UNIVERSIDADE ESTADUAL PAULISTA
“Júlio de Mesquita Filho”
INSTITUTO DE BIOCIÊNCIAS DE BOTUCATU

IDENTIFICAÇÃO DE ALVOS ONCOGÊNICOS NA PRÓSTATA DE RATOS EXPOSTOS A MISTURAS DE FTALATOS DURANTE O DESENVOLVIMENTO PERINATAL: ESTUDO TRANSGERACIONAL

MSc. Ariana Musa de Aquino

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Tese apresentada ao Instituto de Biociências campus de Botucatu, UNESP, como requisito obrigatório para obtenção do título de Doutora pelo programa de Pós-Graduação em Biologia Geral e Aplicada, Área de concentração: *Biologia Celular Estrutural e Funcional.*

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1. Disruptores Endócrinos. 2. Hereditariedade. 3. Exposição materna. 4. Plastificantes. 5. Carcinógenos.

Palavras-chave: Desregulador endrócrino; Efeitos transgeracionais; Exposição materna; Ftalatos; Repercussões tardia.

Dedicatória

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Resumo

Os ftalatos correspondem a um grupo de plastificantes comercialmente utilizados em diferentes meios de produção com maior ou menor risco de exposição a depender do material ao qual estão empregados. Por outro lado, os ftalatos são facilmente liberadas para o meio ambiente, porque não formam ligação covalente. Os estudos que avaliam exposição precoce aos ftalatos, são focados na exposição isolada ou associada a outras diferentes substâncias que atuam como Desreguladores Endócrinos (DEs). Este estudo buscou investigar o efeito da exposição a um conjunto de ftalatos para mimetizar a exposição ambiental e avaliar o efeito da exposição. Além disso, estudos recentes apontam que metabólitos de ftalatos são detectados em amostras de urina de mulheres em idade reprodutiva, gestantes e crianças nas diferentes fases do desenvolvimento, o que comprova a exposição precoce a um conjunto de ftalatos. Paralelamente, estudos avaliando as janelas de susceptibilidade apontam que a exposição aos ftalatos está associada as desordens reprodutivas masculina e feminina, bem como as doenças do indivíduo adulto e senil. Na próstata, o efeito está associado a prejuízos na morfofisiologia da glândula, aumento de stress e inflamação, criado por um microambiente reativo e, maior vulnerabilidade do tecido para alterações relacionadas as patologias prostáticas, que acometem homens em idade reprodutivas e homens senis. Neste sentido, este estