MG development [115]. MicroRNAs regulate post-transcriptional gene expression for establishment of mature MG: - miRNA-101 (regulates differentiation) [151]; - miRNA-212 and miR-132 (MMP expression) [152]; - miRNA-378, miRNA-423−5p and miRNA-7 (milk synthesis) [154]; - miRNA-204 (Areg expression) [157]; - miRNA-152 (AKT pathway during the proliferation) [155]; - miRNA-126 family and miRNA-15a family (expression and secretion of milk proteins) [lange 1998]; - miRNA-101a (COX2 control) [151]; - miRNA-1, miRNA-152, and miRNA-155 (proliferative factors expression) [131];	*Perinatal* - DNA hypermethylation caused by oxidative stress [236]; - alpha-lactalbumin gene changes [239]; - increase the responsiveness of cells to estrogens by hypomethylation of Hoxa10 [232]; - increase miRNA-217 (endothelial cells damage) and miRNA-608 (cell cycle arrest) [250] *Gestational* - alterations in the β–casein gene's methylation [237]

* The alterations mentioned in the third column are related to the specific periods of BPA exposure. HPG: hypothalamic-pituitary-gonadal; MG: mammary gland.

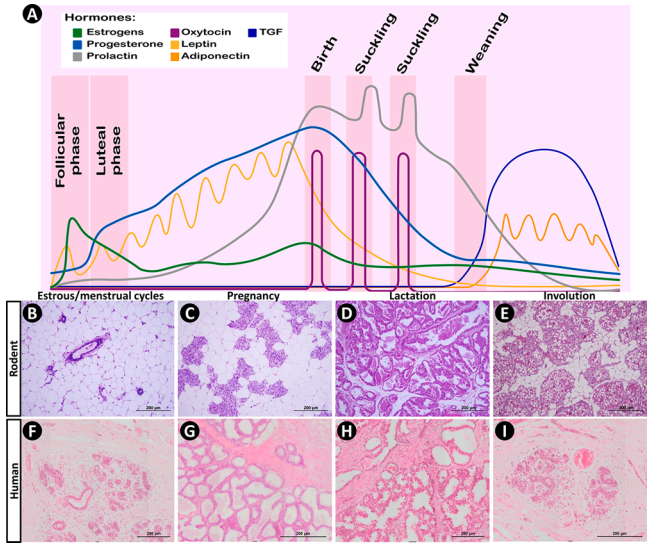

Fig. 1. The histological aspects of postpubertal rodent (*Meriones unguiculatus*) and human mammary gland in response to the main serum hormonal changes (A). During estrous or menstrual cycles (B and F, respectively) subtle morphological changes take place in response mainly to estrogens and progesterone. However, a drastic alteration in epithelial structures occurs in response to progesterone and leptin during gestation (C;G) and in response to prolactin and oxytocin during lactation (D;H). Weaning triggers involution of the epithelium, that responds to an increase in TGF-β and adiponectin and a reduction in other serum hormones (E;I).

nuclear estrogen response elements (EREs) in the DNA, which alters gene expression [51,52]. Co-regulators enhance the transcriptional activity promoted by estrogen-ER-binding, activating (co-activators) or repressing (co-repressors) the gene expressed [38,53,54].

Paracrine signaling occurs when estrogen-ER binding promotes the proliferation of ER-negative epithelial and stromal cells during MG development [55,56]. The stromal paracrine induction pathway acts mainly by modulating the expression and effectiveness of mitogens [57,58]. ERα expression in stromal cells is therefore mandatory for MG development through its interaction with and modulation of growth hormones and cognate receptors [59].

Although estrogen is not directly related to MG involution, its regulation during late lactation and the beginning of tissue remodeling is required. During involution, ER co-regulators are responsible for determining the apoptotic process of the cells, a drastic phenomenon to remodel the structure of the MG. The most important regulation of the involution process is the interaction of the transforming growth factor-beta (TGF-β) pathway and ERα [60]. Although ERα also modifies the TGF-β cascade, the high expression of TGF-β in mammary tissue restrains the ER pathway and activates apoptosis [61]. During the post-menopausal period, involution of the MG adipose tissue is the primary source of estrogen synthesis, assuring high local levels of 17β-estradiol (E2) and ERα-positive epithelial cells despite low serum levels of steroids [62].

2.1.2. Progesterone

Progesterone is mainly secreted by the corpus luteum during the mid-term ovarian cycle, and the placenta during pregnancy [63,64]. Although progesterone and estrogen are interdependent hormones during mammary proliferative activity in the mouse model, the progesterone receptor (PR) requires transcriptional induction by ERα to be expressed in epithelial cells, while PR expression in stromal cells is estrogenic-independent [65,66]. This hormone has two receptor isoforms, PR-A and PR-B, in which PR-A presents high relevance in the uterus development, while PR-B is the main effector of the proliferative process in MG [67,68].

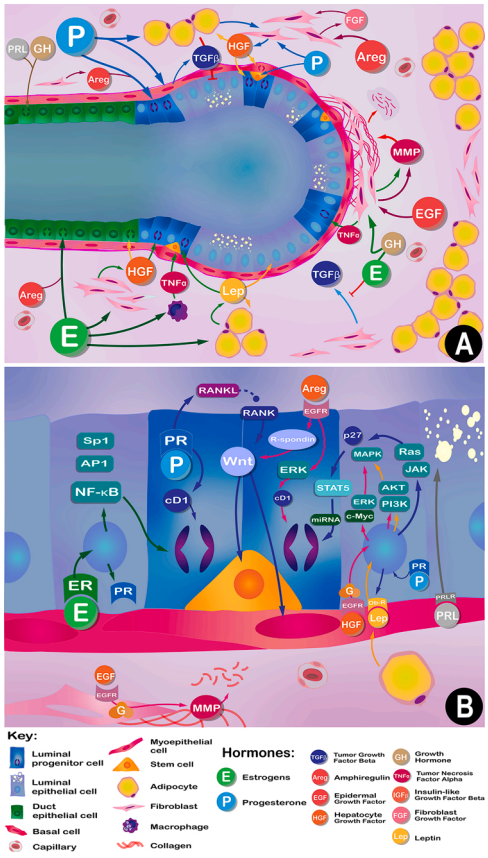

Fig. 2. Pregnancy. (A) The alveolar structure establishment during pregnancy occurs through stromal remodeling and epithelial proliferation. *E* and *P* act as the main hormones that control these changes. *E*-induced pathways: the proliferation of ductal epithelial cells is associated with *Areg* or induced by the synthesis and secretion of hormones by stromal cells – *HGF* by fibroblasts, *Lep* by adipocytes, *TNFα* by fibroblasts and inflammatory cells. Furthermore, *E* promotes stromal remodeling by stimulating the synthesis of *MMPs* by fibroblasts. *P*-induced pathways: in association with *Areg* promotes luminal epithelial proliferation, and stimulates *HGF* synthesis by fibroblasts for the establishment of myoepithelial cells, luminal cell proliferation, and basal cell differentiation. *EGF* acts primarily to stimulate the synthesis of *MMPs* to break down connective fibers. *Areg* acts on fibroblasts by increasing *FGF* synthesis for autocrine and paracrine proliferation signaling. (B) Molecular signaling in mammary development during pregnancy. *E*-receptor *ER* binding triggers the synthesis of progesterone receptor *PR* as well as proliferative signaling through *NF-κB*, *AP1*, and *Sp1*. *P-PR* binding triggers a mitogenic response in luminal epithelial cells by increasing *cD1* and *RANKL-RANK/Wnt* for paracrine signaling in basal and myoepithelial cells. *Areg* also acts via *R-spondin* to trigger a response via *Wnt*, in addition to activating *ERK* and *cD1* for cell proliferation. In addition, *P-PR* signals leptin receptor synthase *Ob-R*. The proliferation of epithelial cells occurs through the expression of *p27* and *MAPK* to trigger a signal via *STAT5* and *miRNA*. The expression of *p27/MAPK* occurs through three main pathways: by progesterone activation, via the *JAK/Ras* pathway; by leptin stimulation through the *PI3K/AKT* pathway; or by *HGF* via *ERK/c-Myc* or leptin-like pathways.

The mechanism of action of progesterone is summarized in two steps: the first involves PR + cells and the performance of cyclins D1 (cyclin D1-dependent mechanism); while the second involves PR- cells and the paracrine function, dependent on receptor activator nuclear transcription factor-kappa B ligand (RANKL), a membrane receptor of the TNF superfamily (RANKL-dependent mechanism) [69]. The second step depends on binding of RANKL to its receptor, RANK, to promote alveolar development [67,70–73]. RANKL is a paracrine mediator expressed only in the presence of higher availability of progesterone and depends on the presence of estrogen, *i.e.*, in PR + ER + cells [74]. PR + epithelial cells

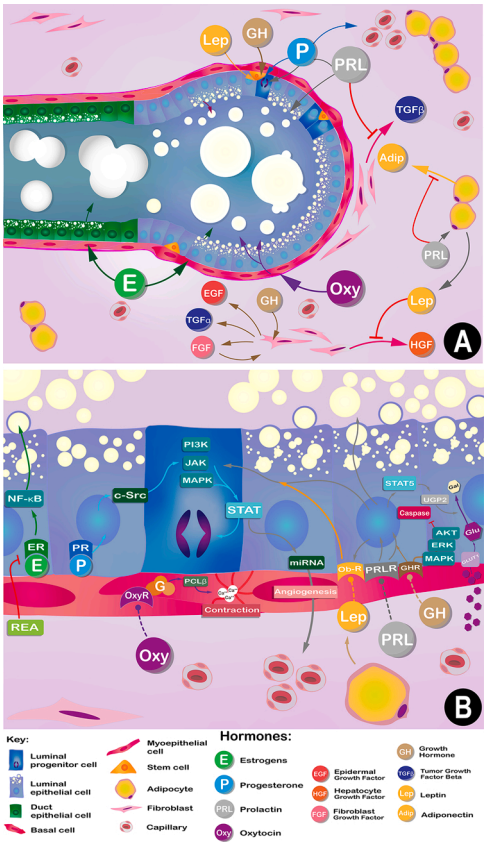

Fig. 3. Lactation. (A) During lactation, in addition to *PRL*, *P* and *E* aid the secretion of milk produced by luminal and ductal cells. *P* acts via induction of *PRL* in the secretion and proliferation of luminal cells, as well as *Lep* and *GH*, to maintain the alveolar structure. To expel milk from the alveoli, oxytocin acts on myoepithelial cells to promote their contraction. In the stroma, *GH* acts to stimulate the production of *EGF* and *FGF* by fibroblasts, while adipocytes are stimulated by prolactin to produce leptin, inhibiting the mitogenic action of *HGF*, and inhibiting the synthesis and activity of *Adip* and *TGFβ*. (B) The molecular pathways of synthesis and secretion, activated during lactation are coordinated by events stimulated by *PRL*. Through *PRL*-receptor (*PRLR*) binding there is a direct stimulation for the synthesis and secretion of milk and conversion of glycogen (*Glu*) into galactose (*Gal*) via *STAT5/UGP2*. In addition, during lactation, anti-apoptotic mechanisms are evoked, activated by *GH*, and triggered by the expression of *MAPK/ERK/AKT*, inhibiting the activity of *Caspase*, and proliferation for maintenance of the mammary gland epithelium through the expression of *PI3K/JAK/MAPK* for *STAT* establishment. Activation of angiogenic mechanisms occurs via *miRNA*. *P-PR*, via *c-Src*, activates cell mitogenic pathways similar to those produced by *PRL* and *Lep* stimulation. *E* helps to secrete milk into the lumen via *NF-κB*, a mechanism that may be inhibited by *REA*. *Oxy* acts on *G* protein-coupled receptors (*OxyR*) in myoepithelial cells to activate *PCLβ*, triggering the contraction of these cells.

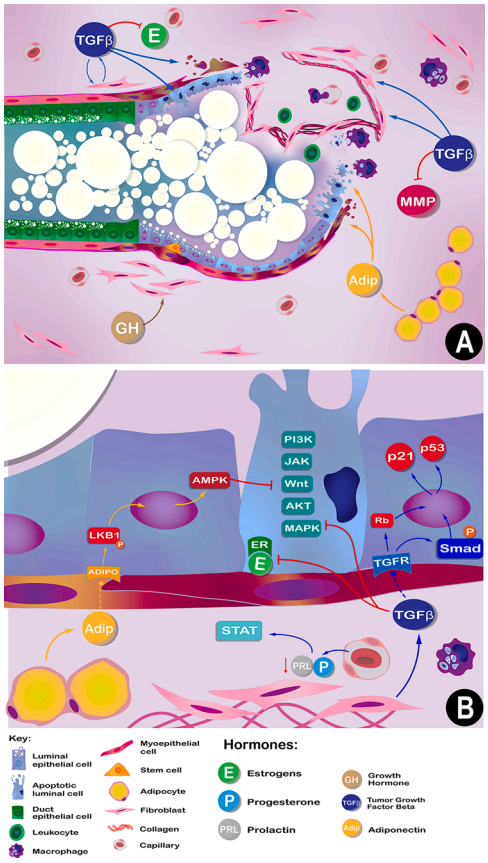

Fig. 4. Involution. (A) During mammary involution, *TGFβ*, like *Adip*, acts as an apoptotic promoter in epithelial cells, inhibiting the proliferative action of *E*. *TGFβ* acts on the proliferation of stromal cells to increase this compartment, inhibiting the action of *MMP* and acting as a chemotactic factor for inflammatory cells. *GH* acts on the stromal compartment for fibroblast proliferation. (B) *TGFβ* and adiponectin act by inhibiting classical proliferative pathways in luminal cells, such as *PI3K, JAK, Wnt, AKT,* and *MAPK*. *Adip* acts specifically through a receptor-binding signal (*ADIPO*) to phosphorylate *LKB1* to increase the synthesis of *AMPK*, a proliferative inhibitor. Apoptosis in epithelial cells is generated by the activation of *Rb* and via phosphorylation of the *Smad* protein, which activates the expression of *p21* and *p53*, both pro-apoptotic proteins. Furthermore, the drastic reduction in *PRL* and *P* acts as a signal for the increase in *STAT* expression, providing a favorable environment for the gland regression process.

express and secrete WNT4 protein that regulates progesterone paracrine activity through myoepithelial and basal cells [75–77].

The main role of progesterone role is stimulation of growth of terminal end buds (TEBs) and ductal elongation and branching [78]. Epithelial cells of the ductal tree show high sensitivity to progesterone related to its receptor since it increases the side branching process and alveologenesis in the MG and leads to the production of new epithelial PR sites [79]. In addition, progesterone promotes relevant activation of the lactation process, supporting the prolactin pathway.

In pregnancy, progesterone stimulates the synthesis of TGF-β and Wnt proteins which repress milk secretion [17]. With a perinatal decrease in progesterone levels, lipid and protein biosynthesis pathways, such as protein kinase B (AKT) and sterol regulatory element-binding proteins (SREBP), are upregulated [29]. Nonetheless, circulating progesterone serum levels decrease in late pregnancy and post-parturition, signaling for the secretion of milk, stored in luminal cells, in response to the increase in prolactin levels [69]. The upcoming increase in progesterone occurs only at the mid/end of the involution when there is a stimulus for STAT1 and STAT3 phosphorylation that triggers the cell death process [80,81].

2.2. Metabolic hormones and growth factors

2.2.1. Family of epidermal growth factors (EGF)

EGF is a family of proteins that stimulates proliferation response and differentiation in MG cells. Two primary receptor categories are related to EGF binding-signal: human epidermal growth factor receptor 2 (HER2), or ErbB2/*neu* in rodents [82], and a cognate receptor, epidermal growth factor receptor (EGFR), or ErbB1/HER1 [83]. HER-EGF signaling activates the complex phosphoinositide 3-kinases (PI3K)/AKT/mTOR cascade for proliferative and anti-apoptotic processes [84,85]. EGFR also initiates MAPK and STAT pathways, triggered by other hormones and growth factors (Figs. 2 and 3) [86,87].

The EGF-EGFR binding pathway is triggered from the transactivation

of G-protein coupled estrogen receptor (GPER) that increases MMP expression and stimulates the ERK phosphorylation for c-Myc mitogenic activity in the stroma, ducts, and TEBs [88,89]. This GPER-induction of MMPs is regulated by the c-Src pathway that amplifies mRNA expression [86]. Moreover, EGF determines an epithelial expansion toward stroma by a hyaluronan small-fragments type signal in the ECM [88]. Thus, EGF activity in stromal and epithelial compartments is protease-dependent, supporting the epithelial proliferation needs for stromal ECM relaxation [90]. MMPs are responsible not only for the proteolytic activity of extracellular components in the stroma [91] but also to promote the cleavage of the newly synthesized EGF molecule transmembrane precursors [92].

Amphiregulin (Areg) is a transmembrane glycoprotein, a precursor of the EGF family, synthesized in the epithelium and stroma [93]. Areg binds to EGFR type to promote fibroblast proliferative activity, stimulating secretion and autocrine action of fibroblast growth factor [94,95]. It also mediates estrogen-paracrine signals during ductal proliferation and progesterone-dependent morphogenesis in pregnancy [96–98]. Areg acts by increasing DNA synthesis through the ERK pathway that stimulates the upregulation of cyclin D1, modulating the progression of the cell cycle [99]. Furthermore, Areg induces R-spondin protein expression in luminal cells that synergize with Wnt to determine cell fate [100].

2.2.2. Transforming growth factor-β

TGF-β is a cytokine that presents dual activity: antiproliferative or mitogenic effects, depending on the tissue [61]. It modulates cell activity by MAPK and AKT pathways to provoke antiproliferative/apoptotic effects [101]. For involution regulation, TGF-β promotes ECM remodeling by its expression in myoepithelial cells and cross-regulation of the AKT pathway (Fig. 4) [102–104]. The last mechanism involves prolactin crosstalk, which stimulates PI3K/AKT activation for cell survival, inhibiting the TGF-β -apoptotic signaling [105]. After weaning, prolactin levels decrease and cease stimulation of PI3K/AKT, leading to apoptotic fate mediated by TGF-β [101,104]. TGF-β modulates cyclin-dependent kinase (CDK) activity and reduces c-Myc, the protein coded by the *Myc* gene that regulates cell cycle progression and expression in epithelial cells, acting as a ductal elongation inhibitor interrupting the epithelial cell cycle [106]. Indirectly, TGF-β regulates the expression of p21 and p53, which promote cell cycle arrest and pro-apoptotic induction during late lactation and involution [102,107].

TGF-β regulates ER+/PR + cell fate is provided by activation of its cognate receptor, which promotes the nuclear accumulation of "small mothers against decapentaplegic" (Smad), a signal protein for cell development regulated by TGF-β, in its phosphorylated form [108]. In addition, estrogens and TGF-β interact during MG development. Estrogens present a TGF-β inhibition route, influencing Smad proteins through ERα signaling, promoting a ubiquitin-proteasome pathway for degradation of Smad [60]. This is a non-genomic mechanism of estrogens and TGF-β crosstalk, and an important co-regulation pathway which is not well established.

2.3. Adipokines

Adipokines are a class of hormonal-cytokines secreted mainly by adipocytes, which play an essential role in breast development [109]. Several adipokines, such as ghrelin, interleukin 6 (IL-6), adiponectin, and leptin are involved in mammary stroma remodeling and cell proliferation mechanisms [56].

Leptin and adiponectin act in opposite ways during the development, lactation, and involution of the MG. Leptin induces cell-cell interaction between adipocytes and epithelial cells, promoting epithelium proliferation and differentiation (Figs. 2 and 3) [109,110]. Through this mitogenic ability, leptin also expands the stem and progenitor cell population in the epithelial compartment by stimulating the PI3K/AKT pathway [111]. Extramammary leptin is a significant effector in mice mammary tissue by stimulation of the hypothalamic-pituitary-ovarian axis to produce estrogens [112]. Although leptin acts as an HGF inhibitor, in the presence of prolactin, it promotes mitogenic pathways [113]. In cows, prolactin increases leptin receptor (Ob-R) expression and leptin mRNA expression during lactation [114,115]. Ob-R binding triggers most intracellular proliferation pathways, such as MAPK, PI3K, JAK/-STAT, and mTOR [116], and regulates lactogenesis and fatty acid synthesis in bovine MG [114].

Adiponectin, contrarily, activates antiproliferative and pro-apoptotic pathways, promoting 5′ adenosine monophosphate-activated protein kinase (AMPK) cascade [117,118], which inhibits proliferative pathways, *i.e.*, MAPK, AKT, Wnt, JAK, and PI3K (Fig. 4) [119–121]. Prolactin inhibits adiponectin release in the breast [122]. This lactation hormone and growth hormone regulate adiponectin transcription and its two receptors, ADIPOR1 and ADIPOR2 [118,122,123]. Both hormones modulate STAT5 binding to adiponectin gene promoter, aiming to decrease its expression [124]. Thus, adiponectin mRNA expression decreases during lactation when prolactin and GH are at high circulating levels [123].

2.4. Epigenetic Regulation and miRNA

The epigenetic regulation of MG development is crucial to specific aspects of cell differentiation, notably in the epithelial compartment [125]. Processes related to DNA methylation (DNAm), histone modifications, and miRNA expression play crucial roles in pathway crosstalk mechanisms, previously explained, and repressing/silencing of the expression of essential genes [126]. Briefly, DNAm and histone modifications alter the chromatin state, enabling the inhibition or promotion of genes during mammary development, whereas miRNA impacts mainly the post-transcriptional process [125,127,128].

2.4.1. DNA methylation

In general, DNAm is an epigenetic tool by which cells "lock" genes; this means that when methylation occurs, gene silencing takes place [125]. However, the impacts of DNAm vary according to the context since it may also modulate active gene transcription [129]. In MG, when DNAm takes place in tumor-suppressing genes it leads to carcinogenesis [130]. DNAm is mediated by DNA methyltransferase enzymes (DMTs) occurring in CpG (cytosine-phosphate-guanine) islands [126]. During MG development, the expression of DMTs increases (for hypermethylation) or decreases (for hypomethylation) in cells linked to epithelial-luminal differentiation, such as stem and progenitor cells [127]. DNAm in specific genomic regions contributes to the differentiation and self-renewal processes observed by reducing Ki-67 and SOX transcription factor expression in stem and luminal progenitor cells [131]. Thus, DNAm is associated with silencing genomic regions related to cell fate or expression of genes that determine the cell lineage. During lactation, genes linked to milk proteins are demethylated and start to be expressed in luminal epithelial cells [125]. Furthermore, methylation/demethylation events in DNA regulate the expression of oxytocin receptors in the MG, indirectly modulating this hormone action in lactation [132].

2.4.2. Histone modifications

Molecular alterations in histones, such as acetylation, ubiquitination, methylation, and demethylation, regulate DNA accessibility and transcription of specific chromatin sites [133]. These post-translational modifications present a variable role in progenitor, basal, and luminal epithelial cells during MG development [134,135]. Thus, histone-modifier proteins in mammary tissue regulate epithelial-mesenchymal transition and cellular hierarchy [127]; histone methyltransferases and demethylases control these modifications. The maintenance of this activity is performed by polycomb repressive complexes (PRCs), *i.e.*, protein groups that mediate the transcriptional signal of essential proteins for cell fate. The most commonly studied PRCs in MG are the histone-lysine N-methyltransferase EZH2, which

regulates differentiation in TEBs and progesterone-induced proliferation, and the K demethylases group (KDM), which mediates luminal progenitor cell differentiation [136–139]. This regulation also indirectly impacts the pathway of paracrine differentiation induced by EGF and TGF-β [134].

2.4.3. miRNA

miRNAs are a family of RNAs that do not code known proteins but can silence gene expression through translational suppression [140]. They are produced in the nucleus and go through a series of modifications in the cytoplasm [141]. The fact that a single miRNA may target several genes means that the downstream effects can considerably affect many cell functions [142]. The existence of oncosuppressor miRNA has been described and these are downregulated in cancer [143]. The primary mechanism of miRNAs action is the post-translational targeting of specific sequences of mRNA, which leads to the suppression of the encoded protein [144]. miRNAs can either act on cells in which they have been transcribed or affect other cells and tissues from their translocation by exosome vesicles [145]. After being transcribed, miRNA binds to the Argonaute protein (AGO)/RNA-induced silencing (RISC) complex, between the translated region (open reading frame - ORF) or after the stop codon (3′prime untranslated region - 3′UTR), which keeps it silenced until its activation [146].

In the MG, miRNAs have been described to have specific physiological impacts: they regulate post-transcriptional expression of genes directly involved in mammary duct branching and acini formation (Fig. 2) [147,148]. The mechanisms involved in duct elongation are matrix metalloproteinase expression levels which are also related to the expression of miRNA [149]. Specific miRNAs are expressed according to the developmental stage of the MG [128,150]. They attenuate the expression of methyltransferases and acetyltransferases that impact two epigenetic mechanisms: DNAm [151], and histones acetylation [152]. The potential of miRNA under normal and/or pathological conditions is due to the inhibition of mRNA translation or transcript degradation (see review - Klinge (2015)). During breast development and lactation, miRNA acts mainly by inhibiting the expression of mRNAs and proteins linked to cell death and/or methyltransferases that suppress proliferative signals (Fig. 3) [154]. The Smad complex, generated by the stimulus of exogenous TGF, recruits co-activators in the nucleus and induces acetylation and expression of the TGF gene in stromal cells [155].

During involution, cyclooxygenase-2 (Cox-2) controls cell proliferation and the progress of MG to the regression phase, modulated by miRNA families [148]. In the murine model, downregulation of miRNA affects transcription and translation of MAPK, CDKs, and proliferative factor receptors [156]. Several miRNAs reduce the stability or translation of ERα mRNA, decreasing the influence of estrogen in cells [153].

3. Bisphenol A

BPA is one of the most widely studied EDs. Humans and a wide range of species are commonly exposed to BPA in everyday life. BPA is a xenoestrogen directly related to alterations in reproductive organs [6,157].

From a toxicokinetic point of view, the oral ingestion of BPA precedes transformation during the liver first pass metabolism, which converts it into BPA-glucuronide and sulfate conjugates, through a series of oxidation and hydroxylation reactions in hepatocytes due to the presence of cytochrome P450 [158]. Thus, the great majority of ingested BPA is metabolized within 5 h of its ingestion. Despite glucuronides have no estrogenic activity, they contribute to adipogenesis, as demonstrated in a murine preadipocyte cell line (3T3L1) [159] but other oxidative metabolites may have endocrine impacts, such as estrogenic activity [160]. Bisphenol S and Bisphenol F, two analogues that were proposed to replace BPA, have a similar metabolism [161]. BPA is considered a metabolic-endocrine disruptor with access to multiple cell levels (from cellular machinery to epigenetic fields) and multiple organs [162–165].

Although the hepatic metabolism of BPA is known to be quite fast in humans, unconjugated molecules have been detected in blood and urine samples, which means that non-oral routes, such as skin absorption, for example, may circumvent liver and intestinal metabolism [166]. According to Vandenberg et al., it is, however, difficult to identify all the sources of non-oral exposure to xenoestrogens [167]. In addition, bioaccumulation in fatty tissues is considerable due to its lipophilic feature [168]. Efforts by the Consortium Linking Academic and Regulatory Insights on BPA Toxicity (CLARITY-BPA) have described a list of target-organs affected by BPA according to exposure doses [165].

In vivo experiments describing the impacts of BPA in the MG have applied a range of different doses. Frequently, doses are representative concentrations of ubiquitous human exposure, according to public health organizations (FDA, EPA, WHO; [169]). In this review we focused on discussing results from papers that have applied either doses considered safe or doses that are commonly found in human blood or urinary samples. However, it is important to clarify that non-monotonic dose response relations in MG physiology and morphology have been assigned to BPA exposure. In summary, there is a consensus that BPA may cause effects at low doses that are not present at high doses [170,171]. Thus, it is crucial to take into consideration the possibility of a non-monotonic dose-response when comparing results obtained in different experimental designs, since the impacts of low doses may not be predicted by those of high doses. The main mechanisms by which BPA acts in the MG tissue and some consequences of exposure during windows of susceptibility will be discussed below.

3.1. Changes in serum hormone availability

The serum concentration of hormones is a factor that actively impacts MG homeostasis and dynamics (Fig. 5A). BPA exposed adult rats (25 mg/kg/day) showed increases in the serum levels of E2 and progesterone; in these animals, there was a higher generation of reactive oxygen species (ROS) and lower presence of antioxidants in granulosa cells, leading to an increase in inflammatory markers in the ovary [172]. Thus, BPA impacts steroidogenesis through direct influence in ovarian cells, which alters serum steroid concentrations [173]. Perinatal and prepubertal exposures to BPA (25 and 250 ng/kg/day) play a role in disrupting the hypothalamic-pituitary-gonadal axis and/or the metabolic homeostasis of hormones in mouse models [174]. Thus, these alterations may lead to disruption effects, since BPA (50−5000 mg/kg/day) was also proven to increase E2 bioavailability by inhibiting enzymes that inactivate this hormone [175–178]. Contrarily, perinatally treated F1 female rats (0.05 mg/kg/day or 20 mg/kg/day) showed reduced serum levels of E2 and progesterone during gestation [179]. Perinatal exposure to BPA (0.5 or 10 mg/kg/day) also triggered a disrupting process in female CD-1 mouse offspring through the increase in length of estrous cycles, impacting the MG homeostasis, and inducing accelerated morphogenesis of the epithelial structures [180].

BPA (0−3000 μg/kg/day) induced a dose-dependent decrease serum levels of leptin and adiponectin in mouse [181]. Obesity disorders are linked to modulation at the cellular level caused by BPA (Fig. 5A) – related to insulin and leptin resistance [182] and to the effect in human and murine preadipocyte differentiation [159] –, and changes in the pancreatic cell cycle, increasing the serum insulin level in dams exposed (10 or 100 ug/kg/day) during pregnancy [183,184].

3.2. Changes in hormone receptor machinery and expression

Variations in serum estrogen concentrations impact the MG homeostasis mechanisms. However, similarly to what happens in response to variations in endogenous estrogen levels, disrupting pollutants influence the expression and activity of hormone receptors (Fig. 5B) [185,186].

Xenoestrogens, similarly to endogenous estrogens, diffuse the cell membrane and bind to nuclear ERs (ERα and ERβ) [187]. Therefore,

hormone receptor binding has been considered the central toxicity mechanism by which these chemicals impact homeostasis of the organism [188]. When available in high concentrations, BPA also binds to androgen receptors, antagonizing their functions [168]. Even though BPA binds to ER, with an approximately 10^{-4} fold lower affinity than E2 [189], it reduces the ability of E2 to activate ERα+ cells [190]. Since BPA presents more affinity to ERβ [191–193], it may affect signaling of ERα by E2 [194]. Both receptor interactions have been proposed as responsible for triggering hormone-dependent tumors in the breast [195,196].

E2-ER binding is based on the affinity of the molecule to the ligand-binding domain of the receptor; for exogenous compounds, the presence of a para-hydroxy group assures this interaction [197]. Through binding, conformational changes in ER protein activate it, enabling genomic and non-genomic mechanisms [6]. However, studies comparing the binding features of compounds to ERs have shown that while E2 binds in similar ways to ERα and ERβ, other chemicals do this in different manners [198]. BPA was proven to exert an allosteric modulation, binding to a specific receptor site to increase agonist and antagonist functions [199]. A molecular modeling study revealed that BPA binding to the ERα "ligand-binding domain" disturbs the receptor-DNA recognition process [188], causing long-lasting epigenetic alterations that impact gene expression [200].

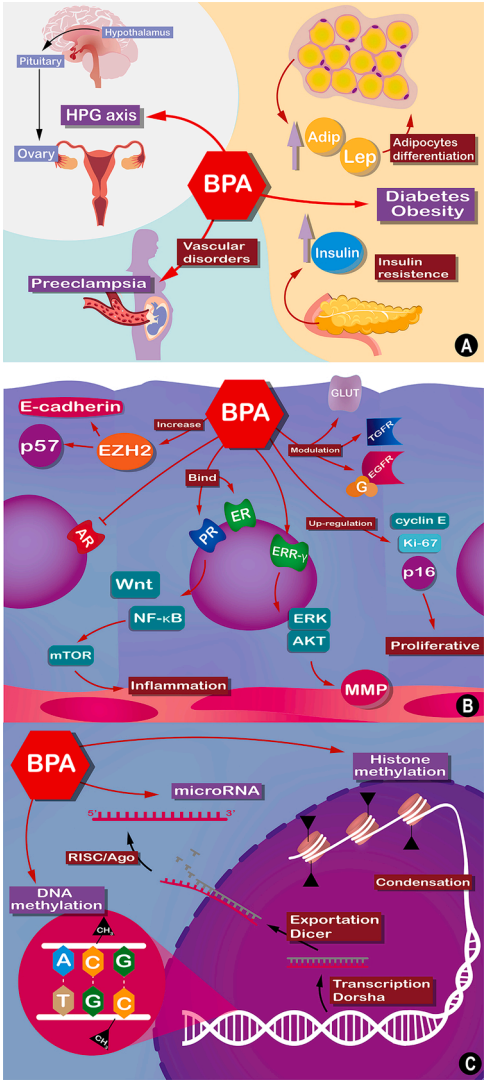

Fig. 5. Mechanisms by which bisphenol A induces mammary gland alterations. (A) Short- and long-term changes in morphological and, consequently, pathological aspects of the mammary gland. (B) Molecular pathways disrupted by BPA: binding to *ER* and *PR* to trigger inflammatory response through *mTOR* via *Wnt/NF-κB*; binding *ERR-γ* to trigger *ERK/AKT* pathways for stromal remodeling by *MMP* expression; modulating membrane receptors, such as *TFGR*, *EGFR*, and proteins, such as *GLUT*; and increasing *EZH2* protein synthesis, as an epigenetic disruption mechanism, and/or up-regulating proliferative proteins (*cyclin E, Ki-67, p16*). (C) Epigenetic mechanisms disrupted by BPA.

The ligand-receptor binding may trigger three different mechanisms. A genomic mechanism occurs based on binding of the activated receptor to EREs, specific nucleotide sequences in the DNA located near estrogen-regulated genes [201]. The second consists of an indirect genomic mechanism, which involves changes in gene expression as well, however, through interaction with other transcription factors, not EREs [202]. A non-genomic mechanism has also been described, based on the interaction of the ligand-receptor association with kinase receptors to influence other signaling pathways, impacting cell proliferation by stimulating ERα or reducing ERβ signaling [203].

The agonistic effect of xenoestrogens mimics the transcriptional activation promoted by endogenous estrogens. It is worth considering that the agonist/antagonist activity created by EDs depends on the target gene and cell type in question [201]. Among the wide range of estrogen target tissues – comprising female and male reproductive tracts, bone, and the cardiovascular system – is the MG [204]. As previously stated, estrogen plays a significant role in the MG, among other hormones, for cell differentiation and proliferation [2]. The expression varied throughout the lifetime in a gerbil model, responding to the current hormonal profile [205].

3.3. Interference in molecular pathways

Perinatal BPA exposure (250 ng/kg/day) in CD-1 and C57Bl6 mice increases MG estrogen sensitivity [206]. This sensitiveness implicates changes in the expression of proteins related to proliferation, such as Ki-67 [207], which presents an increase in its cytoplasmic expression related to breast tumors [208] and upregulation of p16 and cyclin E, to induce proliferation [209]. In contrast, BPA (5 mg/kg/day) also increases the caspase-3 cascade related to the involution process in albino rat mammary tissue [207]. Caspase is a cell death protein that could switch the normal apoptotic process response to necrotic activation [210].

EGFR response in breast cells is BPA-induced (1 μM, cell culture) through the GPER/EGFR transduction pathway [211]. ERK is rapidly activated by the GPER cascade after BPA exposure (10 μM, cell culture) in breast cancer cell lines [212]. Furthermore, Perrot-Applanat et al. hypothesize that BPA and exogenous estrogens [206] could induce overexpression of Areg in TEBs of different species during the proliferative period [213]. Thus, BPA can increase MG sensitivity to EGFs, even in cells that lack ER, through access to the GPER cascade [211], which interestingly shows a BPA-regulation of EGF via non-estrogenic pathways in MG. Furthermore, in MG of rat offspring prenatally exposed to BPA (250 μg/kg/day), a higher expression of AKT/ERK and a lower expression of TGF-β provokes modulation of proliferative signaling [214]. BPA also interacts with estrogen-related receptor γ (ERR-γ), an orphan receptor to which BPA binds strongly [168]. BPA-induced ERR-γ activity (10 nM, cell culture) can modulate MMPs by ERK/AKT cascade, increasing the expression of MMP-2 and MMP-9 [215] and, consequently, impacting the composition and organization of MG tissue.

It is known that BPA modulates multiple receptor targets by epigenetic reprogramming [164]. From a tumorigenic perspective, BPA (1−10 nM, cell culture) up-regulates VEGF expression in breast tumor cell lines, supporting invasiveness and malignancy by means of an ER-dependent mechanism that promotes angiogenesis in vivo [216]. By inducing high sensitivity to progesterone, perinatal exposure BPA (5 mg/kg/day) in C57/Bl6 mice triggers Wnt-4 and Nf-kB in MG, stimulating a proliferative process in MSC [217] and an increase in RANKL expression that participates in progesterone signaling of MG in exposed rats (0, 25, 250ug/kg/day, dose-dependent) [218]. Through NF-kB and

mTOR signaling, perinatal exposure to BPA diluted in drinking water (10 μg/mL/day) also promotes tissue inflammation and autophagic activity in MG from rats female offspring [219], creating a suitable microenvironment for cancer establishment [11].

In general, exposure to BPA is linked to the incidence of epigenetic alterations, related to the production of ROS and oxidative stress [220]. Changes in the modulation of oxidative mediator synthesis lead to disruption in the functions of imposing cellular organelles, such as mitochondria [221,222], and triggering of cell signaling pathways that induce the expression of apoptotic proteins [223]. In women in pre- and post-menopause phases, exposure to BPA increases the production of inflammatory and oxidative markers, with postmenopausal women being more susceptible due to their lower concentrations of serum E2 [224].

3.4. Epigenetic and miRNA alterations

There is evidence of epigenetic involvement in the majority of morphological alterations caused by increased exposure to EDs. Epigenetic alterations are caused by environmental or hereditary stable gene expression changes without modification in DNA sequences [225]. Perinatal exposure to factors that shift gene expression patterns may cause epigenomic reprogramming in mammals during embryogenesis [226]. This window of susceptibility is highly responsive to EDs, which control the epigenetic inheritance of adult diseases by modulating DNA epigenetic changes in reproductive cells [227]. These changes may shift the expression pattern by silencing or activating genes based mainly on methylation and/or modifications in histones [228].

Since DNAm, post-translational histone modification and noncoding RNAs are key regulators during MG development and differentiation, the impact of endocrine disruptors on these events will be discussed (Fig. 5C).

3.4.1. Changes in methylation patterns and histones

DNAm was confirmed to be sensitive to environmental exposure, and the methylation patterns vary according to cell types [168]. Previous experiments have confirmed this by the shift in mice offspring coat color after maternal exposure to a BPA formulated diet (50 mg/kg) [229]. Usually, a decrease in DNAm is present when perinatal exposure to this ED occurs, but no changes were observed after adult CD-1 mice exposure (5 mg/kg) [230], suggesting that methylation effects depend on the developmental phase of exposure.

In vitro experiments revealed that BPA (0.1 and 0.01 nM) changes the methylation pattern of MCF-10 F cells, by increasing the expression of genes involved in DNA repair and decreasing the expression of genes involved in apoptosis [231]. In MCF-7 cells, BPA exposure (0.1 and 10 nM) can alter the methylation pattern in several ER-dependent regions compared to BPS and BPF [232]. This disruptor is known to promote hypermethylation of oncogenes and hypomethylation of tumor suppressor genes [233]. The potential effect of neonatal exposure of rats to BPA (2.4 ug/day) on methyltransferase gene expression may be involved in this mechanism [234]. Alteration in methyltransferase expression is a known mechanism of action of BPA in metabolic disorders. In rats, for example, DNA hypermethylation after BPA perinatal exposure (50 μg/kg/day) was attributed to oxidative stress [235].

In pregnant exposed rats, BPA (6 or 52 mg/kg/day) changed the β–casein gene methylation pattern, promoting alterations in the secretion of this protein [236]. Epigenetic changes involving DNAm and remodeling of chromatin have been related to the process of carcinogenesis. Exposure during the perinatal window also impacts the methylation patterns of offspring MG: significant differences in the methylation pattern of MG are shown on the 50th postnatal day when comparing control and perinatally exposed (250 μg/kg/day) rats; changes in the initiation site of the alpha-lactalbumin gene are noteworthy [237], contributing to the development of neoplastic lesions in adulthood.

Environmental BPA-exposure that leads to carcinogenic changes may also be induced by histone methylation [238], which causes gene silencing. As an example of histone methyltransferase, PRC2 is known to induce gene silencing and tumorigenesis. Its catalytic subunit, EZH2, is responsible for providing the methyltransferase activity [239]. In addition to increasing EZH2 mRNA and protein expression in MCF7 cells, BPA also increases the EZH2 mRNA and protein expression in the MG from in utero exposed adult mice [238]. EZH2-mediated methylation also impacts p57 and the E-cadherin genes, thus influencing cell cycle regulation and cell-cell adhesion, impacting cell proliferation [240] and cancer invasiveness [241]. In addition, the Homeobox A10 gene (Hoxa10), which encodes the homeobox transcription factor protein, was hypomethylated in mice after in utero exposure to BPA, causing alterations in gene expression programming and in the binding of ER to the ERE of Hoxa10, increasing the responsiveness of cells to estrogens [230].

3.4.2. Changes in microRNA expression

Among the factors that modulate miRNA expression, hormone signaling is of great importance, as estrogens and androgens are the main modulators of miRNAs in the reproductive system [242]. The cleavage of pri-miRNA into precursor miRNAs during its biogenesis in the nucleus may be repressed by ERα interactions [243]. Technically, ERα and androgen receptors affect this process through their gene transcription regulation [153]. In human cell lines, E2 induces alterations in miRNAs, as demonstrated by sequencing techniques [244]. Estrogen binds to ERα and ERβ to activate response elements in some miRNA promoters, i.e., miRNA-21, -155, and -124 [245].

Therefore, exposure to endocrine disruptors such as xenoestrogens may also affect the expression of certain types of miRNAs. This modulation even attributes them with a role as biomarkers since the quantities present in extracellular fluids may reflect environmental modifications [142,246]. The disruptors DDT and BPA (10 μM) are known to downregulate miR-21 in MCF-7 cells [247]. The effects of miRNAs on the estrogen activation pathway may also influence ER coactivators [153,247]. In goat mammary epithelial cells, for example, miR-135b down--regulated prolactin [248]. In ewes, embryonic exposure to BPA (0.5 mg/kg/day) upregulated miRNAs related to endothelial cell damage (miR-217) and cell cycle arrest (miR-608) in adult offspring [249]. Goats and ewes are models of constant mammary gland activity, due to their use for persistent milk production; this aspect obviously characterizes their MG as different within regards to human and rodent models, which are the most widely discussed herein. However, they are a very interesting model for obtaining mammary epithelial cell lines [250].

Therefore, the impacts of ED exposure during phases where MG shows considerable growth and differentiation may involve long-term epigenetic modifications through miRNAs. Specifically, in breast cancer, miRNA expression is related to the type of neoplasia developed by the patient, which, consequently, is a valuable tool to detect and evaluate stages in breast cancer, since serum analysis can be performed [251]. Thus, since EDs are regulators of miRNAs expression due to hormone receptor binding, there may be a relation between ED exposure and breast cancer susceptibility.

4. What are the consequences of these disturbances during the developmental windows?

BPA exposure and a normal environment are compared in Table 1, which summarizes the consequences of disruption during different windows of susceptibility. Morphological alterations in the epithelium and a higher susceptibility to breast cancer are the major impacts of ED exposure during perinatal development. Higher numbers of TEBs, dilated ducts, and alveolar structures [252] are MG alterations present after gestational exposure. In adulthood, the impacts of perinatal exposure to BPA include an increase in collagen fibers and in the incidence of hyperplasia [207,236,253], inflammation [254,255], and the presence of proliferative cells [256,257]. In the MG, EDs increase susceptibility to malignant alterations, especially during critical periods of development, when tissue is prone to growth and differentiation [258].

The morphological alterations in the gland during these windows are based on tissue remodeling occasioned by hormonal signaling [259]. According to Fenton and Birnbaum, EDs may cause irreversible impacts in tissue architecture related to hormone binding and receptor activation, but not limited to this mechanism [13].

Thus, it is reasonable to connect the possible impacts of chemicals that cause hormonal balance changes and alter the long-term homeostasis of the gland. It is important to bear in mind that perinatal exposure to chemicals (a window of susceptibility) implicates transgenerational effects by impacting the development of primordial germ cells [260], leading to impacts on embryo development. These effects are transmitted not due to DNA mutations but due to epigenetic changes in the germline or embryonic context-dependent transmission cells during development [10]. In addition, epigenomic changes caused by in utero exposure induce long-term alterations in mammary morphology since mammary stem cell differentiation, lineage determination, and development are functions regulated epigenetically, employing DNAm and miRNA [125].

The period of perinatal development is critical regarding these influences so that epigenetic changes in parents can be transmitted to children [164]. According to Skinner et al., the sequence of events that culminates in pathological effects starts in the epigenetic action, passing through alterations in the reading of DNA, protein expression, and organ physiology until it causes tissue disorder [261].

Yaoi et al. report that maternal exposure to BPA can change approximately 0.3 % of the CpG regions, causing hyper or hypomethylation in the developing brain's genetic material [262]. These authors also reinforce that methylation caused by BPA is loco-specific and dependent on the developmental stage of the organ. Regarding possibly affected genes, it is believed that they involve deregulation of, for example, genes encoding hormone receptors, altering their expression, or long-term activity [263]. Specifically, in MG cells, some genes known to be affected by BPA in vitro action are involved in DNA repair (BRCA1, BRCA2, BARD1, CtIP, RAD51, and BRCC3) and the regulation of apoptosis (PDCD5 and BCL2L11) [231].

During the post-weaning involution window of susceptibility, MG is characterized by a pro-inflammatory environment that may facilitate metastasis and tumor development [264]. The weaning period may also lead to a critical window of estrogenic signaling, consisting of a risk factor to the development of breast cancer, which can be worsened in the presence of EDs [265]. When sensitized by EDs such as BPA, this pro-neoplastic microenvironment develops characteristics for the growth of local tumors by hormonal imbalance or interferences in molecular pathways, to accelerate the apoptosis or overstimulate proliferative activity [29,52]. In postmenopausal women, serum BPA was positively associated with elevated mammographic density, confirming a relation between EDs and a higher breast cancer risk [177]. Both molecular and physiological changes converge or start from an epigenetic point of view, and epigenetic regulation (or disruption) affects MG structure [152].

5. Final remarks and conclusion

The MG comprises a complex organ where a dynamic development/involution system is present to allow the necessary plasticity for the early nourishment of mammals. It constitutes a warning system for risk assessment of EDs, which disturb MG homeostasis and cause long-term alterations mainly related to malignant pathologies. Although some aspects have been elucidated regarding the mechanisms involved in these processes, much remains to be unveiled, especially because cohort and epidemiological studies are the only options to evaluate human effects, and experimental designs for animals do not usually mimic all the possible exposure routes.

Reviewing the literature led us to raise a particular concern regarding the lack of studies about the impacts of gestational and lactational exposure in mothers, since most studies focus on perinatal and pubertal exposures. It is also important to emphasize that BPA acts not only on ERs but also on multiple hormone receptors and epigenetic targets in cells, triggering a plethora of adverse effects. Thus, BPA is a metabolic-endocrine disruptor that is dose-dependent – different dosages of exposure can trigger different effects in organs – and have effects on multiple cell levels – interfering in processes from metabolism to morphology.

In conclusion, MG development windows are orchestrated by several molecules and pathways that respond to the effects of ubiquitous industrialized chemical compounds. BPA contributes to the establishment of the MG neoplastic predisposition by acting through several mechanisms. This review synthesizes some lessons learned in the scientific field, contributing to understanding of MG dynamics and responses to deleterious factors.

Declaration of Competing Interest

The authors declare no conflict of interest.

Acknowledgements

This paper was supported by grants from the Brazilian agencies: Fundação de Amparo à Pesquisa do Estado de São Paulo – Brazil (FAPESP) – Process number 2020/01240-9, the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brazil (CAPES) – Finance Code 001, and by the National Council for Scientific and Technological Development – Brazil (CNPq) – Finance Code 302938/2020-6. The authors' laboratory was funded by FAPESP (Grant no. 2018/23383-6).

References

[1] R. Strange, K.C. Westerlind, A. Ziemiecki, A.C. Andres, Proliferation and apoptosis in mammary epithelium during the rat oestrous cycle, Acta Physiol. Oxf. (Oxf) 190 (2007) 137–149, https://doi.org/10.1111/j.1748-1716.2007.01704.x.
[2] H. Macias, L. Hinck, Mammary gland development, Wiley Interdiscip. Rev. Dev. Biol. 1 (2012) 533–557, https://doi.org/10.1002/wdev.35.
[3] J. Stingl, Estrogen and progesterone in normal mammary gland development and in Cancer, Horm. Cancer 2 (2011) 85–90, https://doi.org/10.1007/s12672-010-0055-1.
[4] K.M. Rodgers, J.O. Udesky, R.A. Rudel, J.G. Brody, Environmental chemicals and breast cancer: an updated review of epidemiological literature informed by biological mechanisms, Environ. Res. 160 (2018) 152–182, https://doi.org/10.1016/j.envres.2017.08.045.
[5] T. Damstra, S. Barlow, A. Bergman, R. Kavlock, G. Van Der Kraak, Global assessment of the state-of-the-science of endocrine disruptors, Geneva World Heal, Organ. (2002) 11–32.
[6] P.D. Darbre, What are endocrine disrupters and where are they found?. Endocr. Disrupt. Hum. Heal Elsevier, 2015, pp. 3–26.
[7] M.V. Maffini, B.S. Rubin, C. Sonnenschein, A.M. Soto, Endocrine disruptors and reproductive health: the case of bisphenol-A, Mol. Cell. Endocrinol. 254–255 (2006) 179–186, https://doi.org/10.1016/j.mce.2006.04.033.
[8] I. Badamasi, R. Odong, C. Masembe, Threats posed by xenoestrogenic chemicals to the aquatic ecosystem, fish reproduction and humans: a review, African J. Aquat. Sci. (2020) 1–16.
[9] E.-E. Commission, Report of the Proceedings of the European Workshop on the Impact of Endocrine Disrupters on Human Health and Wildlife (EUR 17549), Eur. Comm. DG XII, Brussels, Belgium, 1996.
[10] E. Diamanti-Kandarakis, J.-P. Bourguignon, L.C. Giudice, R. Hauser, G.S. Prins, A. M. Soto, R.T. Zoeller, A.C. Gore, Endocrine-disrupting chemicals: an Endocrine Society scientific statement, Endocr. Rev. 30 (2009) 293–342.
[11] J.L. Inman, C. Robertson, J.D. Mott, M.J. Bissell, Mammary gland development: cell fate specification, stem cells and the microenvironment, Dev. 142 (2015) 1028–1042, https://doi.org/10.1242/dev.087643.
[12] B.A. Cohn, P.M. Cirillo, M.B. Terry, DDT and Breast Cancer: Prospective Study of Induction Time and Susceptibility Windows, J. Natl. Cancer Inst. 111 (2019) 803–810, https://doi.org/10.1093/jnci/djy198.
[13] S.E. Fenton, L.S. Birnbaum, Timing of environmental exposures as a critical element in breast Cancer risk, J. Clin. Endocrinol. Metab. 100 (2015) 3245–3250, https://doi.org/10.1210/jc.2015-2848.
[14] R.C. Humphreys, M. Krajewska, S. Krnacik, R. Jæger, H. Weiher, S. Krajewski, J. C. Reed, J.M. Rosen, Apoptosis in the terminal endbud of the murine mammary gland: a mechanism of ductal morphogenesis, Development. 122 (1996) 4013–4022.
[15] Y.J. Topper, C.S. Freeman, Multiple hormone interactions in the developmental biology of the mammary gland, Physiol. Rev. 60 (1980) 1049–1106, https://doi.org/10.1152/physrev.1980.60.4.1049.

[16] J.J. Heindel, B. Blumberg, M. Cave, R. Machtinger, A. Mantovani, M.A. Mendez, A. Nadal, P. Palanza, G. Panzica, R. Sargis, L.N. Vandenberg, F. Vom Saal, Metabolism disrupting chemicals and metabolic disorders, Reprod. Toxicol. 68 (2017) 3–33, https://doi.org/10.1016/j.reprotox.2016.10.001.

[17] M.C. Rudolph, J.L. McManaman, L. Hunter, T. Phang, M.C. Neville, Functional development of the mammary gland: use of expression rofiling and trajectory clustering to reveal changes in gene expression during pregnancy, lactation, and involution, J. Mammary Gland Biol, Neoplasia. 8 (2003) 287–307, https://doi.org/10.1023/B:JOMG.0000010030.73983.57.

[18] W. Imagawa, V.K. Pedchenko, J. Helber, H. Zhang, Hormone/growth factor interactions mediating epithelial/stromal communication in mammary gland development and carcinogenesis, J. Steroid Biochem. Mol. Biol. 80 (2002) 213–230, https://doi.org/10.1016/S0960-0760(01)00188-1.

[19] C.D. LaPlante, L.N. Vandenberg, Data describing lack of effects of 17α-ethinyl estradiol on mammary gland morphology in female mice exposed during pregnancy and lactation, Data Br. 14 (2017) 337–343, https://doi.org/10.1016/j.dib.2017.07.062.

[20] K. Almholt, K.A. Green, A. Juncker-Jensen, B.S. Nielsen, L.R. Lund, J. Rømer, Extracellular proteolysis in transgenic mouse models of breast Cancer, J. Mammary Gland Biol. Neoplasia 12 (2007) 83–97, https://doi.org/10.1007/s10911-007-9040-x.

[21] T. Stein, J.S. Morris, C.R. Davies, S.J. Weber-Hall, M.-A. Duffy, V.J. Heath, A. K. Bell, R.K. Ferrier, G.P. Sandilands, B.A. Gusterson, Involution of the mouse mammary gland is associated with an immune cascade and an acute-phase response, involving LBP, CD14 and STAT3, Breast Cancer Res. 6 (2003) R75, https://doi.org/10.1186/bcr753.

[22] D.C. Radisky, L.C. Hartmann, Mammary Involution and Breast Cancer Risk: Transgenic Models and Clinical Studies, J. Mammary Gland Biol. Neoplasia 14 (2009) 181–191, https://doi.org/10.1007/s10911-009-9123-y.

[23] N. Kanaya, G. Chang, X. Wu, K. Saeki, L. Bernal, H.-J. Shim, J. Wang, C. Warden, T. Yamamoto, J. Li, J.-S. Park, T. Synold, S. Vonderfecht, M. Rakoff, S. L. Neuhausen, S. Chen, Single-cell RNA-sequencing analysis of estrogen- and endocrine-disrupting chemical-induced reorganization of mouse mammary gland, Commun. Biol. 2 (2019) 406, https://doi.org/10.1038/s42003-019-0618-9.

[24] P.A. Masso-welch, K.M. Darcy, N.C. Stangle-castor, M.M. Ip, A developmental atlas of rat mammary gland histology, J. Mammary Gland Biol. Neoplasia 5 (2000) 165–185, https://doi.org/10.1023/a:1026491221687.

[25] J.E. Visvader, J. Stingl, Mammary stem cells and the differentiation hierarchy: current status and perspectives, Genes Dev. 28 (2014) 1143–1158, https://doi.org/10.1101/gad.242511.114.

[26] K.A. Green, C.H. Streuli, Apoptosis regulation in the mammary gland, Cell. Mol. Life Sci. 61 (2004) 1867–1883, https://doi.org/10.1007/s00018-004-3366-y.

[27] W. Zhang, P. Qian, X. Zhang, M. Zhang, H. Wang, M. Wu, X. Kong, S. Tan, K. Ding, J.K. Perry, Z. Wu, Y. Cao, P.E. Lobie, T. Zhu, Autocrine/paracrine human growth hormone-stimulated microRNA 96-182-183 cluster promotes epithelial-mesenchymal transition and invasion in breast cancer, J. Biol. Chem. 290 (2015) 13812–13829, https://doi.org/10.1074/jbc.M115.653261.

[28] J.I. Englund, H. Cojoc, L. Blaas, A. Ritchie, N. Pentinmikko, J. Döhla, P. Munne, M. Patarroyo, J. Klefström, J. Ivaska, P. Katajisto, Laminin alpha 5 is necessary for mammary epithelial growth and function by maintaining luminal epithelial cell identity, BioRxiv (2019), https://doi.org/10.1101/2019.12.27.889451, 2019.12.27.889451.

[29] M.K. Jena, S. Jaswal, S. Kumar, A.K. Mohanty, Molecular mechanism of mammary gland involution: an update, Dev. Biol. 445 (2019) 145–155, https://doi.org/10.1016/j.ydbio.2018.11.002.

[30] J. Li, H. Sun, M.L. Feltri, A.M. Mercurio, Integrin β4 regulation of PTHrP underlies its contribution to mammary gland development, Dev. Biol. 407 (2015) 313–320, https://doi.org/10.1016/j.ydbio.2015.09.015.

[31] S. Pamarthy, L. Mao, G.K. Katara, S. Fleetwood, A. Kulshreshta, A. Gilman-Sachs, K.D. Beaman, The V-atpase a2 isoform controls mammary gland development through notch and TGF-β signaling, Cell Death Dis. 7 (2016) 1–13, https://doi.org/10.1038/cddis.2016.347.

[32] T.Y. Feinberg, R.G. Rowe, T.L. Saunders, S.J. Weiss, Functional roles of mmp14 and mmp15 in early postnatal mammary gland development, Dev. 143 (2016) 3956–3968, https://doi.org/10.1242/dev.136259.

[33] M.M. Richert, K.L. Schwertfeger, J.W. Ryder, S.M. Anderson, An atlas of mouse mammary gland development, J. Mammary Gland Biol. Neoplasia 5 (2000) 227–241.

[34] S.Z. Haslam, The ontogeny of mouse mammary gland responsiveness to ovarian steroid hormones, Endocrinology. 125 (1989) 2766–2772, https://doi.org/10.1210/endo-125-5-2766.

[35] S. Mallepell, A. Krust, P. Chambon, C. Brisken, Paracrine signaling through the epithelial estrogen receptor α is required for proliferation and morphogenesis in the mammary gland, Proc. Natl. Acad. Sci. U. S. A. 103 (2006) 2196–2201, https://doi.org/10.1073/pnas.0510974103.

[36] S. Truchet, E. Honvo-Houéto, Physiology of milk secretion, best pract, Res. Clin. Endocrinol. Metab. 31 (2017) 367–384, https://doi.org/10.1016/j.beem.2017.10.008.

[37] O.M. Conneely, B. Mulac-Jericevic, J.P. Lydon, Progesterone-dependent regulation of female reproductive activity by two distinct progesterone receptor isoforms, Steroids. 68 (2003) 771–778, https://doi.org/10.1016/S0039-128X(03)00126-0.

[38] C. Brisken, S. Park, T. Vass, J.P. Lydon, B.W. O'Malley, R.A. Weinberg, A paracrine role for the epithelial progesterone receptor in mammary gland development, Proc. Natl. Acad. Sci. U. S. A. 95 (1998) 5076–5081, https://doi.org/10.1073/pnas.95.9.5076.

[39] M.C. Neville, J. Morton, S. Umemura, Lactogenesis: The transition from pregnancy to lactation, Pediatr. Clin. North Am. 48 (2001) 35–52, https://doi.org/10.1016/S0031-3955(05)70284-4.

[40] M.C. Neville, S.M. Anderson, J.L. McManaman, T.M. Badger, M. Bunik, N. Contractor, T. Crume, D. Dabelea, S.M. Donovan, N. Forman, D.N. Frank, J. E. Friedman, J.B. German, A. Goldman, D. Hadsell, M. Hambidge, K. Hinde, N. D. Horseman, R.C. Hovey, E. Janoff, N.F. Krebs, C.B. Lebrilla, D.G. Lemay, P. S. MacLean, P. Meier, A.L. Morrow, J. Neu, L.A. Nommsen-Rivers, D.J. Raiten, M. Rijnkels, V. Seewaldt, B.D. Shur, J. Van Houten, P. Williamson, Lactation and neonatal nutrition: defining and refining the critical questions, J. Mammary Gland Biol. Neoplasia 17 (2012) 167–188, https://doi.org/10.1007/s10911-012-9261-5.

[41] G. Gimpl, F. Fahrenholz, The oxytocin receptor system: structure, function, and regulation, Physiol. Rev. 81 (2001) 629–683, https://doi.org/10.1152/physrev.2001.81.2.629.

[42] M.S. Soloff, Oxytocin Receptors and Mammary Myoepithelial Cells, J. Dairy Sci. 65 (1982) 326–337, https://doi.org/10.3168/jds.S0022-0302(82)82194-2.

[43] A. Masedunskas, Y. Chena, R. Stussman, R. Weigert, I.H. Mather, Kinetics of milk lipid droplet transport, growth, and secretion revealed by intravital imaging: Lipid droplet release is intermittently stimulated by oxytocin, Mol. Biol. Cell 28 (2017) 935–946, https://doi.org/10.1091/mbc.E16-11-0776.

[44] R. Clarke, Introduction and overview: Sex steroids in the mammary gland, J. Mammary Gland Biol, Neoplasia. 5 (2000) 245–250, https://doi.org/10.1023/A:1009527609611.

[45] S. Saji, E.V. Jensen, S. Nilsson, T. Rylander, M. Warner, J.-Åke Gustafsson, Estrogen receptors α and β in the rodent mammary gland, Proc. Natl. Acad. Sci. U. S. A. 97 (2000) 337–342. http://www.ncbi.nlm.nih.gov/pmc/articles/PMC26664/.

[46] S.E. Fenton, The mammary gland: a tissue sensitive to environmental exposures, Rev. Environ. Health 24 (2009) 319–325, https://doi.org/10.1515/REVEH.2009.24.4.319.

[47] M. Jia, K. Dahlman-Wright, J.Å. Gustafsson, Estrogen receptor alpha and beta in health and disease, Best Pract. Res. Clin. Endocrinol. Metab. 29 (2015) 557–568, https://doi.org/10.1016/j.beem.2015.04.008.

[48] G. Canul-Medina, C. Fernandez-Mejia, Morphological, hormonal, and molecular changes in different maternal tissues during lactation and post-lactation, J. Physiol. Sci. 69 (2019) 825–835, https://doi.org/10.1007/s12576-019-00714-4.

[49] G.V. Dall, S. Hawthorne, Y. Seyed-Razavi, J. Vieusseux, W. Wu, J.A. Gustafsson, D. Byrne, L. Murphy, G.P. Risbridger, K.L. Britt, Estrogen receptor subtypes dictate the proliferative nature of the mammary gland, J. Endocrinol. 237 (2018) 323–336, https://doi.org/10.1530/JOE-17-0582.

[50] C. Förster, S. Mäkela, A. Wärri, S. Kietz, D. Becker, K. Hultenby, M. Warner, J. Å. Gustafsson, Involvement of estrogen receptor β in terminal differentiation of mammary gland epithelium, Proc. Natl. Acad. Sci. U. S. A. 99 (2002) 15578–15583, https://doi.org/10.1073/pnas.192561299.

[51] M. Palaniappan, L. Nguyen, S.L. Grimm, Y. Xi, Z. Xia, W. Li, C. Coarfa, The genomic landscape of estrogen receptor α binding sites in mouse mammary gland, PLoS One 14 (2019) 1–22, https://doi.org/10.1371/journal.pone.0220311.

[52] A. Nadal, E. Fuentes, C. Ripoll, S. Villar-Pazos, M. Castellano-Muñoz, S. Soriano, J. Martinez-Pinna, I. Quesada, P. Alonso-Magdalena, Extranuclear-initiated estrogenic actions of endocrine disrupting chemicals: Is there toxicology beyond paracelsus? J. Steroid Biochem. Mol. Biol. 176 (2018) 16–22, https://doi.org/10.1016/j.jsbmb.2017.01.014.

[53] B. Manavathi, V.S.K. Samanthapudi, V.N.R. Gajulapalli, Estrogen receptor coregulators and pioneer factors: the orchestrators of mammary gland cell fate and development, Front. Cell Dev. Biol. 2 (2014) 1–13, https://doi.org/10.3389/fcell.2014.00034.

[54] C. Brisken, B. O'Malley, Hormone action in the mammary gland, Perspect. Biol. Med. 4 (2010) 1–16, https://doi.org/10.1101/cshperspect.a013086.

[55] L. Gagniac, M. Rusidzé, F. Boudou, S. Cagnet, M. Adlanmerini, P. Jeannot, N. Gaide, F. Giton, A. Besson, A. Weyl, P. Gourdy, I. Raymond-Letron, J.F. Arnal, C. Brisken, F. Lenfant, Membrane expression of the estrogen receptor ERα is required for intercellular communications in the mammary epithelium, Dev. 147 (2020), https://doi.org/10.1242/dev.182303.

[56] Ż. Dziegelewska, M. Gajewska, Stromal-epithelial interactions during mammary gland development, Stromal Cells - Struct. Funct. Ther. Implic. (2019), https://doi.org/10.5772/intechopen.80405.

[57] A.L. Pavlovich, S. Manivannan, C.M. Nelson, Adipose stroma induces branching morphogenesis of engineered epithelial tubules, Tissue Eng. - Part A. 16 (2010) 3719–3726, https://doi.org/10.1089/ten.tea.2009.0836.

[58] H.Z. Zhang, J.M. Bennett, K.T. Smith, N. Sunil, S.Z. Haslam, Estrogen mediates mammary epithelial cell proliferation in serum-free culture indirectly via mammary stroma-derived hepatocyte growth factor, Endocrinology. 143 (2002) 3427–3434, https://doi.org/10.1210/en.2002-220007.

[59] L.M. Arendt, J.A. Rudnick, P.J. Keller, C. Kuperwasser, Stroma in breast development and disease, Semin. Cell Dev. Biol. 21 (2010) 11–18, https://doi.org/10.1016/j.semcdb.2009.10.003.

[60] K.B.R. Ewan, H.A. Oketch-Rabah, S.A. Ravani, G. Shyamala, H.L. Moses, M. H. Barcellos-Hoff, Proliferation of estrogen receptor-α-positive mammary epithelial cells is restrained by transforming growth factor-β1 in adult mice, Am. J. Pathol. 167 (2005) 409–417, https://doi.org/10.1016/S0002-9440(10)62985-9.

[61] A.M. Band, M. Laiho, Crosstalk of TGF-β and estrogen receptor signaling in breast cancer, J. Mammary Gland Biol. Neoplasia 16 (2011) 109–115, https://doi.org/10.1007/s10911-011-9203-7.

[62] W.H. Cleland, C.R. Mendelson, E.R. Simpson, Effect of aging and obesity on aromatase activity of human adipose cells, J. Clin. Endocrinol. Metab. 60 (1985) 174–177, https://doi.org/10.1093/ajcn/45.1.290.
[63] L. Hennighausen, G.W. Robinson, Signaling pathways in mammary gland development, Dev. Cell 1 (2001) 467–475.
[64] R. Sitruk-Ware, M. El-Etr, Progesterone and related progestins: potential new health benefits, Climacteric. 16 (2013) 69–78, https://doi.org/10.3109/13697137.2013.802556.
[65] Y. Feng, D. Manka, K.U. Wagner, S.A. Khan, Estrogen receptor-α expression in the mammary epithelium is required for ductal and alveolar morphogenesis in mice, Proc. Natl. Acad. Sci. U. S. A. 104 (2007) 14718–14723, https://doi.org/10.1073/pnas.0706933104.
[66] S. Wang, L.J. Counterman, S.Z. Haslam, Progesterone action in normal mouse mammary gland, Endocrinology. 127 (1990) 2183–2189, https://doi.org/10.1210/endo-127-5-2183.
[67] B. Mulac-Jericevic, J.P. Lydon, F.J. DeMayo, O.M. Conneely, Defective mammary gland morphogenesis in mice lacking the progesterone receptor B isoform, Proc. Natl. Acad. Sci. 100 (2003) 9744–9749.
[68] A.E. Obr, D.P. Edwards, The biology of progesterone receptor in the normal mammary gland and in breast cancer, Mol. Cell. Endocrinol. 357 (2012) 4–17, https://doi.org/10.1016/j.mce.2011.10.030.
[69] M. Beleut, R.D. Rajaram, M. Caikovski, A. Ayyanan, D. Germano, Y. Choi, P. Schneider, C. Brisken, Two distinct mechanisms underlie progesterone-induced proliferation in the mammary gland, Proc. Natl. Acad. Sci. U. S. A. 107 (2010) 2989–2994, https://doi.org/10.1073/pnas.0915148107.
[70] Y. Cao, G. Bonizzi, T.N. Seagroves, F.R. Greten, R. Johnson, E.V. Schmidt, M. Karin, IKKα provides an essential link between RANK signaling and cyclin D1 expression during mammary gland development, Cell. 107 (2001) 763–775, https://doi.org/10.1016/S0092-8674(01)00599-2.
[71] A. Mukherjee, S.M. Soyal, J. Li, Y. Ying, B. He, F.J. Demayo, J.P. Lydon, Targeting RANKL to a specific subset of murine mammary epithelial cells induces ordered branching morphogenesis and alveologenesis in the absence of progesterone receptor expression, FASEB J. 24 (2010) 4408–4419, https://doi.org/10.1096/fj.10-157982.
[72] E. Gonzalez-Suarez, D. Branstetter, A. Armstrong, H. Dinh, H. Blumberg, W. C. Dougall, RANK overexpression in transgenic mice with mouse mammary tumor virus promoter-controlled RANK increases proliferation and impairs alveolar differentiation in the mammary epithelia and disrupts lumen formation in cultured epithelial acini, Mol. Cell. Biol. 27 (2007) 1442–1454, https://doi.org/10.1128/mcb.01298-06.
[73] M. Sisay, G. Mengistu, D. Edessa, The RANK/RANKL/OPG System in Tumorigenesis and Metastasis of Cancer Stem Cell: Potential Therapeutic Targets for Anticancer Therapy, J. Cancer Sci. Ther. 09 (2017) 3801–3810, https://doi.org/10.4172/1948-5956.1000451.
[74] B. Trabert, M.E. Sherman, N. Kannan, F.Z. Stanczyk, Progesterone and breast cancer, Endocr. Rev. 68 (2020) 715–717, https://doi.org/10.1210/endrev/bnz001.
[75] C. Brisken, A. Heineman, T. Chavarria, B. Elenbaas, J. Tan, S.K. Dey, J. A. McMahon, A.P. McMahon, R.A. Weinberg, Essential function of Wnt-4 in mammary gland development downstream of progesterone signaling, Genes Dev. 14 (2000) 650–654, https://doi.org/10.1101/gad.14.6.650.
[76] R.D. Rajaram, D. Buric, M. Caikovski, A. Ayyanan, J. Rougemont, J. Shan, S. J. Vainio, O. Yalcin-Ozuysal, C. Brisken, Progesterone and W nt4 control mammary stem cells via myoepithelial crosstalk, EMBO J. 34 (2015) 641–652, https://doi.org/10.15252/embj.201490434.
[77] C. Brisken, V. Scabia, Progesterone signalling in the normal breast and its implications for cancer, J. Mol. Endocrinol. (2020), https://doi.org/10.1530/JME-20-0091. JME-20-0091.
[78] M.D. Aupperlee, A.A. Drolet, S. Durairaj, W. Wang, R.C. Schwartz, S.Z. Haslam, Strain-specific differences in the mechanisms of progesterone regulation of murine mammary gland development, Endocrinology. 150 (2009) 1485–1494, https://doi.org/10.1210/en.2008-1459.
[79] J. Dinny Graham, C.L. Clarke, Physiological action of progesterone in target tissues, Endocr. Rev. 18 (1997) 502–519, https://doi.org/10.1210/er.18.4.502.
[80] R.S. Chapman, P.C. Lourenco, E. Tonner, D.J. Flint, S. Selbert, K. Takeda, S. Akira, A.R. Clarke, C.J. Watson, Suppression of epithelial apoptosis and delayed mammary gland involution in mice with a conditional knockout of Stat3, Genes Dev. 13 (1999) 2604–2616, https://doi.org/10.1101/gad.13.19.2604.
[81] R.S. Chapman, E.K. Duff, P.C. Lourenco, E. Tonner, D.J. Flint, A.R. Clarke, C. J. Watson, A novel role for IRF-1 as a suppressor of apoptosis, Oncogene. 19 (2000) 6386–6391, https://doi.org/10.1038/sj.onc.1204016.
[82] M.J. Wieduwilt, M.M. Moasser, The epidermal growth factor receptor family: biology driving targeted therapeutics, Cell. Mol. Life Sci. 65 (2011) 1566–1584, https://doi.org/10.1007/s00018-008-7440-8.The.
[83] E.P.M. Timmermans-Sprang, A. Gracanin, J.A. Mol, Molecular signaling of progesterone, growth hormone, Wnt, and HER in mammary glands of dogs, rodents, and humans: New treatment target identification, Front. Vet. Sci. 4 (2017), https://doi.org/10.3389/fvets.2017.00053.
[84] T. Helikar, N. Kochi, B. Kowal, M. Dimri, M. Naramura, S.M. Raja, V. Band, H. Band, J.A. Rogers, A. Comprehensive, Multi-scale dynamical model of ErbB receptor signal transduction in human mammary epithelial cells, PLoS One 8 (2013), https://doi.org/10.1371/journal.pone.0061757.
[85] N.E. Hynes, G. MacDonald, ErbB receptors and signaling pathways in cancer, Curr. Opin. Cell Biol. 21 (2009) 177–184, https://doi.org/10.1016/j.ceb.2008.12.010.
[86] S. Xu, S. Yu, D. Dong, L.T.O. Lee, G protein-coupled estrogen receptor: a potential therapeutic target in Cancer, Front. Endocrinol. (Lausanne). 10 (2019) 1–12, https://doi.org/10.3389/fendo.2019.00725.
[87] E.R. Prossnitz, M. Barton, The G-protein-coupled estrogen receptor GPER in health and disease, Nat. Rev. Endocrinol. 7 (2011) 715–726, https://doi.org/10.1038/nrendo.2011.122.
[88] C. Tolg, H. Yuan, S.M. Flynn, K. Basu, J. Ma, K.C.K. Tse, B. Kowalska, D. Vulkanesku, M.K. Cowman, J.B. McCarthy, E.A. Turley, Hyaluronan modulates growth factor induced mammary gland branching in a size dependent manner, Matrix Biol. 63 (2017) 117–132, https://doi.org/10.1016/j.matbio.2017.02.003.
[89] E.J. Filardo, Epidermal growth factor receptor (EGFR) transactivation by estrogen via the G-protein-coupled receptor, GPR30: a novel signaling pathway with potential significance for breast cancer, J. Steroid Biochem. Mol. Biol. 80 (2002) 231–238, https://doi.org/10.1016/S0960-0760(01)00190-X.
[90] J.A. McCubrey, L.S. Steelman, W.H. Chappell, S.L. Abrams, E.W.T. Wong, F. Chang, B. Lehmann, D.M. Terrian, M. Milella, A. Tafuri, F. Stivala, M. Libra, J. Basecke, C. Evangelisti, A.M. Martelli, R.A. Franklin, Roles of the Raf/MEK/ERK pathway in cell growth, malignant transformation and drug resistance, Biochim. Biophys. Acta - Mol. Cell Res. 1773 (2007) 1263–1284, https://doi.org/10.1016/j.bbamcr.2006.10.001.
[91] R. Khokha, Z. Werb, Mammary gland reprogramming: metalloproteinases couple form with function, Cold Spring Harb, Perspect. Biol. Med. 3 (2011) 1–19, https://doi.org/10.1101/cshperspect.a004333.
[92] M.P. Sanderson, P.J. Dempsey, A.J. Dunbar, Control of ErbB signaling through metalloprotease mediated ectodomain shedding of EGF-like factors, Growth Factors 24 (2006) 121–136, https://doi.org/10.1080/08977190600634373.
[93] N.J. Kenney, R. -P Huang, G.R. Johnson, J. -X Wu, D. Okamura, W. Matheny, E. Kordon, W.J. Gullick, G. Plowman, G.H. Smith, D.S. Salomon, E.D. Adamson, Detection and location of amphiregulin and Cripto-1 expression in the developing postnatal mouse mammary gland, Mol. Reprod. Dev. 41 (1995) 277–286, https://doi.org/10.1002/mrd.1080410302.
[94] E.A. Peterson, E.C. Jenkins, K.A. Lofgren, N. Chandiramani, H. Liu, E. Aranda, M. Barnett, P.A. Kenny, Amphiregulin is a critical downstream effector of estrogen signaling in ERα-positive breast cancer, Cancer Res. 75 (2015) 4830–4838, https://doi.org/10.1158/0008-5472.CAN-15-0709.
[95] S.P.H. Mao, M. Park, R.M. Cabrera, J.R. Christin, G.S. Karagiannis, M.H. Oktay, D. M.W. Zaiss, S.I. Abrams, W. Guo, J.S. Condeelis, P.A. Kenny, J.E. Segall, Loss of amphiregulin reduces myoepithelial cell coverage of mammary ducts and alters breast tumor growth, Breast Cancer Res. 20 (2018) 1–14, https://doi.org/10.1186/s13058-018-1057-0.
[96] N.C. Luetteke, T.H. Qiu, S.E. Fenton, K.L. Troyer, R.F. Riedel, A. Chang, D.C. Lee, Targeted inactivation of the EGF and amphiregulin genes reveals distinct roles for EGF receptor ligands in mouse mammary gland development, Development. 126 (1999) 2739–2750.
[97] H.L. LaMarca, J.M. Rosen, Estrogen regulation of mammary gland development and breast cancer: amphiregulin takes center stage, Breast Cancer Res. 9 (2007) 4–6, https://doi.org/10.1186/bcr1740.
[98] A. Kariagina, J. Xie, J.R. Leipprandt, S.Z. Haslam, Amphiregulin mediates estrogen, progesterone, and EGFR signaling in the normal rat mammary gland and in hormone-dependent rat mammary cancers, Horm. Cancer 1 (2010) 229–244, https://doi.org/10.1007/s12672-010-0048-0.
[99] M. Sisto, L. Lorusso, G. Ingravallo, S. Lisi, Exocrine gland morphogenesis: insights into the role of amphiregulin from development to disease, Arch. Immunol. Ther. Exp. (Warsz). 65 (2017) 477–499, https://doi.org/10.1007/s00005-017-0478-2.
[100] C. Cai, A. Geng, M. Wang, L. Yang, Q.C. Yu, Y.A. Zeng, Amphiregulin mediates the hormonal regulation on Rspondin-1 expression in the mammary gland, Dev. Biol. 458 (2020) 43–51, https://doi.org/10.1016/j.ydbio.2019.10.006.
[101] Y.E. Zhang, Non-Smad pathways in TGF-β signaling, Cell Res. 19 (2009) 128–139.
[102] N.S. Jahchan, Y.H. You, W.J. Muller, K. Luo, Transforming growth factor-β regulator SnoN modulates mammary gland branching morphogenesis, postlactational involution, and mammary tumorigenesis, Cancer Res. 70 (2010) 4204–4213, https://doi.org/10.1158/0008-5472.CAN-10-0135.
[103] A.V. Nguyen, J.W. Pollard, Transforming growth factor β3 induces cell death during the first stage of mammary gland involution, Development. 127 (2000) 3107–3118.
[104] R.S. Muraoka-Cook, I. Shin, J.Y. Yi, E. Easterly, M.H. Barcellos-Hoff, J. M. Yingling, R. Zent, C.L. Arteaga, Activated type I TGFβ receptor kinase enhances the survival of mammary epithelial cells and accelerates tumor progression, Oncogene. 25 (2006) 3408–3423, https://doi.org/10.1038/sj.onc.1208964.
[105] J.P. Bailey, K.M. Nieport, M.P. Herbst, S. Srivastava, R.A. Serra, N.D. Horseman, Prolactin and transforming growth factor-β signaling exert opposing effects on mammary gland morphogenesis, involution, and the Akt-forkhead pathway, Mol. Endocrinol. 18 (2004) 1171–1184, https://doi.org/10.1210/me.2003-0345.
[106] J. Donovan, J. Slingerland, Cell cycle arrest by transforming growth factor β.pDf, Breast Cancer Res. 2 (2000) 116–124.
[107] S. Zhang, M. Ekman, N. Thakur, S. Bu, P. Davoodpour, S. Grimsby, S. Tagami, C. H. Heldin, M. Landström, TGFβ1-induced activation of ATM and p53 mediates apoptosis in a Smad7-dependent manner, Cell Cycle 5 (2006) 2787–2795, https://doi.org/10.4161/cc.5.23.3523.
[108] C.A. Mitz, A.M. Viloria-Petit, TGF-beta signalling in bovine mammary gland involution and a comparative assessment of MAC-T and BME-UV1 cells as in vitro models for its study, PeerJ (2019) (2019) 1–21, https://doi.org/10.7717/peerj.6210.
[109] M.F. Palin, C. Farmer, C.R.A. Duarte, Triennial lactation symposium/bolfa: adipokines affect mammary growth and function in farm animals, J. Anim. Sci. 95 (2017) 5689–5700, https://doi.org/10.2527/jas2017.1777.

[110] A. Brenot, I. Hutson, C. Harris, Epithelial-adipocyte interactions are required for mammary gland development, but not for milk production or fertility, Dev. Biol. 458 (2020) 153–163, https://doi.org/10.1016/j.ydbio.2019.11.001.
[111] I. Tenvooren, M.Z. Jenks, H. Rashid, K.L. Cook, J.K. Muhlemann, C. Sistrunk, J. Holmes, K. Wang, K. Bonin, K. Hodges, H.W. Lo, A. Shaikh, I.G. Camarillo, S. A. Lelièvre, V. Seewaldt, P.A. Vidi, Elevated leptin disrupts epithelial polarity and promotes premalignant alterations in the mammary gland, Oncogene 38 (2019) 3855–3870, https://doi.org/10.1038/s41388-019-0687-8.
[112] F.F. Chehab, M.E. Lim, R. Lu, Correction of the sterility defect in homozygous obese female mice by treatment with the human recombinant leptin, Nat. Genet. 12 (1996) 318–320, https://doi.org/10.1038/ng0396-318.
[113] D. Yamaji, A. Kamikawa, M.M. Soliman, T. Ito, M.M. Ahmed, K. Makondo, A. Watanabe, M. Saito, K. Kimura, Leptin inhibits hepatocyte growth factor-induced ductal morphogenesis of bovine mammary epithelial cells, Jpn. J. Vet. Res. 54 (2007) 183–189.
[114] Y. Feuermann, S.J. Mabjeesh, A. Shamay, Leptin affects prolactin action on milk protein and fat synthesis in the bovine mammary gland, J. Dairy Sci. 87 (2004) 2941–2946, https://doi.org/10.3168/jds.S0022-0302(04)73425-6.
[115] Y. Feuermann, A. Shamay, S.J. Mabjeesh, Leptin up-regulates the lactogenic effect of prolactin in the bovine mammary gland in vitro, J. Dairy Sci. 91 (2008) 4183–4189, https://doi.org/10.3168/jds.2008-0988.
[116] A.R.G. Wylie, Leptin in farm animals: Where are we and where can we go? Animal. 5 (2011) 246–267, https://doi.org/10.1017/S1751731110001540.
[117] J. Boudeau, A.F. Baas, M. Deak, N.A. Morrice, A. Kieloch, M. Schutkowski, A. R. Prescott, H.C. Clevers, D.R. Alessi, MO25α/β interact with STRADα/β enhancing their ability to bind, activate and localize LKB1 in the cytoplasm, EMBO J. 22 (2003) 5102–5114, https://doi.org/10.1093/emboj/cdg490.
[118] Y. Wang, K.S.L. Lam, J.Y. Xu, G. Lu, L.Y. Xu, G.J.S. Cooper, A. Xu, Adiponectin inhibits cell proliferation by interacting with several growth factors in an oligomerization-dependent manner, J. Biol. Chem. 280 (2005) 18341–18347, https://doi.org/10.1074/jbc.M501149200.
[119] D.R. Alessi, K. Sakamoto, J.R. Bayascas, LKB1-dependent signaling pathways RID C-9450-2011, Annu. Rev. Biochem. 75 (2006) 137–163, https://doi.org/10.1146/annurev.biochem.75.103004.142702.
[120] W. Jeong, H. Bae, W. Lim, F.W. Bazer, G. Song, Adiponectin: A prosurvival and proproliferation signal that increases bovine mammary epithelial cell numbers and protects them from endoplasmic reticulum stress responses, J. Anim. Sci. 95 (2017) 5278–5289, https://doi.org/10.2527/jas2017.1885.
[121] G.S. Christodoulatos, N. Spyrou, J. Kadillari, S. Psallida, M. Dalamaga, The role of adipokines in breast Cancer: current evidence and perspectives, Curr. Obes. Rep. 8 (2019) 413–433, https://doi.org/10.1007/s13679-019-00364-y.
[122] T. Brandebourg, E. Hugo, N. Ben-Jonathan, Adipocyte prolactin: regulation of release and putative functions, Diabetes, Obes. Metab. 9 (2007) 464–476, https://doi.org/10.1111/j.1463-1326.2006.00671.x.
[123] Y. Ohtani, T. Yonezawa, S.H. Song, T. Takahashi, A. Ardiyanti, K. Sato, A. Hagino, S. Gun Roh, K. Katoh, Gene expression and hormonal regulation of adiponectin and its receptors in bovine mammary gland and mammary epithelial cells, Anim. Sci. J. 82 (2011) 99–106, https://doi.org/10.1111/j.1740-0929.2010.00805.x.
[124] U.A. White, J. Maier, P. Zhao, A.J. Richard, J.M. Stephens, The modulation of adiponectin by STAT5-activating hormones, Am. J. Physiol. - Endocrinol. Metab. 310 (2016) E129–E136, https://doi.org/10.1152/ajpendo.00068.2015.
[125] M. Rijnkels, E. Kabotyanski, M.B. Montazer-Torbati, C.H. Beauvais, Y. Vassetzky, J.M. Rosen, E. Devinoy, The epigenetic landscape of mammary gland development and functional differentiation, J. Mammary Gland Biol. Neoplasia 15 (2010) 85–100, https://doi.org/10.1007/s10911-010-9170-4.
[126] H. Holliday, L.A. Baker, S.R. Junankar, S.J. Clark, A. Swarbrick, Epigenomics of mammary gland development, Breast Cancer Res. 20 (2018) 1–11, https://doi.org/10.1186/s13058-018-1031-x.
[127] R. Maruyama, S. Choudhury, A. Kowalczyk, M. Bessarabova, B. Beresford-Smith, T. Conway, A. Kaspi, Z. Wu, T. Nikolskaya, V.F. Merino, P.K. Lo, X.S. Liu, Y. Nikolsky, S. Sukumar, I. Haviv, K. Polyak, Epigenetic regulation of cell type-specific expression patterns in the human mammary epithelium, PLoS Genet. 7 (2011), https://doi.org/10.1371/journal.pgen.1001369.
[128] R. Xuan, T. Chao, A. Wang, F. Zhang, P. Sun, S. Liu, M. Guo, G. Wang, Z. Ji, J. Wang, M. Cheng, Characterization of microRNA profiles in the mammary gland tissue of dairy goats at the late lactation, dry period and late gestation stages, PLoS One 15 (2020) 1–25, https://doi.org/10.1371/journal.pone.0234427.
[129] P.A. Jones, Functions of DNA methylation: islands, start sites, gene bodies and beyond, Nat. Rev. Genet. 13 (2012) 484–492, https://doi.org/10.1038/nrg3230.
[130] R. Kanwal, K. Gupta, S. Gupta, in: M. Verma (Ed.), Cancer Epigenetics: An Introduction BT - Cancer Epigenetics: Risk Assessment, Diagnosis, Treatment, and Prognosis, Springer New York, New York, NY, 2015, pp. 3–25, https://doi.org/10.1007/978-1-4939-1804-1_1.
[131] R. Pathania, S. Ramachandran, S. Elangovan, R. Padia, P. Yang, S. Cinghu, R. Veeranan-Karmegam, P. Arjunan, J.P. Gnana-Prakasam, F. Sadanand, L. Pei, C. S. Chang, J.H. Choi, H. Shi, S. Manicassamy, P.D. Prasad, S. Sharma, V. Ganapathy, R. Jothi, M. Thangaraju, DNMT1 is essential for mammary and cancer stem cell maintenance and tumorigenesis, Nat. Commun. 6 (2015), https://doi.org/10.1038/ncomms7910.
[132] S. Mamrut, H. Harony, R. Sood, H. Shahar-Gold, H. Gainer, Y.J. Shi, L. Barki-Harrington, S. Wagner, DNA methylation of specific CpG sites in the promoter region regulates the transcription of the mouse oxytocin receptor, PLoS One 8 (2013), https://doi.org/10.1371/journal.pone.0056869.
[133] A.J. Bannister, T. Kouzarides, Regulation of chromatin by histone modifications, Cell Res. 21 (2011) 381–395, https://doi.org/10.1038/cr.2011.22.
[134] D. Pellacani, M. Bilenky, N. Kannan, A. Heravi-Moussavi, D.J.H.F. Knapp, S. Gakkhar, M. Moksa, A. Carles, R. Moore, A.J. Mungall, M.A. Marra, S.J. M. Jones, S. Aparicio, M. Hirst, C.J. Eaves, Analysis of normal human mammary epigenomes reveals cell-specific active enhancer states and associated transcription factor networks, Cell Rep. 17 (2016) 2060–2074, https://doi.org/10.1016/j.celrep.2016.10.058.
[135] C. Dravis, C.Y. Chung, N.K. Lytle, J. Herrera-Valdez, G. Luna, C.L. Trejo, T. Reya, G.M. Wahl, Epigenetic and transcriptomic profiling of mammary gland development and tumor models disclose regulators of cell state plasticity, Cancer Cell 34 (2018) 466–482, https://doi.org/10.1016/j.ccell.2018.08.001, e6.
[136] E.M. Michalak, K. Nacerddine, A. Pietersen, V. Beuger, I. Pawlitzky, P. Cornelissen-Steijger, E. Wientjens, E. Tanger, J. Seibler, M. Van Lohuizen, J. Jonkers, Polycomb group gene Ezh2 regulates mammary gland morphogenesis and maintains the luminal progenitor pool, Stem Cells 31 (2013) 1910–1920, https://doi.org/10.1002/stem.1437.
[137] B. Pal, T. Bouras, W. Shi, F. Vaillant, J.M. Sheridan, N. Fu, K. Breslin, K. Jiang, M. E. Ritchie, M. Young, G.J. Lindeman, G.K. Smyth, J.E. Visvader, Global Changes in the Mammary Epigenome Are Induced by Hormonal Cues and Coordinated by Ezh2, Cell Rep. 3 (2013) 411–426, https://doi.org/10.1016/j.celrep.2012.12.020.
[138] K.H. Yoo, S. Oh, K. Kang, C. Wang, G.W. Robinson, K. Ge, L. Hennighausen, Histone demethylase KDM6A controls the mammary luminal lineage through enzyme-independent mechanisms, Mol. Cell Biol. 36 (2016) 2108–2120, https://doi.org/10.1128/MCB.00089-16.
[139] L. Qin, Y. Xu, X. Yu, M.J. Toneff, D. Li, L. Liao, J.D. Martinez, Y. Li, J. Xu, The histone demethylase Kdm 3a is required for normal epithelial proliferation ductal elongation and tumor growth in the mouse mammary gland, Oncotarget. 8 (2017) 84761–84775, https://doi.org/10.18632/oncotarget.21380.
[140] L. Song, R.S. Tuan, MicroRNAs and cell differentiation in mammalian development, Birth Defects Res, Part C Embryo Today Rev. 78 (2006) 140–149, https://doi.org/10.1002/bdrc.20070.
[141] V.N. Kim, J. Han, M.C. Siomi, Biogenesis of small RNAs in animals, Nat. Rev. Mol. Cell Biol. 10 (2009) 126–139, https://doi.org/10.1038/nrm2632.
[142] R. Sabry, J. Yamate, L. Favetta, J. LaMarre, MicroRNAs: potential targets and agents of endocrine disruption in female reproduction, J. Toxicol. Pathol. 32 (2019) 213–221, https://doi.org/10.1293/tox.2019-0054.
[143] B. Zeng, Y. Li, Y. Feng, M. Lu, H. Yuan, Z. Yi, Y. Wu, T. Xiang, H. Li, G. Ren, Downregulated miR-1247-5p associates with poor prognosis and facilitates tumor cell growth via DVL1/Wnt/β-catenin signaling in breast cancer, Biochem. Biophys. Res. Commun. 505 (2018) 302–308, https://doi.org/10.1016/j.bbrc.2018.09.103.
[144] T. Kehl, C. Backes, F. Kern, T. Fehlmann, N. Ludwig, E. Meese, H.-P. Lenhof, A. Keller, About miRNAs, miRNA seeds, target genes and target pathways, Oncotarget. 8 (2017), 107167.
[145] C. Braicu, C. Tomuleasa, P. Monroig, A. Cucuianu, I. Berindan-Neagoe, G.A. Calin, Exosomes as divine messengers: Are they the Hermes of modern molecular oncology? Cell Death Differ. 22 (2015) 34–45, https://doi.org/10.1038/cdd.2014.130.
[146] E.C. Lai, Micro RNAs are complementary to 3′ UTR sequence motifs that mediate negative post-transcriptional regulation, Nat. Genet. 30 (2002) 363–364, https://doi.org/10.1038/ng865.
[147] Z. Ji, R. He, T. Chao, R. Xuan, S. Liu, G. Wang, J. Wang, Chi-miR-143-3p promotes apoptosis of mammary gland epithelial cells from dairy goats by targeting Ndfip1, DNA Cell Biol. 38 (2019) 1188–1196, https://doi.org/10.1089/dna.2019.4830.
[148] T. Tanaka, S. Haneda, K. Imakawa, S. Sakai, K. Nagaoka, A microRNA, miR-101a, controls mammary gland development by regulating cyclooxygenase-2 expression, Differentiation. 77 (2009) 181–187, https://doi.org/10.1016/j.diff.2008.10.001.
[149] A. Ucar, V. Vafaizadeh, H. Jarry, J. Fiedler, P.A.B. Klemmt, T. Thum, B. Groner, K. Chowdhury, MiR-212 and miR-132 are required for epithelial stromal interactions necessary for mouse mammary gland development, Nat. Genet. 42 (2010) 1101–1108, https://doi.org/10.1038/ng.709.
[150] J. Wang, Y. Bian, Z. Wang, D. Li, C. Wang, Q. Li, X. Gao, MicroRNA-152 regulates DNA methyltransferase 1 and is involved in the development and lactation of mammary glands in dairy cows, PLoS One 9 (2014) 1–11, https://doi.org/10.1371/journal.pone.0101358.
[151] S. Guil, M. Esteller, DNA methylomes, histone codes and miRNAs: tying it all together, Int. J. Biochem. Cell Biol. 41 (2009) 87–95, https://doi.org/10.1016/j.biocel.2008.09.005.
[152] E. Devinoy, M. Rijnkels, Preface: Epigenetics in mammary gland biology and cancer, J. Mammary Gland Biol. Neoplasia 15 (2010) 1–4, https://doi.org/10.1007/s10911-010-9171-3.
[153] C.M. Klinge, miRNAs regulated by estrogens, tamoxifen, and endocrine disruptors and their downstream gene targets, Mol. Cell. Endocrinol. 418 (2015) 273–297, https://doi.org/10.1016/j.mce.2015.01.035.
[154] B.C. Melnik, G. Schmitz, MicroRNAs: Milk's epigenetic regulators, Best Pract, Res. Clin. Endocrinol. Metab. 31 (2017) 427–442, https://doi.org/10.1016/j.beem.2017.10.003.
[155] C.H. Heldin, M. Landström, A. Moustakas, Mechanism of TGF-β signaling to growth arrest, apoptosis, and epithelial-mesenchymal transition, Curr. Opin. Cell Biol. 21 (2009) 166–176, https://doi.org/10.1016/j.ceb.2009.01.021.
[156] C. Wang, Q. Li, Identification of Differentially Expressed MicroRNAs During the Development of Chinese Murine Mammary Gland, J. Genet. Genomics 34 (2007) 966–973, https://doi.org/10.1016/S1673-8527(07)60109-X.

[157] D.D. Seachrist, K.W. Bonk, S.M. Ho, G.S. Prins, A.M. Soto, R.A. Keri, A review of the carcinogenic potential of bisphenol A, Reprod. Toxicol. 59 (2016) 167–182, https://doi.org/10.1016/j.reprotox.2015.09.006.
[158] J. Michałowicz, Bisphenol A – sources, toxicity and biotransformation, Environ. Toxicol. Pharmacol. 37 (2014) 738–758, https://doi.org/10.1016/j.etap.2014.02.003.
[159] J.G. Boucher, A. Boudreau, S. Ahmed, E. Atlas, In vitro effects of bisphenol a β-D-Glucuronide (BPA-G) on adipogenesis in human and murine preadipocytes, Environ. Health Perspect. 123 (2015) 1287–1293.
[160] J.S. Kang, J.-S. Choi, W.-K. Kim, Y.-J. Lee, J.-W. Park, Estrogenic potency of bisphenol S, polyethersulfone and their metabolites generated by the rat liver S9 fractions on a MVLN cell using a luciferase reporter gene assay, Reprod. Biol. Endocrinol. 12 (2014) 102, https://doi.org/10.1186/1477-7827-12-102.
[161] D. Gramec Skledar, L. Peterlin Mašič, Bisphenol A and its analogs: Do their metabolites have endocrine activity? Environ. Toxicol. Pharmacol. 47 (2016) 182–199, https://doi.org/10.1016/j.etap.2016.09.014.
[162] G.S. Prins, H.B. Patisaul, S.M. Belcher, L.N. Vandenberg, CLARITY-BPA academic laboratory studies identify consistent low-dose Bisphenol A effects on multiple organ systems, Basic Clin. Pharmacol. Toxicol. 125 (2019) 14–31, https://doi.org/10.1111/bcpt.13125.
[163] B.S. Rubin, A. Bisphenol, An endocrine disruptor with widespread exposure and multiple effects, J. Steroid Biochem. Mol. Biol. 127 (2011) 27–34, https://doi.org/10.1016/j.jsbmb.2011.05.002.
[164] G. Mileva, S.L. Baker, A.T.M. Konkle, C. Bielajew, Bisphenol-A: epigenetic reprogramming and effects on reproduction and behavior, Int. J. Environ. Res. Public Health 11 (2014) 7537–7561, https://doi.org/10.3390/ijerph110707537.
[165] L.N. Vandenberg, P.A. Hunt, A.C. Gore, Endocrine disruptors and the future of toxicology testing — lessons from CLARITY–BPA, Nat. Rev. Endocrinol. 15 (2019) 366–374, https://doi.org/10.1038/s41574-019-0173-y.
[166] R.W. Stahlhut, W.V. Welshons, S.H. Swan, Bisphenol A data in NHANES suggest longer than expected half-life, substantial nonfood exposure, or both, Environ. Health Perspect. 117 (2009) 784–789.
[167] L.N. Vandenberg, I. Chahoud, J.J. Heindel, V. Padmanabhan, F.J.R. Paumgartten, G. Schoenfelder, Urinary, circulating, and tissue biomonitoring studies indicate widespread exposure to bisphenol A, Environ. Health Perspect. 118 (2010) 1055–1070, https://doi.org/10.1289/ehp.0901716.
[168] S. Basak, M.K. Das, A.K. Duttaroy, Plastics derived endocrine-disrupting compounds and their effects on early development, Birth Defects Res. (2020), https://doi.org/10.1002/bdr2.1741 n/a.
[169] L.N. Vandenberg, T. Colborn, T.B. Hayes, J.J. Heindel, D.R. Jacobs, D.H. Lee, T. Shioda, A.M. Soto, F.S. Vom Saal, W.V. Welshons, R.T. Zoeller, J.P. Myers, Hormones and endocrine-disrupting chemicals: low-dose effects and nonmonotonic dose responses, Endocr. Rev. 33 (2012) 378–455, https://doi.org/10.1210/er.2011-1050.
[170] M. Montévil, N. Acevedo, C.M. Schaeberle, M. Bharadwaj, S.E. Fenton, A.M. Soto, A combined morphometric and statistical approach to assess non-monotonicity in the developing mammary gland of rats in the CLARITY-BPA study, BioRxiv (2019) (2012) 1–21, https://doi.org/10.1101/783019.
[171] C.E. Hill, J.P. Myers, L.N. Vandenberg, Nonmonotonic dose–Response curves occur in dose ranges that are relevant to regulatory decision-making, Dose-Response. 16 (2018) 1–4, https://doi.org/10.1177/1559325818798282.
[172] O. Banerjee, S. Singh, S.K. Prasad, A. Bhattacharjee, A. Banerjee, A. Banerjee, A. Saha, B.K. Maji, S. Mukherjee, Inhibition of catalase activity with 3-amino-1,2,4-triazole intensifies bisphenol A (BPA)-induced toxicity in granulosa cells of female albino rats, Toxicol. Ind. Health 34 (2018) 787–797, https://doi.org/10.1177/0748233718795744.
[173] A. Mansur, M. Adir, G. Yerushalmi, A. Hourvitz, H. Gitman, Y. Yung, R. Orvieto, R. Machtinger, Does BPA alter steroid hormone synthesis in human granulosa cells in vitro? Hum. Reprod. 31 (2016) 1562–1569, https://doi.org/10.1093/humrep/dew088.
[174] C.M. Markey, P.R. Wadia, B.S. Rubin, C. Sonnenschein, A.M. Soto, Long-term effects of fetal exposure to low doses of the xenoestrogen bisphenol-A in the female mouse genital tract, Biol. Reprod. 72 (2005) 1344–1351, https://doi.org/10.1095/biolreprod.104.036301.
[175] E.C.R. Leonel, S.G.P. Campos, L.H.A. Guerra, C.M. Bedolo, P.S.L. Vilamaior, M. F. Calmon, P. Rahal, C.A. Amorim, S.R. Taboga, Impact of perinatal bisphenol A and 17β estradiol exposure: comparing hormone receptor response, Ecotoxicol. Environ. Saf. 188 (2020), 109918, https://doi.org/10.1016/j.ecoenv.2019.109918.
[176] M.H.A. Kester, S. Bulduk, H. van Toor, D. Tibboel, W. Meinl, H. Glatt, C.N. Falany, M.W.H. Coughtrie, A.G. Schuur, A. Brouwer, T.J. Visser, Potent inhibition of estrogen sulfotransferase by hydroxylated metabolites of polyhalogenated aromatic hydrocarbons reveals alternative mechanism for estrogenic activity of endocrine disrupters, J. Clin. Endocrinol. Metab. 87 (2002) 1142–1150, https://doi.org/10.1210/jcem.87.3.8311.
[177] B.L. Sprague, A. Trentham-Dietz, C.J. Hedman, J. Wang, J.D. Hemming, J. M. Hampton, D.S. Buist, E.J. Aiello Bowles, G.S. Sisney, E.S. Burnside, Circulating serum xenoestrogens and mammographic breast density, Breast Cancer Res. 15 (2013) R45, https://doi.org/10.1186/bcr3432.
[178] A. Konieczna, D. Rachoń, K. Owczarek, P. Kubica, A. Kowalewska, B. Kudłak, A. Wasik, J. Namieśnik, Serum bisphenol A concentrations correlate with serum testosterone levels in women with polycystic ovary syndrome, Reprod. Toxicol. 82 (2018) 32–37, https://doi.org/10.1016/j.reprotox.2018.09.006.
[179] A.A. Martínez-Peña, J. Rivera-Baños, L.L. Méndez-Carrillo, M.I. Ramírez-Solano, A. Galindo-Bustamante, J.C. Páez-Franco, S. Morimoto, L. González-Mariscal, M. E. Cruz, C.A. Mendoza-Rodríguez, Perinatal administration of bisphenol A alters the expression of tight junction proteins in the uterus and reduces the implantation rate, Reprod. Toxicol. 69 (2017) 106–120, https://doi.org/10.1016/j.reprotox.2017.02.009.
[180] Y. Nikaido, K. Yoshizawa, N. Danbara, M. Tsujita-Kyutoku, T. Yuri, N. Uehara, A. Tsubura, Effects of maternal xenoestrogen exposure on development of the reproductive tract and mammary gland in female CD-1 mouse offspring, Reprod. Toxicol. 18 (2004) 803–811, https://doi.org/10.1016/j.reprotox.2004.05.002.
[181] J.C.J. van Esterik, M.E.T. Dollé, M.H. Lamoree, S.P.J. van Leeuwen, T. Hamers, J. Legler, L.T.M. van der Ven, Programming of metabolic eff ects in C57BL/6JxFVB mice by exposure to bisphenol A during gestation and lactation, Toxicology (2014) 40–52.
[182] R. Meli, A. Monnolo, C. Annunziata, C. Pirozzi, M.C. Ferrante, Oxidative stress and BPA toxicity: an antioxidant approach for male and female reproductive dysfunction, Antioxidants. 9 (2020), https://doi.org/10.3390/antiox9050405.
[183] P. Alonso-Magdalena, M. García-Arévalo, I. Quesada, Á. Nadal, Effects of gestational exposure to bisphenol-A on the development of diabetes in mothers, Toxicol. Lett. 259 (2016) S31–S32, https://doi.org/10.1016/j.toxlet.2016.07.650.
[184] P. Alonso-Magdalena, M. García-Arévalo, I. Quesada, Á. Nadal, Bisphenol-A treatment during pregnancy in mice: a new window of susceptibility for the development of diabetes in mothers later in life, Endocrinology. 156 (2015) 1659–1670, https://doi.org/10.1210/en.2014-1952.
[185] I. Ceccarelli, D. Della Seta, P. Fiorenzani, F. Farabollini, A.M. Aloisi, Estrogenic chemicals at puberty change ERα in the hypothalamus of male and female rats, Neurotoxicol. Teratol. 29 (2007) 108–115.
[186] C. Gupta, Reproductive malformation of the male offspring following maternal exposure to estrogenic chemicals (44516), Proc. Soc. Exp. Biol. Med. 224 (2000) 61–68.
[187] M. Beklova, R. Kizek, V. Adam, Molecular biology of beta-estradiol-estrogen receptor complex binding to estrogen response element and the effect on cell proliferation, Neuroendocrinol. Lett. 34 (2013) 2.
[188] Y. Liu, K. Qu, Y. Hai, C. Zhao, Bisphenol A (BPA) binding on full-length architectures of estrogen receptor, J. Cell. Biochem. 119 (2018) 6784–6794.
[189] D.M. Sheehan, Activity of Environmentally Relevant Low Doses of Endocrine Disruptors and the Bisphenol A Controversy: Initial Results Confirmed (44515), 2000.
[190] H. Hiroi, O. Tsutsumi, M. Momoeda, Y. Takai, Y. Osuga, Y. Taketani, Differential Interactions of Bisphenol A and 17 beta-estradiol with Estrogen Receptor alpha (ERa) and ER-b, Endocr. J. 46 (1999) 773–778.
[191] J.B. Matthews, K. Twomey, T.R. Zacharewski, In vitro and in vivo interactions of bisphenol A and its metabolite, bisphenol A glucuronide, with estrogen receptors α and β, Chem. Res. Toxicol. 14 (2001) 149–157, https://doi.org/10.1021/tx0001833.
[192] E.J. Routledge, R. White, M.G. Parker, J.P. Sumpter, Differential effects of xenoestrogens on coactivator recruitment by estrogen receptor (ER) α and ERβ, J. Biol. Chem. 275 (2000) 35986–35993, https://doi.org/10.1074/jbc.M006777200.
[193] Y.B. Wetherill, B.T. Akingbemi, J. Kanno, J.A. McLachlan, A. Nadal, C. Sonnenschein, C.S. Watson, R.T. Zoeller, S.M. Belcher, In vitro molecular mechanisms of bisphenol A action, Reprod. Toxicol. 24 (2007) 178–198, https://doi.org/10.1016/j.reprotox.2007.05.010.
[194] J. Xu, Y. Osuga, T. Yano, Y. Morita, X. Tang, T. Fujiwara, Y. Takai, H. Matsumi, K. Koga, Y. Taketani, O. Tsutsumi, Bisphenol A induces apoptosis and G2-to-M arrest of ovarian granulosa cells, Biochem. Biophys. Res. Commun. 292 (2002) 456–462, https://doi.org/10.1006/bbrc.2002.6644.
[195] N. Jalal, A.R. Surendranath, J.L. Pathak, S. Yu, C.Y. Chung, Bisphenol A (BPA) the mighty and the mutagenic, Toxicol. Reports. 5 (2018) 76–84.
[196] A. Konieczna, A. Rutkowska, D. Rachoń, Health risk of exposure to Bisphenol A (BPA), Rocz. Państwowego Zakładu Hig. 66 (2015) 5–11. http://www.ncbi.nlm.nih.gov/pubmed/25813067.
[197] A.C.W. Pike, Lessons learnt from structural studies of the oestrogen receptor, best Pract, Res. Clin. Endocrinol. Metab. 20 (2006) 1–14, https://doi.org/10.1016/j.beem.2005.09.002.
[198] G.G.J.M. Kuiper, B. Carlsson, K. Grandien, E. Enmark, J. Häggblad, S. Nilsson, J.-A. Gustafsson, Comparison of the ligand binding specificity and transcript tissue distribution of estrogen receptors $α$ and $β$, Endocrinology 138 (1997) 863–870, https://doi.org/10.1210/endo.138.3.4979.
[199] T.W. Schwartz, B. Holst, Allosteric enhancers, allosteric agonists and ago-allosteric modulators: where do they bind and how do they act? Trends Pharmacol. Sci. 28 (2007) 366–373.
[200] E.M. Jorgensen, M.H. Alderman III, H.S. Taylor, Preferential epigenetic programming of estrogen response after in utero xenoestrogen (bisphenol-A) exposure, FASEB J. 30 (2016) 3194–3201.
[201] C.M. Klinge, Estrogen receptor interaction with estrogen response elements, Nucleic Acids Res. 29 (2001) 2905–2919, https://doi.org/10.1093/nar/29.14.2905.
[202] P. Webb, P. Nguyen, C. Valentine, G.N. Lopez, G.R. Kwok, E. McInerney, B. S. Katzenellenbogen, E. Enmark, J.-A. Gustafsson, S. Nilsson, P.J. Kushner, The Estrogen Receptor Enhances AP-1 Activity by Two Distinct Mechanisms with Different Requirements for Receptor Transactivation Functions, Mol. Endocrinol. 13 (1999) 1672–1685, https://doi.org/10.1210/mend.13.10.0357.
[203] M. Marino, P. Ascenzi, Membrane association of estrogen receptor $α$ and $β$ influences 17$β$-estradiol-mediated cancer cell proliferation, Steroids 73 (2008) 853–858, https://doi.org/10.1016/j.steroids.2007.12.003.
[204] J.F. Couse, J. Lindzey, K. Grandien, J.-A. Gustafsson, K.S. Korach, Tissue distribution and quantitative analysis of estrogen Receptor-α (ERα) and estrogen

Receptor-β (ERβ) messenger ribonucleic acid in the wild-type and ER$α $-Knockout mouse, Endocrinology. 138 (1997) 4613–4621, https://doi.org/10.1210/endo.138.11.5496.
[205] E.C.R. Leonel, L.R. Falleiros, S.G.P. Campos, S.R. Taboga, Histological and immunohistochemical characterization of the Mongolian gerbil's mammary gland during gestation, lactation and involution, Acta Histochem. (2017), https://doi.org/10.1016/j.acthis.2017.02.003.
[206] P.R. Wadia, L.N. Vandenberg, C.M. Schaeberle, B.S. Rubin, C. Sonnenschein, A. M. Soto, Perinatal bisphenol A exposure increases estrogen sensitivity of the mammary gland in diverse mouse strains, Environ. Health Perspect. 115 (2007) 592–598, https://doi.org/10.1289/ehp.9640.
[207] M.A.A. Ibrahim, R.H. Elbakry, N.A. Bayomy, Effect of bisphenol A on morphology, apoptosis and proliferation in the resting mammary gland of the adult albino rat, Int. J. Exp. Pathol. 97 (2016) 27–36, https://doi.org/10.1111/iep.12164.
[208] D. Faratian, A. Munro, C. Twelves, J. Bartlett, Membranous and cytoplasmic staining of Ki67 is associated with HER2 and ER status in invasive breast carcinoma, Histopathology. 54 (2009) 254–257, https://doi.org/10.1111/j.1365-2559.2008.03191.x.
[209] X.-Y. Qin, T. Fukuda, L. Yang, H. Zaha, H. Akanuma, Q. Zeng, J. Yoshinaga, H. Sone, Effects of bisphenol A exposure on the proliferation and senescence of normal human mammary epithelial cells, Cancer Biol. Ther. 13 (2012) 296–306.
[210] Y.C. Li, Y.H. Kuan, F.M. Huang, Y.C. Chang, The role of DNA damage and caspase activation in cytotoxicity and genotoxicity of macrophages induced by bisphenol-A-glycidyldimethacrylate, Int. Endod. J. 45 (2012) 499–507, https://doi.org/10.1111/j.1365-2591.2011.02001.x.
[211] M. Pupo, A. Pisano, R. Lappano, M.F. Santolla, E.M. de Francesco, S. Abonante, C. Rosano, M. Maggiolini, Bisphenol A induces gene expression changes and proliferative effects through GPER in breast cancer cells and cancer-associated fibroblasts, Environ. Health Perspect. 120 (2012) 1177–1182, https://doi.org/10.1289/ehp.1104526.
[212] S. Dong, S. Terasaka, R. Kiyama, Bisphenol A induces a rapid activation of Erk1/2 through GPR30 in human breast cancer cells, Environ. Pollut. 159 (2011) 212–218, https://doi.org/10.1016/j.envpol.2010.09.004.
[213] M. Perrot-Applanat, M. Kolf-Clauw, C. Michel, C. Beausoleil, Alteration of mammary gland development by bisphenol a and evidence of a mode of action mediated through endocrine disruption, Mol. Cell. Endocrinol. 475 (2018) 29–53, https://doi.org/10.1016/j.mce.2018.06.015.
[214] A.M. Betancourt, J.A. Mobley, J. Russo, C.A. Lamartiniere, Proteomic analysis in mammary glands of rat offspring exposed in utero to bisphenol A, J. Proteomics 73 (2010) 1241–1253, https://doi.org/10.1016/j.jprot.2010.02.020.
[215] X.-L. Zhang, N. Liu, S.-F. Weng, H.-S. Wang, Bisphenol a increases the migration and invasion of triple-negative breast Cancer cells via oestrogen-related receptor gamma, Basic Clin. Pharmacol. Toxicol. 119 (2016) 389–395, https://doi.org/10.1111/bcpt.12591.
[216] H. Buteau-Lozano, G. Velasco, M. Cristofari, P. Balaguer, M. Perrot-Applanat, Xenoestrogens modulate vascular endothelial growth factor secretion in breast cancer cells through an estrogen receptor-dependent mechanism, J. Endocrinol. 196 (2008) 399–412, https://doi.org/10.1677/JOE-07-0198.
[217] A. Ayyanan, O. Laribi, S. Schuepbach-Mallepell, C. Schrick, M. Gutierrez, T. Tanos, G. Lefebvre, J. Rougemont, Ö. Yalcin-Ozuysal, C. Brisken, Perinatal exposure to bisphenol a increases adult mammary gland progesterone response and cell number, Mol. Endocrinol. 25 (2011) 1915–1923, https://doi.org/10.1210/me.2011-1129.
[218] S. Jenkins, N. Raghuraman, I. Eltoum, M. Carpenter, J. Russo, C.A. Lamartiniere, Oral exposure to Bisphenol A increases dimethylbenzanthraceneo-induced mammary cancer in rats, Environ. Health Perspect. 117 (2009) 910–915, https://doi.org/10.1289/ehp.11751.
[219] Y. Meng, Z. Yannan, L. Ren, S. Qi, W. Wei, J. Lihong, Adverse reproductive function induced by maternal BPA exposure is associated with abnormal autophagy and activating inflamation via mTOR and TLR4/NF-κB signaling pathways in female offspring rats, Reprod. Toxicol. 96 (2020) 185–194, https://doi.org/10.1016/j.reprotox.2020.07.001.
[220] C.J. Marsit, Influence of environmental exposure on human epigenetic regulation, J. Exp. Biol. 218 (2015) 71–79, https://doi.org/10.1242/jeb.106971.
[221] K. Wang, Z. Zhao, W. Ji, Bisphenol A induces apoptosis, oxidative stress and inflammatory response in colon and liver of mice in a mitochondria-dependent manner, Biomed. Pharmacother. 117 (2019), https://doi.org/10.1016/j.biopha.2019.109182.
[222] D. Pfeifer, Y.M. Chung, M.C.T. Hu, Effects of low-dose bisphenol A on DNA damage and proliferation of breast cells: the role of c-Myc, Environ. Health Perspect. 123 (2015) 1271–1279, https://doi.org/10.1289/ehp.1409199.
[223] N.R. Gassman, Induction of oxidative stress by bisphenol a and its pleiotropic effects, Environ. Mol. Mutagen. 58 (2017) 60–71, https://doi.org/10.1002/em.
[224] Y.J. Yang, Y.C. Hong, S.Y. Oh, M.S. Park, H. Kim, J.H. Leem, E.H. Ha, Bisphenol A exposure is associated with oxidative stress and inflammation in postmenopausal women, Environ. Res. 109 (2009) 797–801, https://doi.org/10.1016/j.envres.2009.04.014.
[225] S.L. Berger, T. Kouzarides, R. Shiekhattar, A. Shilatifard, An operational definition of epigenetics, Genes Dev. 23 (2009) 781–783.
[226] C. Faulk, J.H. Kim, O.S. Anderson, M.S. Nahar, T.R. Jones, M.A. Sartor, D. C. Dolinoy, Detection of differential DNA methylation in repetitive DNA of mice and humans perinatally exposed to bisphenol A, Epigenetics 11 (2016) 489–500, https://doi.org/10.1080/15592294.2016.1183856.
[227] S. Basak, V. Srinivas, A.K. Duttaroy, Bisphenol-A impairs cellular function and alters DNA methylation of stress pathway genes in first trimester trophoblast cells, Reprod. Toxicol. 82 (2018) 72–79.
[228] M. Senyildiz, E.F. Karaman, S.S. Bas, P.A. Pirincci, S. Ozden, Effects of BPA on global DNA methylation and global histone 3 lysine modifications in SH-SY5Y cells: an epigenetic mechanism linking the regulation of chromatin modifiying genes, Toxicol. In Vitro 44 (2017) 313–321, https://doi.org/10.1016/j.tiv.2017.07.028.
[229] O.S. Anderson, M.S. Nahar, C. Faulk, T.R. Jones, C. Liao, K. Kannan, C. Weinhouse, L.S. Rozek, D.C. Dolinoy, Epigenetic responses following maternal dietary exposure to physiologically relevant levels of bisphenol A, Environ. Mol. Mutagen. 53 (2012) 334–342, https://doi.org/10.1002/em.21692.
[230] J.G. Bromer, Y. Zhou, M.B. Taylor, L. Doherty, H.S. Taylor, Bisphenol-A exposure in utero leads to epigenetic alterations in the developmental programming of uterine estrogen response, FASEB J. 24 (2010) 2273–2280, https://doi.org/10.1096/fj.09-140533.
[231] S.V. Fernandez, Y. Huang, K.E. Snider, Y. Zhou, T.J. Pogash, J. Russo, Expression and DNA methylation changes in human breast epithelial cells after bisphenol A (BPA) exposure, Int. J. Oncol. 41 (2012) 369–377, https://doi.org/10.3892/ijo.2012.1444.
[232] Z. Awada, R. Nasr, R. Akika, V. Cahais, C. Cuenin, M. Zhivagui, Z. Herceg, A. Ghantous, N.K. Zgheib, DNA methylome-wide alterations associated with estrogen receptor-dependent effects of bisphenols in breast cancer, Clin. Epigenetics 11 (2019) 138, https://doi.org/10.1186/s13148-019-0725-y.
[233] X. Wang, Y. Wei, T. Guo, J. Yuan, R. Zhao, Epigenetic effect of long-term bisphenol A exposure on human breast adenocarcinoma cells, Toxicol. Environ. Chem. 100 (2018) 258–266.
[234] T. Doshi, S.S. Mehta, V. Dighe, N. Balasinor, G. Vanage, Hypermethylation of estrogen receptor promoter region in adult testis of rats exposed neonatally to bisphenol A, Toxicology 289 (2011) 74–82, https://doi.org/10.1016/j.tox.2011.07.011.
[235] H.M.A. El Henafy, M.A. Ibrahim, S.A. Abd El Aziz, E.M. Gouda, Oxidative Stress and DNA methylation in male rat pups provoked by the transplacental and translactational exposure to bisphenol A, Environ. Sci. Pollut. Res. 27 (2020) 4513–4519, https://doi.org/10.1007/s11356-019-06553-5.
[236] G.A. Altamirano, J.G. Ramos, A.L. Gomez, E.H. Luque, M. Muñoz-de-Toro, L. Kass, Perinatal exposure to bisphenol A modifies the transcriptional regulation of the β-Casein gene during secretory activation of the rat mammary gland, Mol. Cell. Endocrinol. 439 (2017) 407–418, https://doi.org/10.1016/j.mce.2016.09.032.
[237] E. Dhimolea, P.R. Wadia, T.J. Murray, M.L. Settles, J.D. Treitman, C. Sonnenschein, T. Shioda, A.M. Soto, Prenatal exposure to BPA alters the epigenome of the rat mammary gland and increases the propensity to neoplastic development, PLoS One 9 (2014), https://doi.org/10.1371/journal.pone.0099800 e99800–e99800.
[238] L.F. Doherty, J.G. Bromer, Y. Zhou, T.S. Aldad, H.S. Taylor, In utero exposure to diethylstilbestrol (DES) or bisphenol-A (BPA) increases EZH2 expression in the mammary gland: an epigenetic mechanism linking endocrine disruptors to breast cancer, Horm. Cancer 1 (2010) 146–155.
[239] A. Kuzmichev, K. Nishioka, H. Erdjument-Bromage, P. Tempst, D. Reinberg, Histone methyltransferase activity associated with a human multiprotein complex containing the Enhancer of Zeste protein, Genes Dev. 16 (2002) 2893–2905.
[240] T. Zhao Ping, X. Wang Liang, Y. Han Min, Knockdown of p57 gene inhibits breast cancer cell proliferation, Oncol. Lett. 16 (2018) 55–58, https://doi.org/10.3892/ol.2018.8605.
[241] H.C. Bruner, P.W.B. Derksen, Loss of E-Cadherin-Dependent cell–Cell adhesion and the development and progression of Cancer, cold spring harb, Perspect. Biol. Med. 10 (2018), https://doi.org/10.1101/cshperspect.a029330.
[242] L.F. Gulyaeva, N.E. Kushlinskiy, Regulatory mechanisms of microRNA expression, J. Transl. Med. 14 (2016) 143.
[243] K. Yamagata, S. Fujiyama, S. Ito, T. Ueda, T. Murata, M. Naitou, K. Takeyama, Y. Minami, B.W. O'Malley, S. Kato, Retraction notice to: maturation of MicroRNA is hormonally regulated by a nuclear receptor, Mol. Cell 54 (2014) 536, https://doi.org/10.1016/j.molcel.2014.04.018.
[244] G. Di Leva, C. Piovan, P. Gasparini, A. Ngankeu, C. Taccioli, D. Briskin, D. G. Cheung, B. Bolon, L. Anderlucci, H. Alder, G. Nuovo, M. Li, M.V. Iorio, M. Galasso, S. Ramasamy, G. Marcucci, D. Perrotti, K.A. Powell, A. Bratasz, M. Garofalo, K.P. Nephew, C.M. Croce, Estrogen mediated-activation of miR-191/425 cluster modulates tumorigenicity of breast Cancer cells depending on estrogen receptor status, PLoS Genet. 9 (2013), e1003311, https://doi.org/10.1371/journal.pgen.1003311.
[245] C.M. Klinge, Estrogen regulation of MicroRNA expression, Curr. Genomics 10 (2009) 169–183, https://doi.org/10.2174/138920209788185289.
[246] K. Vrijens, V. Bollati, T.S. Nawrot, MicroRNAs as potential signatures of environmental exposure or effect: a systematic review, Environ. Health Perspect. 123 (2015) 399–411, https://doi.org/10.1289/ehp.1408459.
[247] S.L. Tilghman, M.R. Bratton, H.C. Segar, E.C. Martin, L.V. Rhodes, M. Li, J. A. McLachlan, T.E. Wiese, K.P. Nephew, M.E. Burow, Endocrine disruptor regulation of microRNA expression in breast carcinoma cells, PLoS One 7 (2012), https://doi.org/10.1371/journal.pone.0032754.
[248] Z. Chen, J. Luo, C. Zhang, Y. Ma, S. Sun, T. Zhang, J.J. Loor, Mechanism of prolactin inhibition of miR-135b via methylation in goat mammary epithelial cells, J. Cell. Physiol. 233 (2018) 651–662, https://doi.org/10.1002/jcp.25925.
[249] A. Veiga-Lopez, L.J. Luense, L.K. Christenson, V. Padmanabhan, Developmental Programming: Gestational Bisphenol-A Treatment Alters Trajectory of Fetal

Ovarian Gene Expression, Endocrinology 154 (2013) 1873–1884, https://doi.org/10.1210/en.2012-2129.
[250] H. Zhang, T. Liu, B. Li, K. Zhang, D. Wang, Y. Liu, L. Ge, Y. Jiang, F. Su, Establishment of a stable β-Casein protein-secreted laoshan dairy goat mammary epithelial cell line, Front. Vet. Sci. 7 (2020) 501, https://doi.org/10.3389/fvets.2020.00501.
[251] C. Roth, B. Rack, V. Müller, W. Janni, K. Pantel, H. Schwarzenbach, Circulating microRNAs as blood-based markers for patients with primary and metastatic breast cancer, Breast Cancer Res. 12 (2010) R90, https://doi.org/10.1186/bcr2766.
[252] L. Hilakivi-Clarke, S. de Assis, A. Warri, Exposures to synthetic estrogens at different times during the life, and their effect on breast cancer risk, J. Mammary Gland Biol. Neoplasia 18 (2013) 25–42.
[253] C. Wormsbaecher, A.R. Hindman, A. Avendano, M. Cortes-Medina, C.E. Jones, A. Bushman, L. Onua, C.E. Kovalchin, A.R. Murphy, H.L. Helber, A. Shapiro, K. Voytovitch, X. Kuang, R. Aguilar-Valenzuela, J.L. Leight, J.W. Song, C.J. Burd, In utero estrogenic endocrine disruption alters the stroma to increase extracellular matrix density and mammary gland stiffness, Breast Cancer Res. 22 (2020) 1–12, https://doi.org/10.1186/s13058-020-01275-w.
[254] E.C.R. Leonel, S.G.P. Campos, C.M.B. Bedolo, L.H.A. Guerra, P.S.L. Vilamaior, M. F. Calmon, P. Rahal, C.A. Amorim, S.R. Taboga, Perinatal exposure to bisphenol A impacts in the mammary gland morphology of adult Mongolian gerbils, Exp. Mol. Pathol. 113 (2020), 104374, https://doi.org/10.1016/j.yexmp.2020.104374.
[255] C. Fischer, R. Mamillapalli, L.G. Goetz, E. Jorgenson, Y. Ilagan, H.S. Taylor, Bisphenol a (BPA) exposure in utero leads to immunoregulatory cytokine dysregulation in the mouse mammary gland: a potential mechanism programming breast Cancer risk, Horm. Cancer 7 (2016) 241–251, https://doi.org/10.1007/s12672-016-0254-5.
[256] A.P. Tharp, M.V. Maffini, P.A. Hunt, C.A. Vandevoort, C. Sonnenschein, A. M. Soto, Bisphenol A alters the development of the rhesus monkey mammary gland, Proc. Natl. Acad. Sci. U. S. A. 109 (2012) 8190–8195, https://doi.org/10.1073/pnas.1120488109.
[257] L.N. Vandenberg, M.V. Maffini, C.M. Schaeberle, A.A. Ucci, C. Sonnenschein, B. S. Rubin, A.M. Soto, Perinatal exposure to the xenoestrogen bisphenol-A induces mammary intraductal hyperplasias in adult CD-1 mice, Reprod. Toxicol. 26 (2008) 210–219, https://doi.org/10.1016/j.reprotox.2008.09.015.
[258] S.E. Fenton, Endocrine-disrupting compounds and mammary gland development: early exposure and later life consequences, Endocrinology. 147 (2006) s18–s24, https://doi.org/10.1210/en.2005-1131.
[259] H.A. Martinson, T.R. Lyons, E.D. Giles, V.F. Borges, P. Schedin, Developmental windows of breast cancer risk provide opportunities for targeted chemoprevention, Exp. Cell Res. 319 (2013) 1671–1678, https://doi.org/10.1016/j.yexcr.2013.04.018.
[260] M.D. Anway, M.K. Skinner, Epigenetic transgenerational actions of endocrine disruptors, Endocrinology. 147 (2006) s43–s49, https://doi.org/10.1210/en.2005-1058.
[261] M.K. Skinner, M. Manikkam, C. Guerrero-Bosagna, Epigenetic transgenerational actions of environmental factors in disease etiology, Trends Endocrinol. Metab. 21 (2010) 214–222, https://doi.org/10.1016/j.tem.2009.12.007.
[262] T. Yaoi, K. Itoh, K. Nakamura, H. Ogi, Y. Fujiwara, S. Fushiki, Genome-wide analysis of epigenomic alterations in fetal mouse forebrain after exposure to low doses of bisphenol A, Biochem. Biophys. Res. Commun. 376 (2008) 563–567, https://doi.org/10.1016/j.bbrc.2008.09.028.
[263] K. Chiam, W.D. Tilley, L.M. Butler, T. Bianco-Miotto, The dynamic and static modification of the epigenome by hormones: a role in the developmental origin of hormone related cancers, Biochim. Biophys. Acta - Rev. Cancer. 1795 (2009) 104–109, https://doi.org/10.1016/j.bbcan.2008.12.001.
[264] J.M. Faupel-Badger, K.F. Arcaro, J.J. Balkam, A.H. Eliassen, F. Hassiotou, C. B. Lebrilla, K.B. Michels, J.R. Palmer, P. Schedin, A.M. Stuebe, C.J. Watson, M. E. Sherman, Postpartum remodeling, lactation, and breast Cancer risk: summary of a national Cancer institute–sponsored workshop, JNCI J. Natl. Cancer Inst. 105 (2012) 166–174, https://doi.org/10.1093/jnci/djs505.
[265] M.M. Basree, N. Shinde, C. Koivisto, M. Cuitino, R. Kladney, J. Zhang, J. Stephens, M. Palettas, A. Zhang, H.K. Kim, S. Acero-Bedoya, A. Trimboli, D.G. Stover, T. Ludwig, R. Ganju, D. Weng, P. Shields, J. Freudenheim, G.W. Leone, G. M. Sizemore, S. Majumder, B. Ramaswamy, Abrupt involution induces inflammation, estrogenic signaling, and hyperplasia linking lack of breastfeeding with increased risk of breast cancer, Breast Cancer Res. 21 (2019) 80, https://doi.org/10.1186/s13058-019-1163-7.
[266] R. Hart, D.A. Doherty, H. Frederiksen, J.A. Keelan, M. Hickey, D. Sloboda, C. E. Pennell, J.P. Newnham, N.E. Skakkebaek, K.M. Main, The influence of antenatal exposure 1854 to phthalates on subsequent female reproductive development in adolescence: a pilot study, Reproduction 147 (2014) 379–390.
[267] J.F. Ohlstein, A.L. Strong, J.A. McLachlan, J.M. Gimble, M.E. Burow, B. A. Bunnell, Bisphenol a enhances adipogenic differentiation of human adipose stromal/stem cells, J. Mol. Endocrinol. 53 (2014) 345–353, https://doi.org/10.1530/JME-14-0052.
[268] M. Susiarjo, F. Xin, M. Stefaniak, C. Mesaros, R.A. Simmons, M.S. Bartolomei, Bile acids and tryptophan metabolism are novel pathways involved in metabolic abnormalities in BPA-exposed pregnant mice and male offspring, Endocrinology. 158 (2017) 2533–2542, https://doi.org/10.1210/en.2017-00046.
[269] A.L. Gomez, M.B. Delconte, G.A. Altamirano, L. Vigezzi, V.L. Bosquiazzo, L. F. Barbisan, J.G. Ramos, E.H. Luque, M. Muñoz-de-Toro, L. Kass, Perinatal exposure to bisphenol a or diethylstilbestrol increases the susceptibility to develop mammary gland lesions after estrogen replacement therapy in middle-aged rats, Horm. Cancer 8 (2017) 78–89, https://doi.org/10.1007/s12672-016-0282-1.
[270] A. Belfiore, F. Frasca, IGF and insulin receptor signaling in breast cancer, J. 1906 Mammary Gland Biol. Neoplasia. 13 (2008) 381–406, https://doi.org/10.1007/s10911-008-9099-z, 1907.

ANEXO III – Ruiz et al., 2022 (Environmental Toxicology and Pharmacology)

Environmental Toxicology and Pharmacology 89 (2022) 103785

Contents lists available at ScienceDirect

Environmental Toxicology and Pharmacology

journal homepage: www.elsevier.com/locate/etap

Gestational and lactational xenoestrogen exposure disrupts morphology and inflammatory aspects in mammary gland of gerbil mothers during involution

Thalles Fernando Rocha Ruiz [a,1], Ellen Cristina Rivas Leonel [a,b,1], Simone Jacovaci Colleta [a], Carolina Marques Bedolo [a], Silvana Gisele Pegorin de Campos [a], Sebastião Roberto Taboga [a,*]

[a] Department of Biology, Institute of Biosciences, Humanities and Exact Sciences, São Paulo State University (Unesp), Rua Cristóvão Colombo, 2265, Jardim Nazareth, 15054-000 São José do Rio Preto, São Paulo, Brazil
[b] Department of Histology, Embryology and Cell Biology, Institute of Biological Sciences, Federal University of Goiás (UFG), Avenida Esperança, s/n, Câmpus Samambaia, 74690-900 Goiânia, Goiás, Brazil

ARTICLE INFO

Keywords:
Endocrine disruptor
Bisphenol A
Estradiol
Extracellular matrix
Inflammation

ABSTRACT

In the mammary gland (MG), the developmental window for gestational/lactational differentiation and growth is highly vulnerable to hormonal disruption. Here we describe that the MG involution process in female gerbil mothers is delayed by bisphenol A (BPA) exposure during gestation and lactation. The process is directly influenced by changes in expression of extracellular matrix proteases MMP-2, MMP-9, and FAP, and the incidence of collagen and elastin is reduced after 7 and 14 days of weaning. A pro-inflammatory environment in the late involution process was confirmed by higher expression of TNF-α, COX-2 and phospho-STAT3 n the MG stroma, allied to increases in the incidence of macrophages and mast cells. These aspects impacted the proliferative pattern of epithelial cells, which decreased on the 14th post-weaning day. These data confirm that the milk production window of susceptibility is vulnerable to the impact of BPA, which promotes a suggestive protumoral microenvironment during mammary involution.

1. Introduction

Breast cancer is the most commonly diagnosed type of cancer among women worldwide (Jemal et al., 2011) and its incidence has been related to genetic (Kandel et al., 2021) and epigenetic (Doherty et al., 2010) factors. In addition, environmental compounds have been related to mammary gland neoplastic disturbances; having, for example, a detrimental effect on the expression of hormone receptors in this gland, inducing a mechanism of endocrine disruption (Koual et al., 2020). Disturbances in signaling pathways that impact the epithelium/stroma relation have been widely related to endocrine disruptor exposure, especially in plastic and hormonal responsive glands such as the mammary gland (Wadia et al., 2013). The main concern is triggering of neoplastic development, since this promotes oncogenic transformation in tissues in a short- or long-term manner (Miret et al., 2019).

Exposure to endocrine disruptors that exert estrogenic effects, the xenoestrogens, is ubiquitous. Human biomonitoring guidance values for the chemical bisphenol A (BPA), released by plastics, indicate that 230 μg/L and 135 μg/L of this compound are present in the urine of adults and children, respectively (Ougier et al., 2021). Daily contact with BPA induces toxic impacts, such as metabolic diseases, altered liver function, and estrogen action (vom Saal and Vandenberg, 2021). BPA binds to steroid hormone receptors by an inappropriate transcriptional conformation (Acconcia et al., 2015). As a result, the proliferative mechanisms of estrogen receptors are disturbed, leading to epithelial deregulation and lesions (Durando et al., 2006).

When exposure to endocrine disruptors takes place during the developmental phases of a steroid responsive gland the impacts may be worsened (Colborn et al., 1994). Several research groups have focused on studying the impacts of BPA on mammary gland exposure during windows of susceptibility, such as the perinatal (Altamirano et al., 2017; Gomez et al., 2017; Leonel et al., 2020) and pubertal (Schoeters et al.,

* Correspondence to: São Paulo State University (Unesp), Department of Biology, Institute of Biosciences, Humanities and Exact Sciences, Cristóvão Colombo St., 2265, Postal Code, Jardim Nazareth, 15054–000 São José do Rio Preto, São Paulo, Brazil.
E-mail addresses: thalles.ruiz@unesp.br (T.F.R. Ruiz), ellenleonel@ufg.br (E.C.R. Leonel), Brazil.sebastiao.taboga@unesp.br (S.R. Taboga).
[1] T.F.R. Ruiz and E.C.R. Leonel are joint first authors.

https://doi.org/10.1016/j.etap.2021.103785
Received 15 September 2021; Received in revised form 30 November 2021; Accepted 6 December 2021
Available online 9 December 2021

2008; Wang et al., 2014) developmental phases. This is due to the presence of a dynamic environment in the limits between epithelium and stroma, where a basal membrane rupture is present (Cowin and Wysolmerski, 2010), promoting contact between precursor mammary epithelial cells and BPA in the bloodstream.

Conversely, the impacts of this exposure during adult developmental windows of susceptibility, such as the gestational and lactational proliferative/secreting phases have not been well addressed. This mammary gland stage of development is equally unstable and may provide a hyper exposed environment (Terry et al., 2019). This is because, at this moment, cells are preparing to reorganize and establish a completely developed milk productive gland; consequently, the extracellular matrix undergoes deep changes in its composition and structure (Mori et al., 2013), leaving the cells exposed to serum variations in hormones and disrupting chemicals. The next phase, involution, naturally provides a pro-inflammatory environment that may lead to metastasis and tumor development; thus, previous exposure to disruptors could worsen the risk of developing neoplastic lesions (Faupel-Badger et al., 2012).

It is imperative to understand the impacts on tissue homeostasis during vulnerable initial developmental phases. However, the consequences of this exposure during the mammary gland milk production window of susceptibility have been neglected. Even in rodent models, little is known about what happens to the mothers' mammary gland after exposure to a disrupting environment during the secretory window of susceptibility. Furthermore, impacts may be worsened by mechanisms involved in the gland involution after weaning. Thus, the current work aimed to analyze the morphological features and protein expression in Mongolian gerbil involuting mammary gland from mothers exposed to 17β-estradiol and BPA during the gestational and lactational gland developmental phases.

2. Materials and methods

2.1. Ethics and environment

Female Mongolian gerbils (*Meriones unguiculatus*), a species with a natural tendency to develop mammary neoplasia, were used as an experimental model for endocrine disruption (Ruiz et al., 2021b). Animal experimentation was conducted according to the standards defined by the National Council of Animal Experimentation Control (CONCEA, Brazil, www.mctic.gov.br). The manipulations – gavage, weaning, and euthanasia – were approved by the local Ethics Committee on the Use of Animals (CEUA) (IBILCE/UNESP protocol number 113/2015).

Animals were kept in BPA free isolators (Alesco, Monte Mor, SP, Brazil), organized in racks, with ventilation and a controlled environment: light/dark cycle of 12/12 h, room temperature of 23–26 °C, and 50% relative humidity. Fresh water and a commercial chow (Presence, Paulínia, SP, Brazil) were provided ad libitum during the experiment. Virgin female gerbils were housed with fertile males to mate. Pregnancy was confirmed by the presence of sperm cells in the vaginal smears – pregnancy day 0. The pregnant females were the object of study for this experiment, while the males and the offspring were applied for other experiments.

2.2. Animals and treatments

Pregnant gerbils (N = 34) were divided into four groups and subjected to treatments from the 8th gestational day until the end of lactation (39 days of treatment). The manipulation was always performed during the morning (8–10 a.m.) and was suspended only on the parturition day. All pregnant females were subjected to oral gavage and classified as follows: (Control) daily gavage with vehicle corn oil (Mazola, Mairinque, SP, Brazil); (E2) 35 μg/kg of 17β-estradiol (E8515, Sigma Aldrich, St. Louis, MO, USA), applied as a source of natural occurring estrogen (Kuhl, 2005; Pinkerton et al., 2017; Pinto et al., 2008) diluted in 0.1 ml corn oil 3 times/week; (↓BPA) 50 μg/kg bisphenol A (BPA) (239,658, Sigma Aldrich), safe dose recognized by the European Food Safety Authority, diluted in 0.1 ml corn oil daily (Vandenberg et al. 2012; EFSA, 2015); and (↑BPA) 5000 μg/kg BPA (239,658, Sigma Aldrich) diluted in 0.1 ml corn oil daily, referring to an acute exposure.

After weaning, females from each group were kept with no other treatments and were euthanized on either the 7th or the 14th day post weaning, being sorted into two subgroups (n = 5 animals per subgroup of ↓BPA and ↑BPA; n = 4 animals per subgroup of control; n = 3 animals per subgroup of E2) allowing evaluation of two different periods of mammary gland involution. The euthanasia was performed during the morning and comprised sedation with 3 mg/kg xylazine (Anasedan, Ceva, Paulínia, SP, Brazil) and 10 mg/kg ketamine (Cetamin, Syntec, Barueri, SP, Brazil) followed by decapitation.

2.3. Tissue preparation and morphological analysis

The right abdominal mammary gland was removed and immediately fixed in paraformaldehyde 4% for 24 h at 4 °C. Samples were transferred to ethanol 70% and histologically processed in a semi enclosed system according to standard protocols (TP1020, Leica Biosystems, Buffalo Grove IL, USA). The paraffin blocks obtained were sliced (4 μm thick) and hematoxylin and eosin (HE) staining was conducted. Stained slides were digitally scanned (400 × magnification) using a B× 61VS camera (Olympus Corporation, Tokyo, Japan) coupled to an Olympus VS120 Virtual Microscope Slide Scanning System (VS120–S5) from the same company.

2.4. Tissue general morphology analysis

To evaluate the mammary gland regression process, a first analysis of the gland general morphology was performed. For this, sections from three different depths (40 μm of interval) were evaluated from each animal (OlyVIA 3.2, Olympus). Areas of epithelium, stroma surrounding epithelium (fibroblasts + ECM), and adipose tissue occupation were quantified. A percentage of occupation over the total area of the section was calculated and applied to statistical analysis to infer about the involution process.

2.5. Collagen and elastin fiber quantification

To compare collagen fiber deposition rates in the mammary tissue among groups, picrosirius staining was performed. The slides were stained for 1 h in Sirius Red solution (Direct Red 80 in saturated picric acid solution), washed and counterstained with hematoxylin for 10 min, dehydrated, and mounted. For elastin analysis, the slides were stained using the Weigert's Resorcin-Fuchsin technique, by staining with Resorcin-Fuchsin solution followed by picric acid solution washing. Ten random fields (200x magnification) per animal were evaluated and the area occupied by collagen fibers was quantified automatically by ImageJ software (version 1.52a, Wayne Rasband, NIH, USA).

2.6. Histochemistry and Immunohistochemistry

Sections were dewaxed following standard protocols. Histochemistry was performed for identification of mast cells: sections were stained with 1% toluidine blue (pH 4.0). For immunohistochemistry, antigens were retrieved, endogenous peroxidases were blocked in 5% H_2O_2, and nonspecific proteins were blocked using 5% skimmed milk diluted in TBS. Sections were incubated overnight with the following primary antibodies: α-actin (mouse monoclonal, 1A4, 1:100, sc-32251, Santa Cruz Biotechnology, Dallas, TX, USA), MMP-2 (mouse monoclonal, 8B4, 1:50, sc-13595, Santa Cruz Biotechnology), MMP-9 (mouse monoclonal, 2C3, 1:100, sc-21733, Santa Cruz Biotechnology), FAP (fibroblast activation protein) (mouse monoclonal, F11–24, 1:100, sc-65398, Santa Cruz Biotechnology), Phospho-Stat3 (rabbit monoclonal, 1:100, D3A7,

#9145, Cell Signaling, Danvers, MA, USA), Cox2 (rabbit monoclonal, 1:100, D5H5, #12282, Cell Signaling), F4/80 (rabbit monoclonal, 1:100, D2S9R, #70076, Cell Signaling), TNF-α (rabbit monoclonal, 1:100, D2D4, #11948, Cell Signaling), TGF-β1 (rabbit polyclonal, 3c11, 1:100, sc-146, Santa Cruz Biotechnology), active/cleaved caspase-3 (rabbit polyclonal, 1:100, NB100–56113, Novus Biological, Littleton, CO, USA), and Phospho-Histone H3 (P-H-H3, rabbit polyclonal, H3, 1:75,Ser10, 9701, Cell Signaling). Washes in PBS or TBS were performed between steps. The slides were then incubated with a post-primary antibody and Polymer kit (Novolink ™ polymer detection system 1, Leica Biosystems Newcastle Ltd., Newcastle, United Kingdom) according to the manufacturer's descriptions. Detection of positive staining was performed with 3–30 diaminobenzidine tetrahydrochloride (DAB) (Novolink TM DAB, RE7270-CE, Leica Biosystems, Buffalo Grove, USA) and counterstaining with hematoxylin. Compatibility of the primary antibody in mammary tissue of Mongolian gerbils was confirmed in previous studies (Leonel et al., 2020, 2021; Ruiz et al., 2021a).

2.7. Statistical analysis

Results obtained 7- and 14-days post-weaning were compared in each group by the paired sample T test. The difference between the two correlated samples was evaluated among groups.

To compare the results of each post-weaning period between the groups, the data were checked for normality using the Kolmogorov-Smirnov test. Parametric data (collagen area, PHH3, caspase-3, TGF-β, COX-2, TNF-α, p-STAT3, F4/80, mast cells) were analyzed by one-way ANOVA followed by Tukey's test; non-parametric data (stereological data, elastin area, MMP-2, MMP-9, FAP) were analyzed by the Kruskal-Wallis test followed by Dunn's test. Differences were considered statistically significant when $p < 0.05$. Statistical analyses were performed using GraphPad Prism 5.00 for Windows (GraphPad Software, San Diego, CA, USA, www.graphpad.com).

3. Results

3.1. The morphological features of mammary gland involution were impacted by BPA exposure

The area occupied by epithelial structures in the mammary gland showed a decrease from the 7th to the 14th day in control and E2 groups (Fig. 1a-c). However, mothers treated with BPA presented a lower epithelial occupation area on the 7th day, with no significant reduction from the 7th to 14th day (Fig. 1a).

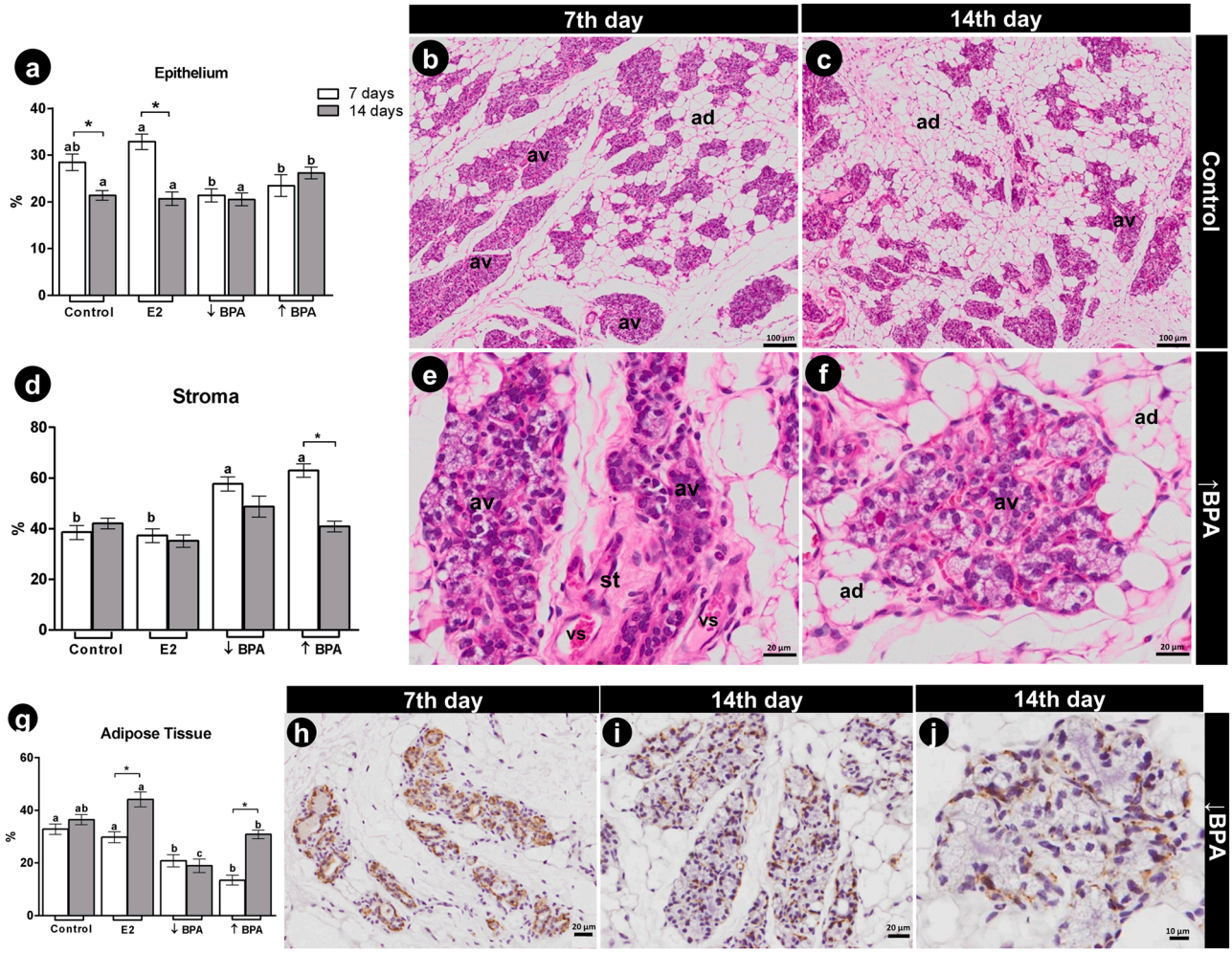

Fig. 1. Morphological features of mammary gland during involution. (a) Percentage of epithelial occupation (mean ± SEM) on 7th and 14th days. Mammary involution from 7th (b) to 14th days (c) in the control group. (d) Stromal area from 7th to14th days of involution. ↑BPA exposure reduced stroma surrounding epithelial structures from 7th (e) to 14th days (f). (g) Adipose tissue area percentage during involution. The appearance of myoepithelial layer thickness, as shown by α-actin staining, changes from the 7th (h) to the 14th (i) post-weaning days, showing a discontinuous pattern on the 14th day (j). Different letters in a, d, and g indicate statistical differences ($p < 0.05$) among groups and * indicate differences between involution periods in the same group (↓BPA and ↑BPA: n = 5 per subgroups; control: n = 4 per subgroups; E2: n = 3 per subgroups). Abbreviations: av: alveoli; ad: adipose tissue; st: stroma; vs: blood vessel. Scale Bars: (b-c) 100 μm; (e-f; h-k) 20 μm; (l) 10 μm.

The non-epithelial compartment was evaluated regarding the occupation rate of adipose tissue and of stroma. Differences in the stromal areas (Fig. 1d) between 7- and 14-days post-lactation were only present in the ↑BPA group (Fig. 1e and f). Both groups treated with BPA showed larger areas of stroma in comparison to all other groups at 7 days post-weaning (Fig. 1 f); no differences between any groups were found for stroma area after 14 days.

The areas of adipose tissue (Fig. 1 g) were larger after 14 days in comparison to 7 days only in the E2 and the ↑BPA treated groups. Both doses of BPA presented a smaller adipose tissue area at 7-days post-weaning in comparison to the control and E2 groups. Control and ↑BPA did not differ statistically at 14 days of involution, whereas ↓BPA was lower in the same period when compared to all groups.

3.2. The thickness and shape of myoepithelial cell layer changed between 7- and 14-days post-weaning

During mammary gland involution, the epithelium acquired a typical morphological aspect that resembles a damaged structure (Fig. 1 h). This is evidenced by α-actin staining for detection of myoepithelial cells. After 14 days, however, alveoli already showed a better-organized structure (Fig. 1i and j). There was a clear reduction in the myoepithelial layer thickness from the 7th to the 14th post-weaning day (Fig. 1 h and j) and the myoepithelial layer showed a discontinuous pattern on the 14th day (Fig. 1j), confirming that at this stage, there was still a clear disruption in the epithelial delimitation that involves the basal layer.

3.3. The incidence of extracellular matrix fibers changed during mammary gland involution

The ECM remodeling was impacted by exposure to BPA and E2. On the 7th day of involution, collagen area percentage (Fig. 2a) was higher in BPA groups compared to control (Fig. 2b and c); at this period, collagen deposition in the E2 group was also higher (Fig. 2d and e) than that of the control. On the 14th day of involution, collagen area in mammary gland exposed to BPA did not differ from the control group (Fig. 2f and g); whereas in the E2 group, it was lower (Fig. 2h and i) compared with all other groups. Elastin area in the ↓BPA group (Fig. 2j) was enhanced in relation to the control group, but there were no differences between ↑BPA and E2 groups and control on the 7th day. On the 14th day of involution, elastin area did not differ among groups. From the 7th to the14th day elastin (Fig. 2k) and collagen presented increased density in the control group, which was not observed in BPA groups. E2 and BPA groups did not present significant differences from 7th to the14th days of involution for elastin fiber area (Fig. 2l-n).

3.4. The expression of MMPs and FAP during mammary gland involution is impacted by BPA exposure

The quantification of ECM remodeling protein expression showed that BPA exposure modulates MMPs and FAP expression in mammary involution. MMP-2 expression (Fig. 3a-d), on the 7th day presented no differences among groups; on the 14th day of involution, MMP-2 expression was slightly increased in the ↑BPA (Fig. 3d) and E2 groups, compared with that in control. On the 7th day MMP-9 expression (Fig. 3e) was low in all treated groups compared to control. Furthermore, the lowest expression of MMP-9 ocurred in ↓BPA and E2 groups on the 7th day. The expression did not change between groups on the 14th day (Fig. 3f and g). From the 7th to 14th days, the MMPs expression decreased only in the control group. while in BPA groups, cells expressing MMPs were spread in stroma surrounding epithelial structures (Fig. 3d and g) and associated to blood vessels.

FAP positive cells (Fig. 3h) were present only in stroma during the involution period. E2 presented drastically increased FAP expression compared to all groups, in both periods of involution (Fig. 3i and j). FAP expression in BPA groups on the 7th and 14th days was lower than E2 but higher than the control group, which presented the lowest rates of FAP expression.

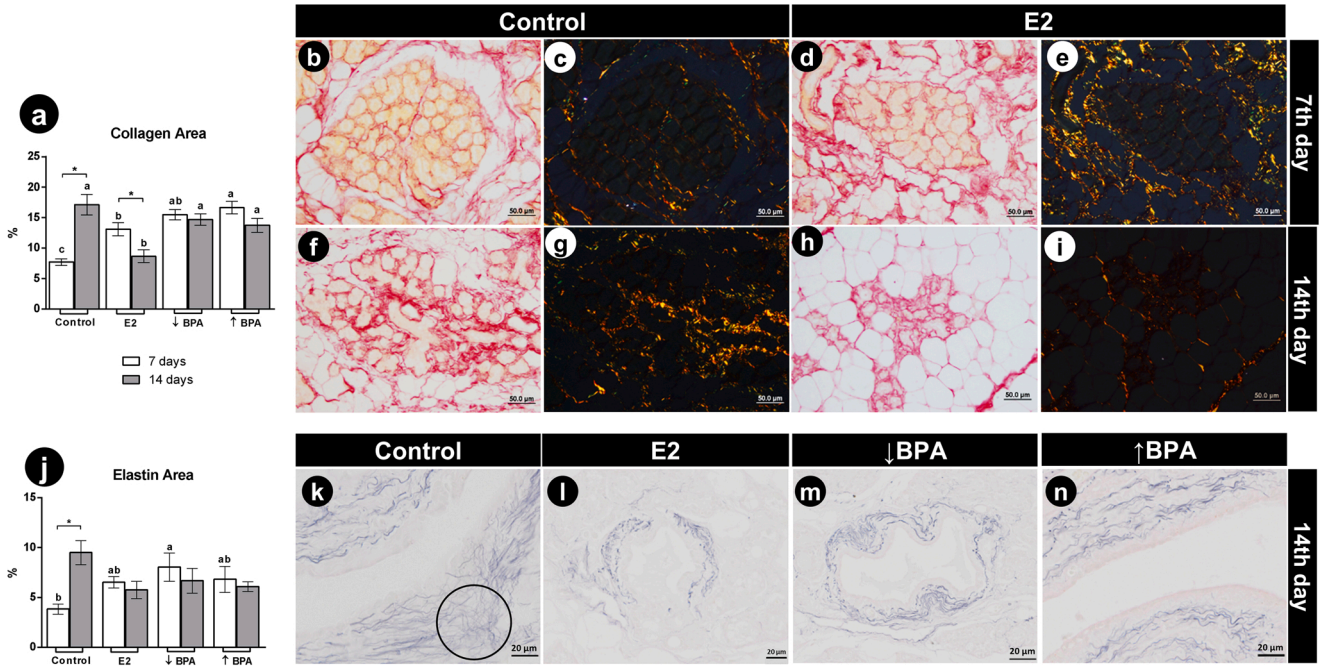

Fig. 2. Extracellular matrix fibers during mammary gland involution. (a) Percentage of collagen area deposition on 7th and 14th days of involution (mean ± SEM). (b-i) Collagen fibers (red). Picrosirius staining demonstrated the collagen deposition at 7 days in control (b, c) and E2 (d, e) groups compared to respective 14 day post-weaning deposition (f, g – control; h, i – E2). (j) Percentage of elastin area on 7th and 14th days of involution (mean ± SEM). (k-n) Resorcin-fucsin staining on 14th day of involution for control (k), E2 (l), ↓BPA (m), and ↑BPA (n) groups. Note the elastin fibers meshwork (circle) in the stroma of the control group (k). Different letters in a and j indicate statistical differences ($p < 0.05$) among groups and * indicate statistical differences between involution periods in the same group (↓BPA and ↑BPA: n = 5 per subgroups; control: n = 4 per subgroups; E2: n = 3 per subgroups). Scale Bars: (b-e; f-i) 50 µm; (k-n) 20 µm.

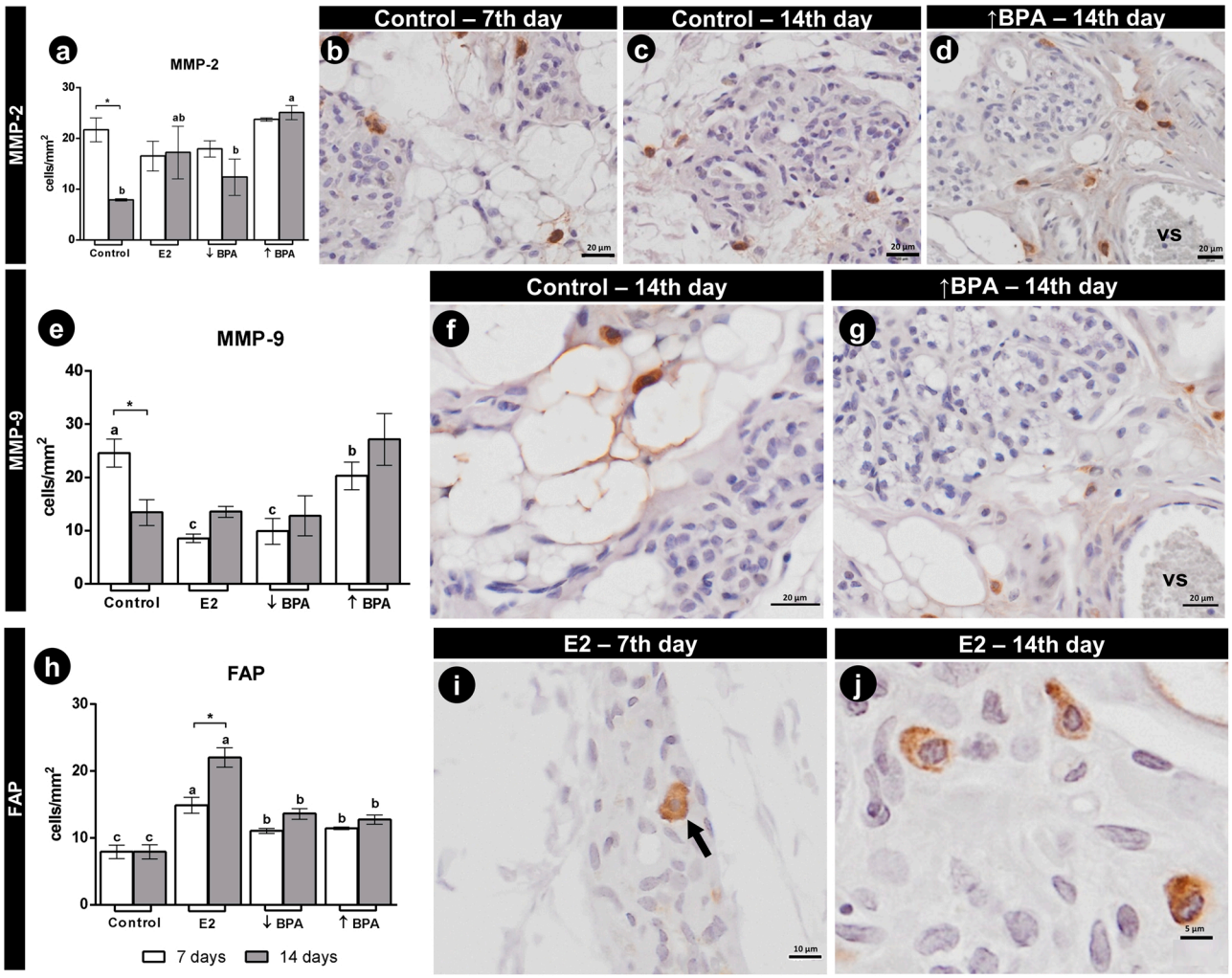

Fig. 3. Extracellular matrix remodeling protein expression during mammary gland involution. MMP-2 (a), MMP-9 (e), and FAP (h) expression (mean of cells/mm2 ± SEM). (b-d) MMP-2 positive cells were found in stoma on 7th (b) and 14th (c) days in control and also in ↑BPA group (d). (f-g) MMP-9 positive cells in adipose tissue in control (f) and perivascular in ↑BPA (g). (i-j) FAP expression: E2 group presented increased expression from 7th (i) to 14th (j) days of involution. Note in (i) the FAP positivity for a mast cell (arrow). Different letters in a, e, and h indicate statistical differences ($p < 0.05$) among groups and * indicate differences between involution periods in the same group (↓BPA and ↑BPA: n = 5 per subgroups; control: n = 4 per subgroups; E2: n = 3 per subgroups). Abbreviation: vs: blood vessel. Scale Bars: (d-i) 20 µm; (j, l) 10 µm; (k) 5 µm.

3.5. The expression of proliferative marker Phospho-Histone H3 was modulated by E2 and BPA exposure

BPA and E2 groups presented high rates of positive cells for PHH3 on the 7th day of involution (Fig. 4a). This scenario changed on the 14th day, when PHH3 expression did not change in the mammary gland of mothers among all groups. Rare PHH3-positive cells were observed in the control group (Fig. 4b). In BPA groups, this proliferative marker was expressed in epithelial (Fig. 4c) and stromal cells (Fig. 4d). The ↓BPA, ↑BPA, and E2 groups showed a decrease in the proliferative activity between the 7th and 14th days of mammary regression, unlike what was observed in the control group, which presented an opposite pattern of PHH3 expression.

3.6. BPA increased TGF-β1 and active Caspase-3 expression in mammary tissue during involution

At 7 days of involution, the number of TGF-β1 positive cells (Fig. 4e) increased in mammary gland of mothers exposed to BPA in comparison to control and E2 groups. Interestingly, this contrast was not maintained on the 14th day of involution, when there were no differences among groups. Only BPA groups and control (Fig. 4f-h) showed differences when comparing 7th to 14th days.

For active caspase-3 (Fig. 4i), BPA groups demonstrated higher rates than control and E2 on the 7th day post-weaning, in which ↑BPA was statistically higher than ↓BPA. Control and E2 groups presented only cytoplasmic staining for caspase-3 (Fig. 4j), whereas BPA groups presented both cytoplasmic and nuclear staining at 7 days (Fig. 4k), and only cytoplasmic staining at 14 days (Fig. 4l). Although a different pattern of intracellular staining was observed, the quantification of positive cells/mm^2 was based on either nuclear or cytoplasmic reaction. From the 7th to the 14th day the number of active caspase-3 positive cells did not change in the control, whereas its expression was reduced in BPA groups and increased in E2 groups.

3.7. BPA and E2 impacted the expression of inflammatory markers during mammary gland involution

Mothers exposed to BPA and E2 presented different expression patterns of inflammatory markers TNFα, COX-2, and p-STAT3. For TNFα (Fig. 5a), control (Fig. 5b) and BPA groups (Fig. 5d) presented lower rates in comparison to the E2 group (Fig. 5c) on the 7th day post-weaning, which showed a high density of positive cells in stroma. This difference was not observed on the 14th day of involution, when none of

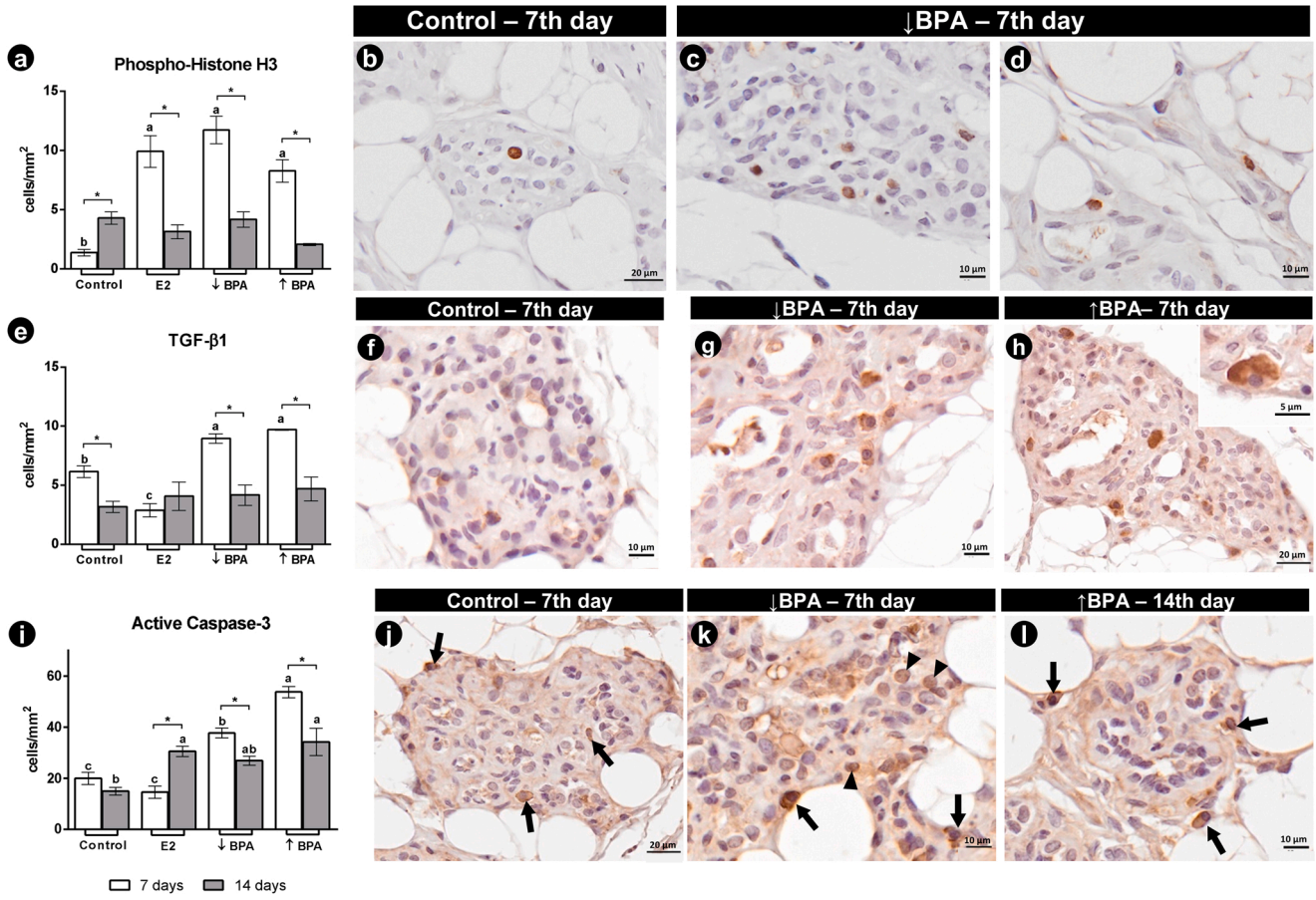

Fig. 4. Proliferative and apoptotic protein expression during mammary gland involution. (a) P-H-H3 positive cells on 7th and 14th days post-weaning (cells/mm2) (mean ± SEM). Control group presented scarce positive cells on 7th day (b); BPA presented epithelial (c) and stromal (d) staining. (e) TGF-β1 positive cells (cells/mm2) (mean ± SEM). Control (f) presented low positivity compared to ↓BPA (g) and ↑BPA (h) on the 7th day. Note that mast cells were stained for TGF-β1 (h-inset). (i) Active caspase-3 positive cells (cells/mm2) (mean ± SEM). At 7-days, the control group presented cytoplasmic staining (arrows) (j), whereas BPA presented cytoplasmic (arrows) and nuclear (arrowheads) staining (k). On the 14th day of involution, only cytoplasmic staining was observed in BPA groups (l). Different letters in a, e, and i indicate statistical differences ($p < 0.05$) among groups and * indicate differences between involution periods in the same group (↓BPA and ↑BPA: n = 5 per subgroups; control: n = 4 per subgroups; E2: n = 3 per subgroups). Scale Bars: (b, h, j) 20 μm; (c, d, f, g, h-inset, k, l) 10 μm.

the groups differed. Furthermore, TNFα positive cells increased in BPA groups from the 7th to 14th days.

COX-2 positive cells (Fig. 5e) presented a higher incidence on the 7th day in the control group (Fig. 5f) which decreased on the 14th day. The expression of COX-2 in mammary gland of mothers exposed to BPA was lower on the 7th day and increased on the 14th day of involution (Fig. 5g-h, respectively). Furthermore, COX-2 did not differ statistically in the E2 group when comparing the 7th and 14th days.

The number of p-STAT3 positive cells (Fig. 5i) remained constant in the control group (Fig. 5j) but was higher on the 7th than 14th day in the E2 group (Fig. 5k). BPA groups showed an increase in the incidence of positive cells between 7 and 14 days of involution (Fig. 5l – ↓BPA; Fig. 5m - ↑BPA). P-STAT3 expression was present as a cytoplasmic and nuclear staining (Fig. 5j-m), which were both considered positive during counts. Lower rates were observed in ↓BPA on the 7th day, when compared to ↑BPA, E2, and control.

3.8. BPA exposure modulated the recruitment of macrophages and mast cells during involution

BPA increased the macrophage population, as shown by F4/80 staining (Fig. 6a), from the 7th to 14th days of involution. Macrophages were scarce in mammary gland tissue on the 7th day in the control (Fig. 6b) and BPA groups and abundant in E2 (Fig. 6c). On the 14th day of involution, ↓BPA presented a high increase in the active macrophage population (Fig. 6d). From the 7th to the 14th days of mammary regression, the macrophage population drastically decreased in E2.

BPA promoted a marked enhancement in mast cell numbers from 7 to 14 days (Fig. 6e). Mast cells in BPA groups were found in epithelial, adipose tissue, and stromal compartments (Fig. 6f, g, and h, respectively). In BPA groups, mast cells were present at intact and degranulated stages. The control group did not present differences between 7 and 14 days. Mast cell recruitment presented opposite modulation in E2 in comparison to BPA, being higher on the 7th than on the 14th day.

4. Discussion

The present experiment revealed that xenoestrogen exposure during the gestational/lactational periods impacts not only neonates', but also the mothers' mammary gland homeostasis in the short-term. Histochemical analysis showed that the normal regression process of the mammary gland is impacted by exposure to BPA and E2, in terms of epithelial and stromal occupation (cells and ECM). Allied to this, the immunohistochemical approach showed that ECM remodeling mechanisms are also impacted by these disruptors in terms of MMPs and FAP expression. Furthermore, indices of cell proliferation and death, as well as inflammatory marker expression, are impacted by this exposure. A schematic representation of the results and discussion is provided in Fig. 7.

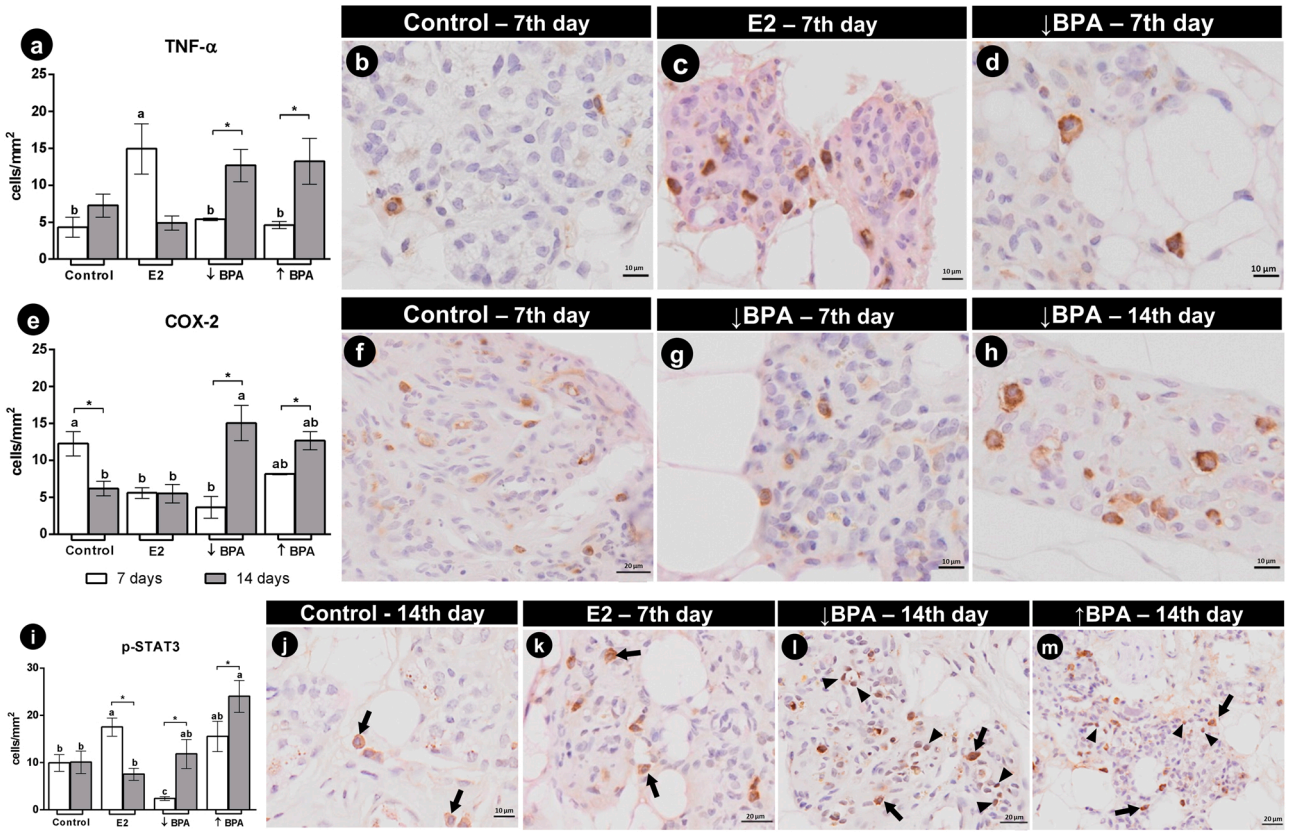

Fig. 5. Inflammatory markers during mammary gland involution. (a) TNFα positive cells (mean ± SEM). On the 7th day, the control group presented low positive cells (b), whereas the E2 group presented high rates of TNFα expression (c). ↓BPA group showed sparse positive cells (d). (e) COX positive cells (mean ± SEM). Control group presented high rates on the 7th day (f). ↓BPA group showed the lowest rates on the 7th day (g), and a drastic increase on the 14th day (h). (i) p-STAT3 expression during involution (mean ± SEM). Arrows indicate stromal cells with cytoplasmic staining in control on the 14th day (j) and E2 on the 7th day (k). Besides stromal cells, ↓BPA (l) and ↑BPA (m) presented epithelial cells with nuclear staining (arrowheads). Different letters in a, e, and i indicate statistical differences ($p < 0.05$) among groups and * indicate differences between involution periods in the same group (↓BPA and ↑BPA: n = 5 per subgroups; control: n = 4 per subgroups; E2: n = 3 per subgroups). Scale Bars: (b, c, g, h, l, m) 10 μm; (d, f, j, k, n, o, p) 20 μm.

4.1. BPA accelerates the mammary gland involution

We have previously described the physiological regression process of Mongolian gerbil mammary gland (Leonel et al., 2017). In that manuscript, the 3rd and 5th post-weaning days were morphologically characterized, and the events of the regression period occurred in a fast manner in comparison to gestational development. One limitation of the present study was that we only analyzed a later period of mammary involution, not describing the possible negative impacts in this first phase (3–5 days post-weaning). These differences could be relevant when associated with exposure to different exogenous estrogenic agents, such as BPA and E2. This relevance is noteworthy in the experimental model applied, which presents neoplasia in hormone-responsive glands and age-related estrogenic sensibilization (Custodio et al., 2008). Based on these results, for better evaluation of the impacts caused by endocrine disruption during gestation and lactation, here we focused on longer involution periods.

The control group showed a reduction in the epithelial compartment between 7 and 14 days, but this was not observed in the BPA groups. This was due to an increase in the apoptotic rate in the epithelial compartment, resulting from high expression of apoptotic proteins such as p-STAT3 and active caspase-3. These proteins are responsible for the initiation of breast involution after milk stasis (Jena et al., 2019), which occurs through the recruitment of phagocytes for tissue remodeling (Hughes et al., 2012) and apoptosis in the epithelial compartment (Chapman et al., 1999; Sargeant et al., 2014).

The second phase of the involution is influenced by the events from the first phase (Hughes and Watson, 2012). At this stage, a physiological ECM remodeling occurs, interfering with the epithelium dynamics and leading to a second apoptotic wave of the compartment (Watson, 2006). E2 exposure led to an epithelial apoptosis delay related to an estrogenic effect (Wärri et al., 2018). However, in the BPA groups, a 2-fold increase was observed in cell death rates in comparison to control. The BPA dose-dependent caspase-3 activity was induced by TGF-β1, which was previously described to establish an apoptotic process (Bailey et al., 2004). This mechanism is often associated with oncogenic activation in tumoral cells (Zhang et al., 2006) supported by an inflammatory microenvironment (Fouad et al., 2014). Our results show that when epithelial cells are subjected to disruption by BPA, they deregulate the cycle by an increase in cell proliferation and in expression of the apoptotic cascade. Thus, it is probable that BPA endocrine deregulation occurs from different cell responses to tissue physiological processes, increasing the sensitivity of mammary gland to hormones and growth factors (Ayyanan et al., 2011; Pupo et al., 2012).

Our results suggest that BPA reprograming of the epithelial cell cycle (Dairkee et al., 2013) extended cell survival time and enhanced lesions, being a hallmark for mammary cancer (Tarullo et al., 2020). In addition, the myoepithelial layer rupture, observed in BPA exposed glands, is an opportunity for invasive ductal carcinoma progression in the mammary gland (Allred et al., 2008). Nevertheless, despite a non-monotonic BPA impact, as previously described (Montévil et al., 2019), p-STAT3 was the main protein that presented different levels of expression in the tissue under low doses of BPA.

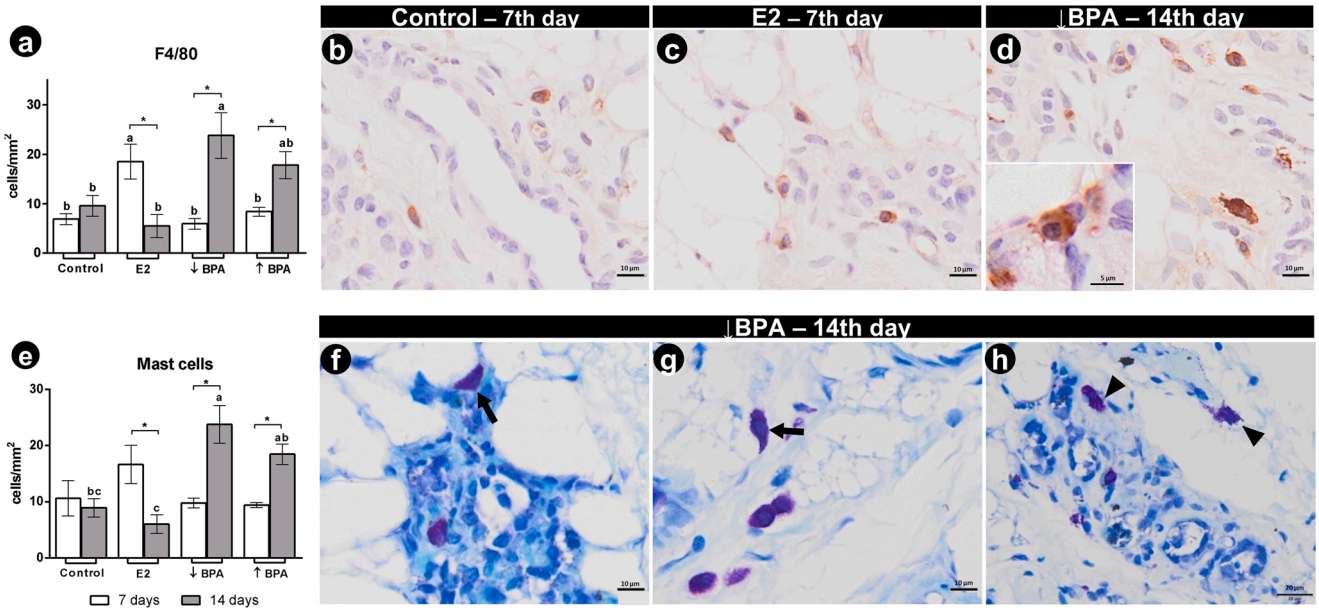

Fig. 6. Macrophage and mast cell incidence during mammary gland involution. (a) Macrophage density (cells/mm2) (mean ± SEM). Macrophages were found in the stromal compartment on the 7th day of involution in control (b) and E2 (c) groups. Macrophage high density was observed in BPA (d) with phagocytic activity in the epithelial compartment (inset). (e) Mast Cell density (cells/mm2) (mean ± SEM). (f-h) Mast cells population: epithelial compartment (f), adipose tissue (g), and in stroma (h). Intact (arrows) and degranulated (arrowheads) mast cells were observed in all compartments. Different letters in a and e indicate statistical differences ($p < 0.05$) among groups and * indicate differences between involution periods in the same group (↓BPA and ↑BPA: n = 5 per subgroups; control: n = 4 per subgroups; E2: n = 3 per subgroups). Scale Bars: (c-g) 10 μm; (h-n) 20 μm.

4.2. BPA alters the stromal compartment and extracellular remodeling proteins in mammary gland during involution

After elimination of epithelial apoptotic cells, stem cells proliferate to reconstruct the epithelium of involuted structures, i.e., terminal-end buds and primary ducts (Maller et al., 2010; Wang et al., 2010). Stroma aids in polarity organization of these cells (Boyd et al., 2007) and is regulated by the estrogenic pathway, which led to a softness condition. Contrarily, findings of BPA groups suggest a stiffness condition. This failure to decrease collagen deposition in mothers treated with BPA may impact the process of breast regression (Simian et al., 2009). Under E2 exposure, ECM softness is promoted by enhanced FAP expression. During involution, fibroblasts and other stromal cells are responsible for the synthesis of proteinases that rearrange the collagen network (Dzięgelewska and Gajewska, 2019). FAP is recognized as a marker for reactive stromal cells (RSC) and carcinoma-associated fibroblasts (CAFs) (Jacob et al., 2012). Despite the absence of differences in the incidence of stroma in the E2 compared to control group, the increase in FAP positive cells may indicate the presence/activation of CAF and RSC, which is favored by the recruitment of macrophages (Tchou et al., 2013). Furthermore, the expression of MMPs in E2 and ↑BPA was supported by higher expression of p-STAT3 (Yuan et al., 2017).

The establishment of a collagen-rich microenvironment in postpartum contribute to pro-tumoral development during breast involution (Provenzano et al., 2006). This invasive condition is promoted by fibrillar collagen, but not by a degraded collagen matrix (Lyons et al., 2011). In the E2 group, however, the presence of FAP determines a degradation activity. The fibrotic phenotype in mammary gland was supported by COX-2 upregulation (Lyons et al., 2011) in BPA groups, mainly on the 14th day of involution.

4.3. BPA aggravates inflammation during mammary gland involution

The expression of inflammatory markers in BPA groups presented an opposite pattern from that observed in control and E2 groups. STAT3 is a first phase marker of mammary gland involution, as previously described (Jena et al., 2019). STAT3 expression is modulated by BPA exposure (Huang et al., 2019) and promotes cell proliferation, typical of breast cancer (Nair et al., 2020). Mammary glands exposed to BPA presented high proliferative activity at 7 days, when p-STAT3 expression was low. However, at 14 days of involution, BPA acts by increasing STAT3 phosphorylation, and hence, decreasing cell proliferation. Of note, BPA exposure can trigger a switch in p-STAT3 and STAT3 expression (Canesi et al., 2005) that could increase the risk of primary breast cancer (Shafei et al., 2018).

E2 is more efficient in binding to estrogen receptors than BPA (Sheehan, 2000). This possibly explains the earlier impacts of E2 exposure (7th day of involution) in the expression of inflammatory markers, being opposite to BPA. The early inflammatory effect promoted by E2 is modulated by TNFα (Xu et al., 2017) and comprises macrophage and mast cell recruitment (Need et al., 2014). However, the significant increase in the incidence of these cells between 7 and 14 days in BPA groups indicates substantial modulation of the inflammatory process (Hennigar et al., 2015), not related to involution. TNFα is a pro-inflammatory cytokine that participates in innate immune response (Perkins, 2007) and is expressed by macrophages when exposed to BPA (Liu et al., 2014).

COX-2 increase in the mammary gland is related to induced-inflammation due to cytokines and hormonal stimuli (Wallace et al., 2019). If its expression is upregulated, it inhibits epithelial cell proliferation and apoptosis (Lu et al., 2005). This inhibition was observed in mammary glands exposed to BPA. In fact, COX-2 is related to chronic inflammation and increases due to high rates of TNFα (Hugo et al., 2015). Post-weaning mammary involution itself is a natural inflammatory microenvironment (Lyons et al., 2011). Thus, enhancement of inflammatory markers represents a risk for post-gestational neoplasia and tumor development (Wallace et al., 2019). Considering this suggestive pro-tumoral microenvironment, BPA modulates the expression and, consequently, the activity of COX-2 in immune-cell recruitment and ECM fibers (Wallace et al., 2019), which may contribute to tumor progression.

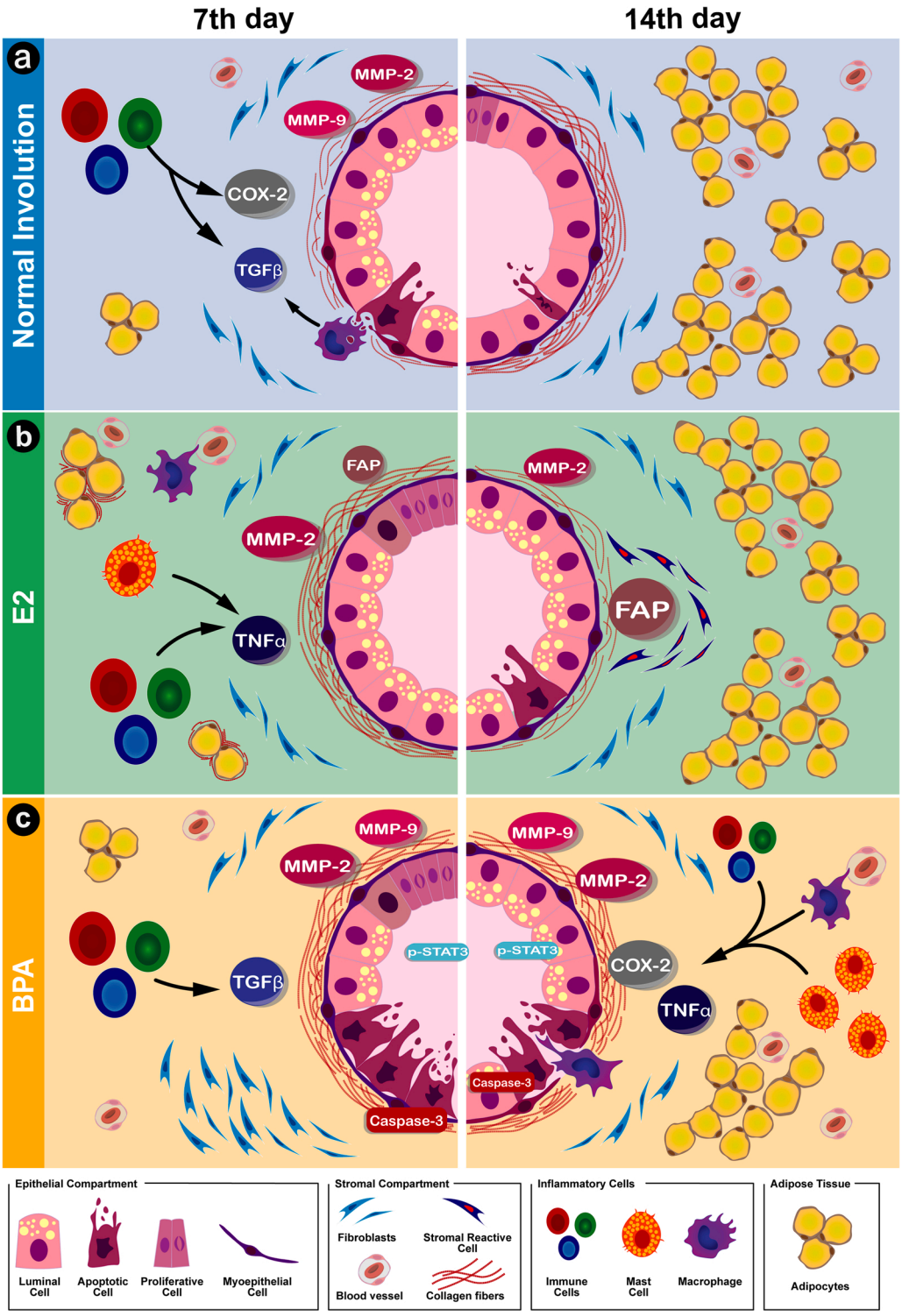

Fig. 7. Morphological features of mammary gland during involution under normal conditions, estradiol, and BPA disruption on 7th and 14th days post-weaning. (a) In a physiological situation, on 7th post-weaning day epithelial cells are apoptotic and stromal cells, such as macrophages and immune cells, express cytokines and COX-2. On the 14th day, the fat pad is reestablished and proliferative activity (PHH3-positive cells) takes place in the epithelial compartment; collagen fibers increase as well. (b) On the 7th day E2 exposure increases proliferation of epithelial cells and the stromal compartment is remodeled by MMP-2, which enhances the expression of TNFα by inflammatory cells, such as mast cells. On the 14th day, E2 induces the presence of apoptotic cells in epithelium and FAP expression in stromal reactive cells. (c) BPA modulates the epithelial compartment through an imbalanced proliferative and apoptotic process on the 7th day, and the stromal compartment is remodeled by MMP-2 and MMP-9. Immune cells express TGFβ in stroma. On the 14th day, TGFβ recruits mast cells, which express COX-2 and TNFα as well as immune cells and macrophages. P-STAT3 is upregulated in both stages in BPA exposed mammary gland.

4.4. BPA recruits mast cells and macrophages during mammary gland involution

Tissue macrophages are recruited in involution to phagocyte the epithelial cells in the apoptotic process, but decrease in number during late involution (Dawson et al., 2020). These cells express MMP-9 (Zollo et al., 2014) in response to the estrogenic pathway, and TNFα, which acts as a signal for new recruitment and activation of pro-inflammatory cells (Fleming et al., 2012). E2 stimulated the recruitment of macrophages on the 7th day. Contrarily, BPA showed a late effect, related to the increase in TNFα from 7th to the 14th day. Another factor that could contribute to macrophage infiltration in BPA groups is the high expression of p-STAT3 (Stein et al., 2003). STAT3 expression changed in ↓BPA and ↑BPA groups from the 7th to 14th day and could stimulate this infiltration by the cited pathway. These inflammatory features and expression of MMPs contribute to create and amplify a suggestive tumorigenic microenvironment by enhancing the macrophage population in BPA groups (Fleming et al., 2012; Xu, 2017).

TGF-β1 is a chemotactic protein for monocytes (Zollo et al., 2014) and mast cells (Ramírez-Valadez et al., 2017). The increased expression

in BPA groups on the 7th day of mammary gland involution was followed by a drastic enhancement in these cell numbers on the 14th day. Elgert and colleagues (1998) associated the loss of antitumoral macrophage activity with TGF-β1 enhancement in tissue, similarly to the mammary gland microenvironment described here on the 7th day in BPA groups. These macrophages could contribute to cancer progression and are found early in breast tumors (Dawson et al., 2020). Tumor-associated macrophages (TAMs) are a myeloid cell type recruited by the tumor site to produce an inflammatory microenvironment for cancer progression and metastasis (Mao et al., 2018; Wani et al., 2014). In the present study, BPA groups showed a correlation of chemoattractant protein TGF-β1 on the 7th day, which allowed the infiltration of TAMs. p-STAT3 in turn supported their recruitment and activation (Clarkson et al., 2006), as observed by the enhancement in COX-2 expression (Gan et al., 2016).

Mast cells trigger the interaction between epithelium and stromal compartments (Durando et al., 2006) and stimulate proliferation in ductal epithelium and the development of carcinoma in situ. Both events were observed in BPA groups during involution (7th to 14th day). As discussed above, the proliferative-apoptotic balance of involution is impaired by BPA exposure and has been deployed in a suggestive preneoplastic and inflammatory microenvironment. Furthermore, the increase in TNFα expression in the E2 group explains the reduction in mast cell numbers, since this is a cytokine that regulates the local degranulation of mast cells in involution (Ramirez et al., 2012).

Exposure to E2 induces a pro-invasive microenvironment, while BPA promotes a fibrotic and inflammatory phenotype. Even though BPA and E2 act in similar pathways, our study showed that inflammatory responses – i.e., recruitment of mast cells and macrophages, proliferation/apoptosis – were opposite between 7 and 14 days of involution. Thus, compared to E2, BPA acts in several other pathways to alter the normal and synthetic-sensitized tissue. This evidence brings to light a perspective for future analysis: estrogenic receptor expression could unveil much of what is still to be clarified, since their affinity varies according to the estrogenic compound. Exposure during the pregnancy and lactation windows of susceptibility induce a progression of features related to the increase in breast cancer incidence (Shafei et al., 2018). BPA exposure contributes to different types of breast cancer (Engin and Engin, 2021) and neoplasia (Sprague et al., 2013) development. The exposure period of the present study should be taken into consideration as a trigger point of pro-tumoral establishment.

5. Conclusion

Gestational and lactational exposure to BPA appears to be crucial for progression of a suggestive pro-tumoral feature and microenvironment of mammary gland, which potentially aggravate the risks of cancer development. The epithelium and stroma of mammary gland during regression (Fig. 7a) negatively respond to E2 (Fig. 7b) and BPA (Fig. 7c) exposure. This pro-tumoral microenvironment is depicted by variations in cell death and proliferation indices and in expression of inflammatory markers, which promote a favorable cancer microenvironment.

Compliance and Ethical Standard

Ethical approval

Experiments adhered to guidelines established by the National Council of Animal Experiment Control (CONCEA, Brazil, www.mctic.gov.br) and were approved by the committee for animal care of the Ethics Committee on the Use of Animals (CEUA) (IBILCE/UNESP protocol number 113/2015).

Funding Sources

This work was supported by the Fundaç ão de Amparo à Pesquisa do Estado de São Paulo – FAPESP (Master fellowship to T.F.R. Ruiz, 2020/01240–9; and research financing, 2018/23383–6); Coordenaç ão de Aperfeiçoamento de Pessoal de N í vel Superior – CAPES (finance code 001); and the National Council for Scientific and Technological Development (CNPq) (Grant Number: 302938/2020/6).

CRediT authorship contribution statement

T.F.R. Ruiz and E.C.R. Leonel contributed equally to this manuscript in the following actions: study conceptualization and methodology, formal analysis, investigation, resources, data curation and writing of both, original draft, and review/editing. S.V. Colleta: investigation, resources, data curation. C.M. Bedolo: formal analysis, investigation, resources, data curation. S.G.P. Campos: writing - original draft, data curation and investigation. S.R. Taboga: conceptualization and methodology, writing - original draft, supervision, project administration and funding acquisition.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Leonel E.C.R reports financial support was provided by Coordination of Higher Education Personnel Improvement. Ruiz T.F.R. reports financial support was provided by State of Sao Paulo Research Foundation. Taboga S.R. reports financial support was provided by State of Sao Paulo Research Foundation. Taboga S.R. reports financial support was provided by National Council for Scientific and Technological Development.

Acknowledgements

The authors would like to thank MSc. Luiz Roberto Falleiros for technical assistance during the development of the experiments.

Funding Information

Fundaç ão de Amparo à Pesquisa do Estado de São Paulo – FAPESP (Master fellowship to T.F.R. Ruiz, 2020/01240–9; and research financing, 2018/23383–6) and Coordenaç ão de Aperfeiçoamento de Pessoal de N í vel Superior – CAPES (finance code 001).

Conflict of Interest

The authors declare that there is no conflict of interest.

References

Acconcia, F., Pallottini, V., Marino, M., 2015. Molecular mechanisms of action of BPA. Dose-Response 13, 1–9. https://doi.org/10.1177/1559325815610582.

Allred, D.C., Wu, Y., Mao, S., Nagtegaal, I.D., Lee, S., Perou, C.M., Mohsin, S.K., O'Connell, P., Tsimelzon, A., Medina, D., 2008. Ductal carcinoma in situ and the emergence of diversity during breast cancer evolution. Clin. Cancer Res. 14, 370–378. https://doi.org/10.1158/1078-0432.CCR-07-1127.

Altamirano, G.A., Ramos, J.G., Gomez, A.L., Luque, E.H., Muñoz-de-Toro, M., Kass, L., 2017. Perinatal exposure to bisphenol A modifies the transcriptional regulation of the β-casein gene during secretory activation of the rat mammary gland. Mol. Cell. Endocrinol. 439, 407–418. https://doi.org/10.1016/j.mce.2016.09.032.

Ayyanan, A., Laribi, O., Schuepbach-Mallepell, S., Schrick, C., Gutierrez, M., Tanos, T., Lefebvre, G., Rougemont, J., Yalcin-Ozuysal, Ö., Brisken, C., 2011. Perinatal exposure to bisphenol A increases adult mammary gland progesterone response and cell number. Mol. Endocrinol 25, 1915–1923. https://doi.org/10.1210/me.2011-1129.

Bailey, J.P., Nieport, K.M., Herbst, M.P., Srivastava, S., Serra, R.A., Horseman, N.D., 2004. Prolactin and transforming growth factor-β signaling exert opposing effects on mammary gland morphogenesis, involution, and the Akt-forkhead pathway. Mol. Endocrinol. 18, 1171–1184. https://doi.org/10.1210/me.2003-0345.

Boyd, N.F., Guo, H., Martin, L.J., Sun, L., Stone, J., Fishell, E., Jong, R.A., Hislop, G., Chiarelli, A., Minkin, S., Yaffe, M.J., 2007. Mammographic density and the risk and detection of breast cancer. N. Engl. J. Med. 356, 227–236. https://doi.org/10.1056/NEJMoa062790.

Canesi, L., Betti, M., Lorusso, L.C., Ciacci, C., Gallo, G., 2005. “In vivo” effects of bisphenol A in Mytilus hemocytes: Modulation of kinase-mediated signalling pathways. Aquat. Toxicol. 71, 73–84. https://doi.org/10.1016/j.aquatox.2004.10.011.

Chapman, R.S., Lourenco, P.C., Tonner, E., Flint, D.J., Selbert, S., Takeda, K., Akira, S., Clarke, A.R., Watson, C.J., 1999. Suppression of epithelial apoptosis and delayed mammary gland involution in mice with a conditional knockout of Stat3. Genes Dev. 13, 2604–2616. https://doi.org/10.1101/gad.13.19.2604.

Clarkson, R.W.E., Boland, M.P., Kritikou, E.A., Lee, J.M., Freeman, T.C., Tiffen, P.G., Watson, C.J., 2006. The genes induced by signal transducer and activators of transcription (STAT)3 and STAT5 in mammary epithelial cells define the roles of these STATs in mammary development. Mol. Endocrinol. 20, 675–685. https://doi.org/10.1210/me.2005-0392.

Colborn, T., vom Saal, F.S., Soto, A.M., 1994. Developmental effects of endocrine-disrupting chemicals in wildlife and humans. Environ. Impact Assess. Rev. 14, 469–489. https://doi.org/10.1016/0195-9255(94)90014-0.

Cowin, P., Wysolmerski, J., 2010. Molecular mechanisms guiding embryonic mammary gland development. Cold Spring Harb. Perspect. Biol. 2 https://doi.org/10.1101/cshperspect.a003251.

Custodio, A.M.G., Santos, F.C.A., Campos, S.G.P., Vilamaior, P.S.L., Góes, R.M., Taboga, S.R., 2008. Aging effects on the mongolian gerbil female prostate (Skene’s paraurethral glands): Structural, ultrastructural, quantitative, and hormonal evaluations. Anat. Rec. 291, 463–474. https://doi.org/10.1002/ar.20637.

Dairkee, S.H., Luciani-Torres, M.G., Moore, D.H., Goodson, W.H., 2013. Bisphenol-A-induced inactivation of the p53 axis underlying deregulation of proliferation kinetics, and cell death in non-malignant human breast epithelial cells. Carcinogenesis 34, 703–712. https://doi.org/10.1093/carcin/bgs379.

Dawson, C.A., Pal, B., Vaillant, F., Gandolfo, L.C., Liu, Z., Bleriot, C., Ginhoux, F., Smyth, G.K., Lindeman, G.J., Mueller, S.N., Rios, A.C., Visvader, J.E., 2020. Tissue-resident ductal macrophages survey the mammary epithelium and facilitate tissue remodelling. Nat. Cell Biol. 22, 546–558. https://doi.org/10.1038/s41556-020-0505-0.

Doherty, L.F., Bromer, J.G., Zhou, Y., Aldad, T.S., Taylor, H.S., 2010. In utero exposure to diethylstilbestrol (DES) or bisphenol-A (BPA) increases EZH2 expression in the mammary gland: An epigenetic mechanism linking endocrine disruptors to breast cancer. Horm. Cancer 1, 146–155. https://doi.org/10.1007/s12672-010-0015-9.

Durando, M., Kass, L., Piva, J., Sonnenschein, C., Soto, A.M., Luque, E.H., Muñoz-De-Toro, M., 2006. Prenatal bisphenol a exposure induces preneoplastic lesions in the mammary gland in wistar rats. Environ. Health Perspect. 115, 80–86. https://doi.org/10.1289/ehp.9282.

Dzięgelewska, Ż., Gajewska, M., 2019. Stromal-epithelial interactions during mammary gland development. Stromal Cells - Struct. Funct. Ther. Implic. https://doi.org/10.5772/intechopen.80405.

EFSA, 2015. Scientific Opinion on the risks to public health related to the presence of bisphenol A (BPA) in foodstuffs. EFSA J. 13 https://doi.org/10.2903/j.efsa.2015.3978.

Elgert, K.D., Alleva, D.G., Mullins, D.W., 1998. Tumor-induced immune dysfunction: The macrophage connection. J. Leukoc. Biol. 64, 275–290. https://doi.org/10.1002/jlb.64.3.275.

Engin, A.B., Engin, A., 2021. The effect of environmental bisphenol A exposure on breast cancer associated with obesity. Environ. Toxicol. Pharmacol. 81, 103544 https://doi.org/10.1016/j.etap.2020.103544.

Faupel-Badger, J.M., Arcaro, K.F., Balkam, J.J., Eliassen, A.H., Hassiotou, F., Lebrilla, C. B., Michels, K.B., Palmer, J.R., Schedin, P., Stuebe, A.M., Watson, C.J., Sherman, M. E., 2012. Postpartum remodeling, lactation, and breast cancer risk: summary of a national cancer institute–Sponsored Workshop. JNCI J. Natl. Cancer Inst. 105, 166–174. https://doi.org/10.1093/jnci/djs505.

Fleming, J.M., Miller, T.C., Kidacki, M., Ginsburg, E., Stuelten, C.H., Stewart, D.A., Troester, M.A., Vonderhaar, B.K., 2012. Paracrine interactions between primary human macrophages and human fibroblasts enhance murine mammary gland humanization in vivo. Breast Cancer Res. 14, 1–18. https://doi.org/10.1186/bcr3215.

Fouad, T.M., Kogawa, T., Reuben, J.M., Ueno, N.T., 2014. The role of inflammation in inflammatory breast cancer. Int. J. Radiat. Biol. 53–73. https://doi.org/10.1007/978-3-0348-0837-8_3.

Gan, L., Qiu, Z., Huang, J., Li, Y., Huang, H., Xiang, T., Wan, J., Hui, T., Lin, Y., Li, H., Ren, G., 2016. Cyclooxygenase-2 in tumor-associated macrophages promotes metastatic potential of breast cancer cells through akt pathway. Int. J. Biol. Sci. 12, 1533–1543. https://doi.org/10.7150/ijbs.15943.

Gomez, A.L., Delconte, M.B., Altamirano, G.A., Vigezzi, L., Bosquiazzo, V.L., Barbisan, L. F., Ramos, J.G., Luque, E.H., Muñoz-de-Toro, M., Kass, L., 2017. Perinatal exposure to bisphenol a or diethylstilbestrol increases the susceptibility to develop mammary gland lesions after estrogen replacement therapy in middle-aged rats. Horm. Cancer 8, 78–89. https://doi.org/10.1007/s12672-016-0282-1.

Hennigar, S.R., Velasquez, V., Kelleher, S.L., 2015. Obesity-induced inflammation is associated with alterations in subcellular zinc pools and premature mammary gland involution in lactating mice. J. Nutr. 145, 1999–2005. https://doi.org/10.3945/jn.115.214122.

Huang, F.M., Chang, Y.C., Lee, S.S., Yang, M.L., Kuan, Y.H., 2019. Expression of pro-inflammatory cytokines and mediators induced by Bisphenol A via ERK-NFκB and JAK1/2-STAT3 pathways in macrophages. Environ. Toxicol. 34, 486–494. https://doi.org/10.1002/tox.22702.

Hughes, K., Watson, C.J., 2012. The role of Stat3 in mammary gland involution: cell death regulator and modulator of inflammation. Horm. Mol. Biol. Clin. Investig. 10, 211–215. https://doi.org/10.1515/hmbci-2012-0008.

Hughes, K., Wickenden, J.A., Allen, J.E., Watson, C.J., 2012. Conditional deletion of Stat3 in mammary epithelium impairs the acute phase response and modulates immune cell numbers during post-lactational regression. J. Pathol. 227, 106–117. https://doi.org/10.1002/path.3961.

Hugo, H.J., Saunders, C., Ramsay, R.G., Thompson, E.W., 2015. New insights on COX-2 in chronic inflammation driving breast cancer growth and metastasis. J. Mammary Gland Biol. Neoplasia 20, 109–119. https://doi.org/10.1007/s10911-015-9333-4.

Jacob, M., Chang, L., Pure, E., 2012. Fibroblast activation protein in remodeling tissues. Curr. Mol. Med. 12, 1220–1243. https://doi.org/10.2174/156652412803833607.

Jemal, A., Bray, F., Center, M.M., Ferlay, J., Ward, E., Forman, D., 2011. Global cancer statistics. CA. Cancer J. Clin. 61, 69–90. https://doi.org/10.3322/caac.20107.

Jena, M.K., Jaswal, S., Kumar, S., Mohanty, A.K., 2019. Molecular mechanism of mammary gland involution: An update. Dev. Biol. 445, 145–155. https://doi.org/10.1016/j.ydbio.2018.11.002.

Kandel, A., Dhillon, S.K., Prabaharan, C.B., Fatnin Binti Hisham, S., Rajamanickam, K., Napper, S., Chidambaram, S.B., Essa, M.M., Yang, J., Sakharkar, M.K., 2021. Identifying kinase targets of PPARγ in human breast cancer. J. Drug Target. 1–18. https://doi.org/10.1080/1061186X.2021.1877719.

Koual, M., Tomkiewicz, C., Cano-Sancho, G., Antignac, J.P., Bats, A.S., Coumoul, X., 2020. Environmental chemicals, breast cancer progression and drug resistance. Environ. Heal. A Glob. Access Sci. Source 19, 1–25. https://doi.org/10.1186/s12940-020-00670-2.

Kuhl, H., 2005. Pharmacology of estrogens and progestogens: influence of different routes of administration. Climacteric 8, 3–63. https://doi.org/10.1080/13697130500148875.

Leonel, E.C.R., Campos, S.G.P., Bedolo, C.M.B., Guerra, L.H.A., Vilamaior, P.S.L., Calmon, M.F., Rahal, P., Amorim, C.A., Taboga, S.R., 2020. Perinatal exposure to bisphenol A impacts in the mammary gland morphology of adult Mongolian gerbils. Exp. Mol. Pathol. 113, 104374 https://doi.org/10.1016/j.yexmp.2020.104374.

Leonel, E.C.R., Ruiz, T.F.R., Bedolo, C.M., Campos, S.G.P., Taboga, S.R., 2021. Inflammatory repercussions in female steroid responsive glands after perinatal exposure to bisphenol A and 17–β estradiol. Cell Biol. Int. https://doi.org/10.1002/cbin.11665.

Leonel, E.C.R.R., Falleiros, L.R., Campos, S.G.P.P., Taboga, S.R., 2017. Histological and immunohistochemical characterization of the Mongolian gerbil’s mammary gland during gestation, lactation and involution. Acta Histochem. 119, 273–283. https://doi.org/10.1016/j.acthis.2017.02.003.

Liu, Y., Mei, C., Liu, H., Wang, H., Zeng, G., Lin, J., Xu, M., 2014. Modulation of cytokine expression in human macrophages by endocrine-disrupting chemical Bisphenol-A. Biochem. Biophys. Res. Commun. 451, 592–598. https://doi.org/10.1016/j.bbrc.2014.08.031.

Lu, S., Yu, G., Zhu, Y., Archer, M.C., 2005. Cyclooxygenase-2 overexpression in MCF-10F human breast epithelial cells inhibits proliferation, apoptosis and differentiation, and causes partial transformation. Int. J. Cancer 116, 847–852. https://doi.org/10.1002/ijc.21142.

Lyons, T.R., O’Brien, J., Borges, V.F., Conklin, M.W., Keely, P.J., Eliceiri, K.W., Marusyk, A., Tan, A.C., Schedin, P., 2011. Postpartum mammary gland involution drives progression of ductal carcinoma in situ through collagen and COX-2. Nat. Med. 17, 1109–1116. https://doi.org/10.1038/nm.2416.

Maller, O., Martinson, H., Schedin, P., 2010. Extracellular matrix composition reveals complex and dynamic stromal-epithelial interactions in the mammary gland. J. Mammary Gland Biol. Neoplasia 15, 301–318. https://doi.org/10.1007/s10911-010-9189-6.

Mao, S.P.H., Park, M., Cabrera, R.M., Christin, J.R., Karagiannis, G.S., Oktay, M.H., Zaiss, D.M.W., Abrams, S.I., Guo, W., Condeelis, J.S., Kenny, P.A., Segall, J.E., 2018. Loss of amphiregulin reduces myoepithelial cell coverage of mammary ducts and alters breast tumor growth. Breast Cancer Res. 20, 1–14. https://doi.org/10.1186/s13058-018-1057-0.

Miret, N.V., Pontillo, C.A., Zárate, L.V., Kleiman de Pisarev, D., Cocca, C., Randi, A.S., 2019. Impact of endocrine disruptor hexachlorobenzene on the mammary gland and breast cancer: The story thus far. Environ. Res. 173, 330–341. https://doi.org/10.1016/j.envres.2019.03.054.

Montévil, M., Acevedo, N., Schaeberle, C.M., Bharadwaj, M., Fenton, S.E., Soto, A.M., 2019. A combined morphometric and statistical approach to assess non-monotonicity in the developing mammary gland of rats in the CLARITY-BPA study. bioRxiv 2012, 1–21. https://doi.org/10.1101/783019.

Mori, H., Lo, A.T., Inman, J.L., Alcaraz, J., Ghajar, C.M., Mott, J.D., Nelson, C.M., Chen, C.S., Zhang, H., Bascom, J.L., Seiki, M., Bissell, M.J., 2013. Transmembrane/cytoplasmic, rather than catalytic, domains of Mmp14 signal to MAPK activation and mammary branching morphogenesis via binding to integrin β1. Development 140, 343–352. https://doi.org/10.1242/dev.084236.

Nair, V.A., Valo, S., Peltomäki, P., Bajbouj, K., Abdel-Rahman, W.M., 2020. Oncogenic potential of bisphenol a and common environmental contaminants in human mammary epithelial cells. Int. J. Mol. Sci. 21 https://doi.org/10.3390/ijms21103735.

Need, E.F., Atashgaran, V., Ingman, W.V., Dasari, P., 2014. Hormonal regulation of the immune microenvironment in the mammary gland. J. Mammary Gland Biol. Neoplasia 19, 229–239. https://doi.org/10.1007/s10911-014-9324-x.

Ougier, E., Zeman, F., Antignac, J.P., Rousselle, C., Lange, R., Kolossa-Gehring, M., Apel, P., 2021. Human biomonitoring initiative (HBM4EU): Human biomonitoring guidance values (HBM-GVs) derived for bisphenol A. Environ. Int. 154, 106563 https://doi.org/10.1016/j.envint.2021.106563.

Perkins, N.D., 2007. Integrating cell-signalling pathways with NF-κB and IKK function. Nat. Rev. Mol. Cell Biol. 8, 49–62. https://doi.org/10.1038/nrm2083.

Pinkerton, J.A.V., Aguirre, F.S., Blake, J., Cosman, F., Hodis, H., Hoffstetter, S., Kaunitz, A.M., Kingsberg, S.A., Maki, P.M., Manson, J.A.E., Marchbanks, P.,

McClung, M.R., Nachtigall, L.E., Nelson, L.M., Pace, D.T., Reid, R.L., Sarrel, P.M., Shifren, J.L., Stuenkel, C.A., Utian, W.H., 2017. The 2017 hormone therapy position statement of the North American Menopause Society. Menopause 24, 728–753. https://doi.org/10.1097/GME.0000000000000921.

Pinto, M.E., Vilamaior, P.S.L., Taboga, S.R., Góes, R.M., 2008. Exposure of young rats to high estrogen doses leads to degeneration of elongated spermatids. Tissue Cell 40, 31–42. https://doi.org/10.1016/j.tice.2007.09.002.

Provenzano, P.P., Eliceiri, K.W., Campbell, J.M., Inman, D.R., White, J.G., Keely, P.J., 2006. Collagen reorganization at the tumor-stromal interface facilitates local invasion. BMC Med 4, 1–15. https://doi.org/10.1186/1741-7015-4-38.

Pupo, M., Pisano, A., Lappano, R., Santolla, M.F., de Francesco, E.M., Abonante, S., Rosano, C., Maggiolini, M., 2012. Bisphenol A induces gene expression changes and proliferative effects through GPER in breast cancer cells and cancer-associated fibroblasts. Environ. Health Perspect. 120, 1177–1182. https://doi.org/10.1289/ehp.1104526.

Ramírez-Valadez, K.A., Vázquez-Victorio, G., Macías-Silva, M., González-Espinosa, C., 2017. Fyn kinase mediates cortical actin ring depolymerization required for mast cell migration in response to TGF-β in mice. Eur. J. Immunol. 47, 1305–1316. https://doi.org/10.1002/eji.201646876.

Ramirez, R.A., Lee, A., Schedin, P., Russell, J.S., Masso-Welch, P.A., 2012. Alterations in mast cell frequency and relationship to angiogenesis in the rat mammary gland during windows of physiologic tissue remodeling. Dev. Dyn. 241, 890–900. https://doi.org/10.1002/dvdy.23778.

Ruiz, T.F.R., Colleta, S.J., Leonel, E.C.R., Taboga, S.R., 2021a. Mammary carcinoma in aged gerbil mothers after endocrine disruption in pregnancy and lactation. Endocr. Relat. Cancer. https://doi.org/10.1530/ERC-21-0198.

Ruiz, T.F.R., Colleta, S.J., Zuccari, D.A.P., de, C., Vilamaior, P.S.L., Leonel, E.C.R., Taboga, S.R., 2021b. Hormone receptor expression in aging mammary tissue and carcinoma from a rodent model after xenoestrogen disruption. Life Sci 285, 120010. https://doi.org/10.1016/j.lfs.2021.120010.

Sargeant, T.J., Lloyd-Lewis, B., Resemann, H.K., Ramos-Montoya, A., Skepper, J., Watson, C.J., 2014. Stat3 controls cell death during mammary gland involution by regulating uptake of milk fat globules and lysosomal membrane permeabilization. Nat. Cell Biol. 16, 1057–1068. https://doi.org/10.1038/ncb3043.

Schoeters, G., Den Hond, E., Dhooge, W., Van Larebeke, N., Leijs, M., 2008. Endocrine disruptors and abnormalities of pubertal development. Basic Clin. Pharmacol. Toxicol 102, 168–175. https://doi.org/10.1111/j.1742-7843.2007.00180.x.

Shafei, A., Matbouly, M., Mostafa, E., Al Sannat, S., Abdelrahman, M., Lewis, B., Muhammad, B., Mohamed, S., Mostafa, R.M., 2018. Stop eating plastic, molecular signaling of bisphenol A in breast cancer. Environ. Sci. Pollut. Res. 25, 23624–23630. https://doi.org/10.1007/s11356-018-2540-y.

Sheehan, D.M., 2000. Activity of environmentally relevant low doses of endocrine disruptors and the bisphenol A controversy: Initial Results Confirmed (44515). Exp. Biol. Med. 224, 57–60.

Simian, M., Bissell, M.J., Barcellos-Hoff, M.H., Shyamala, G., 2009. Estrogen and progesterone receptors have distinct roles in the establishment of the hyperplastic phenotype in PR-A transgenic mice. Breast Cancer Res. 11, 1–11. https://doi.org/10.1186/bcr2408.

Sprague, B.L., Trentham-Dietz, A., Hedman, C.J., Wang, J., Hemming, J.D.C., Hampton, J.M., Buist, D.S.M., Aiello Bowles, E.J., Sisney, G.S., Burnside, E.S., 2013. Circulating serum xenoestrogens and mammographic breast density. Breast Cancer Res. 15 https://doi.org/10.1186/bcr3432.

Stein, T., Morris, J.S., Davies, C.R., Weber-Hall, S.J., Duffy, M.-A., Heath, V.J., Bell, A.K., Ferrier, R.K., Sandilands, G.P., Gusterson, B.A., 2003. Involution of the mouse mammary gland is associated with an immune cascade and an acute-phase response, involving LBP, CD14 and STAT3. Breast Cancer Res. 6, R75. https://doi.org/10.1186/bcr753.

Tarullo, S.E., Hill, R.C., Hansen, K.C., Behbod, F., Borges, V.F., Nelson, A.C., Lyons, T.R., 2020. Postpartum breast cancer progression is driven by semaphorin 7a-mediated invasion and survival. Oncogene 39, 2772–2785. https://doi.org/10.1038/s41388-020-1192-9.

Tchou, J., Zhang, P.J., Bi, Y., Satija, C., Marjumdar, R., Stephen, T.L., Lo, A., Chen, H., Mies, C., June, C.H., Conejo-Garcia, J., Puré, E., 2013. Fibroblast activation protein expression by stromal cells and tumor-associated macrophages in human breast cancer. Hum. Pathol. 44, 2549–2557. https://doi.org/10.1016/j.humpath.2013.06.016.

Terry, M.B., Michels, K.B., Brody, J.G., Byrne, C., Chen, S., Jerry, D.J., Malecki, K.M.C., Martin, M.B., Miller, R.L., Neuhausen, S.L., Silk, K., Trentham-Dietz, A., McDonald, J., Oskar, S., Knight, J., Toro-Campos, R., Cai, X., Rising, C.J., Afanaseva, D., Mullis, M.D., Berry, M.P., Bird, J., Bradfield, C., Gangnon, R., Gould, M., Hampton, J., Lindberg, S., Luongo, S., Rolland, B., Shull, J., Gaudet, M., Thornquist, M., Aupperlee, M.D., Haslam, S.Z., Hoshyar, R., Kariagina, A., Lopes, J. R., Miller, K.J., Morozova, O., Newkirk, C.J., Schwartz, R.C., Thomas, B., Totzkay, D., Xie, F., Silk, K.J., Biro, F.M., Fassler, C.S., Giannini, C.M., Pinney, S., Troester, M.A., Burke, K., Herbstman, J., Kehm, R., Lilge, L., Perera, F., Sahay, D., Tehranifar, P., Walker, D., Zeinomar, N., De Hoz, M., Shepard, P., Binder, A., Bessonneau, V., De La Rosa, V., Ohayon, J., Rudel, R., Corvalan, C., Pereira, A., Pereira, J., Russo, J., Yanrong, S., Shepherd, J., Adams-Campbell, L., Dash, C., Haddad, B., Hamilton, R., Richardson, B., Denic-Roberts, H., Chang, G., Ding, Y.C., Kanaya, N., Rakoff, M., Saeki, K., Serrano, M., Reynolds, P., Dunphy, K., Jerry, J., Symington, A., Vandenberg, L., Schneider, S., Adams, S.A., Brandt, H.M., Friedman, D., Lead, J.R., Kreps, G., Wright, K., Burke-Garcia, A., Fisher, C., 2019. Environmental exposures during windows of susceptibility for breast cancer: A framework for prevention research. Breast Cancer Res. 21, 1–16. https://doi.org/10.1186/s13058-019-1168-2.

Vandenberg, L.N., Colborn, T., Hayes, T.B., Heindel, J.J., Jacobs, D.R., Lee, D.H., Shioda, T., Soto, A.M., vom Saal, F.S., Welshons, W.V., Zoeller, R.T., Myers, J.P., 2012. Hormones and endocrine-disrupting chemicals: Low-dose effects and nonmonotonic dose responses. Endocr. Rev. 33, 378–455. https://doi.org/10.1210/er.2011-1050.

vom Saal, F.S., Vandenberg, L.N., 2021. Update on the health effects of bisphenol a: overwhelming evidence of harm. Endocrinology 162, 1–25. https://doi.org/10.1210/endocr/bqaa171.

Wadia, P.R., Cabaton, N.J., Borrero, M.D., Rubin, B.S., Sonnenschein, C., Shioda, T., Soto, A.M., 2013. Low-dose BPA exposure alters the mesenchymal and epithelial transcriptomes of the mouse fetal mammary gland. PLoS One 8. https://doi.org/10.1371/journal.pone.0063902.

Wallace, T.R., Tarullo, S.E., Crump, L.S., Lyons, T.R., 2019. Studies of postpartum mammary gland involution reveal novel pro-metastatic mechanisms. J. Cancer Metastasis Treat. 2019, 139–148. https://doi.org/10.20517/2394-4722.2019.01.

Wang, D., Gao, H., Bandyopadhyay, A., Wu, A., Yeh, I.-T., Chen, Y., Zou, Y., Huang, C., Walter, C.A., Dong, Q., Sun, L.-Z., 2014. Pubertal bisphenol A exposure alters murine mammary stem cell function leading to early neoplasia in regenerated glands. Cancer Prev. Res. 7, 445–455. https://doi.org/10.1158/1940-6207.CAPR-13-0260.

Wang, X., Sun, L., Maffini, M.V., Soto, A., Sonnenschein, C., Kaplan, D.L., 2010. A complex 3D human tissue culture system based on mammary stromal cells and silk scaffolds for modeling breast morphogenesis and function. Biomaterials 31, 3920–3929. https://doi.org/10.1016/j.biomaterials.2010.01.118.

Wani, N.A., Nasser, M.W., Ahirwar, D.K., Zhao, H., Miao, Z., Shilo, K., Ganju, R.K., 2014. C-X-C motif chemokine 12/C-X-C chemokine receptor type 7 signaling regulates breast cancer growth and metastasis by modulating the tumor microenvironment. Breast Cancer Res. 16, 1–17. https://doi.org/10.1186/bcr3665.

Wärri, A., Cook, K.L., Hu, R., Jin, L., Zwart, A., Soto-Pantoja, D.R., Liu, J., Finkel, T., Clarke, R., 2018. Autophagy and unfolded protein response (UPR) regulate mammary gland involution by restraining apoptosis-driven irreversible changes. Cell Death Discov. 4 https://doi.org/10.1038/s41420-018-0105-y.

Watson, C.J., 2006. Key stages in mammary gland development Involution: Apoptosis and tissue remodelling that convert the mammary gland from milk factory to a quiescent organ. Breast Cancer Res 8, 1–5. https://doi.org/10.1186/bcr1401.

Xu, D., dan, Wang, G., He, X. jing, Wang, J. fa, Yang, B., Sun, Z. peng, Sun, D. bo, He, Q. yu, Zhang, X., Wu, R., 2017. 17β-Estradiol and progesterone decrease MDP induced NOD2 expression in bovine mammary epithelial cells. Vet. Immunol. Immunopathol. 188, 59–64. https://doi.org/10.1016/j.vetimm.2017.04.010.

Xu, R., 2017. Mammary epithelial polarity and macrophage infiltration. Macrophage 4, e1521. https://doi.org/10.1016/j.physbeh.2017.03.040.

Yuan, J., Yang, Y., Gao, Z., Wang, Z., Ji, W., Song, W., Zhang, F., Niu, R., 2017. Tyr23 phosphorylation of Anxa2 enhances STAT3 activation and promotes proliferation and invasion of breast cancer cells. Breast Cancer Res. Treat 164, 327–340. https://doi.org/10.1007/s10549-017-4271-z.

Zhang, S., Ekman, M., Thakur, N., Bu, S., Davoodpour, P., Grimsby, S., Tagami, S., Heldin, C.H., Landström, M., 2006. TGFβ1-induced activation of ATM and p53 mediates apoptosis in a Smad7-dependent manner. Cell Cycle 5, 2787–2795. https://doi.org/10.4161/cc.5.23.3523.

Zollo, M., Di Dato, V., Spano, D., De Martino, D., Liguori, L., Marino, N., Vastolo, V., Navas, L., Garrone, B., Mangano, G., Biondi, G., Guglielmotti, A., Yang, L., Huang, J. J., Ren, X., Gorska, A.E., Chytil, A., Aakre, M., Carbone, D.P., Matrisian, L.M.M., Richmond, A., Lin, P.C., Moses, H.L., Wani, N.A., Nasser, M.W., Ahirwar, D.K., Zhao, H., Miao, Z., Shilo, K., Ganju, R.K., Ungari, M., Ferrero, G., Varotti, E., Gusolfino, M.D., Manotti, L., Tanzi, G., Trombatore, M., Bertoni, R., Stein, T., Morris, J.S., Davies, C.R., Weber-Hall, S.J., Duffy, M.A., Heath, V.J., Bell, A.K., Ferrier, R.K., Sandilands, G.P., Gusterson, B.A., Van Dang, H., Sakai, T., Pham, T.A., Tran, D.H., Yorita, K., Shishido, Y., Fukui, K., Ramírez-Valadez, K.A., Vázquez-Victorio, G., Macías-Silva, M., González-Espinosa, C., Jeffrey, W.Pollard, Liu, Y., Mei, C., Liu, H., Wang, H., Zeng, G., Lin, J., Xu, M., Lilla, J.N., Joshi, R.V., Craik, C.S., Werb, Z., Kupferman, M.E., Fini, M.E., Muller, W.J., Weber, R., Cheng, Y., Muschel, R.J., Mendes, L.O., Amorim, J.P.A., Teixeira, G.R., Chuffa, L.G.A., Fioruci, B.A., Pimentel, T.A., de Mello, W., Padovani, C.R., Pereira, S., Martinez, M., Pinheiro, P.F.F., Oliani, S.M., Martinez, F.E., Huang, F.M., Chang, Y.C., Lee, S.S., Yang, M.L., Kuan, Y.H., Gan, L., Qiu, Z., Huang, J.J., Li, Y., Huang, H., Xiang, T., Wan, J., Hui, T., Lin, Y., Li, H., Ren, G., Elgert, K.D., Alleva, D.G., Mullins, D.W., Durando, M., Kass, L., Piva, J., Sonnenschein, C., Soto, A.M., Luque, E.H., Muñoz-De-Toro, M., Dawson, C.A., Pal, B., Vaillant, F., Gandolfo, L.C., Liu, Z., Bleriot, C., Ginhoux, F., Smyth, G.K., Lindeman, G.J., Mueller, S.N., Rios, A.C., Visvader, J.E., Dasari, P., Sharkey, D.J., Noordin, E., Glynn, D.J., Hodson, L.J., Chin, P.Y., Evdokiou, A., Robertson, S.A., Ingman, W.V., Clarkson, R.W.E., Boland, M.P., Kritikou, E.A., Lee, J.M., Freeman, T.C., Tiffen, P.G., Watson, C.J., Clark, N.M., Bos, P.D., Aoki, M., Pawankar, R., Niimi, Y., Kawana, S., Allavena, P., Sica, A., Solinas, G., Porta, C., Mantovani, A., Kanaya, N., Chang, G., Wu, X., Saeki, K., Bernal, L., Shim, H.-J., Wang, J., Warden, C., Yamamoto, T., Li, J., Park, J.-S., Synold, T., Vonderfecht, S., Rakoff, M., Neuhausen, S.L., Chen, S., Dasari, P., Sharkey, D.J., Noordin, E., Glynn, D.J., Hodson, L.J., Chin, P.Y., Evdokiou, A., Robertson, S.A., Ingman, W.V., Allavena, P., Sica, A., Solinas, G., Porta, C., Mantovani, A., Aoki, M., Pawankar, R., Niimi, Y., Kawana, S., Clark, N.M., Bos, P.D., Clarkson, R.W.E., Boland, M.P., Kritikou, E.A., Lee, J.M., Freeman, T.C., Tiffen, P.G., Watson, C.J., Dawson, C.A., Pal, B., Vaillant, F., Gandolfo, L.C., Liu, Z., Bleriot, C., Ginhoux, F., Smyth, G.K., Lindeman, G.J., Mueller, S.N., Rios, A.C., Visvader, J.E., Durando, M., Kass, L., Piva, J., Sonnenschein, C., Soto, A.M., Luque, E.H., Muñoz-De-Toro, M., Elgert, K.D., Alleva, D.G., Mullins, D.W., Gan, L., Qiu, Z., Huang, J.J., Li, Y., Huang, H., Xiang, T., Wan, J., Hui, T., Lin, Y., Li, H., Ren, G., Huang, F.M., Chang, Y.C., Lee, S.S., Yang, M.L., Kuan, Y.H., Mendes, L.O., Amorim, J.P.A., Teixeira, G.R., Chuffa, L.G.A., Fioruci, B.A., Pimentel, T.A., de Mello, W.,

Padovani, C.R., Pereira, S., Martinez, M., Pinheiro, P.F.F., Oliani, S.M., Martinez, F. E., Kupferman, M.E., Fini, M.E., Muller, W.J., Weber, R., Cheng, Y., Muschel, R.J., Lilla, J.N., Joshi, R.V., Craik, C.S., Werb, Z., Liu, Y., Mei, C., Liu, H., Wang, H., Zeng, G., Lin, J., Xu, M., Jeffrey, W.Pollard, Ramírez-Valadez, K.A., Vázquez-Victorio, G., Macías-Silva, M., González-Espinosa, C., Van Dang, H., Sakai, T., Pham, T.A., Tran, D.H., Yorita, K., Shishido, Y., Fukui, K., Stein, T., Morris, J.S., Davies, C.R., Weber-Hall, S.J., Duffy, M.A., Heath, V.J., Bell, A.K., Ferrier, R.K., Sandilands, G.P., Gusterson, B.A., Ungari, M., Ferrero, G., Varotti, E., Gusolfino, M. D., Manotti, L., Tanzi, G., Trombatore, M., Bertoni, R., Wani, N.A., Nasser, M.W., Ahirwar, D.K., Zhao, H., Miao, Z., Shilo, K., Ganju, R.K., Yang, L., Huang, J.J., Ren, X., Gorska, A.E., Chytil, A., Aakre, M., Carbone, D.P., Matrisian, L.M.M., Richmond, A., Lin, P.C., Moses, H.L., Zollo, M., Di Dato, V., Spano, D., De Martino, D., Liguori, L., Marino, N., Vastolo, V., Navas, L., Garrone, B., Mangano, G., Biondi, G., Guglielmotti, A., 2014. Tumor-induced Immune Dysfunction: The Macrophage Connection. In: J. Biol. Chem, 66, pp. 1777–1783. https://doi.org/10.1007/s10585-012-9473-5.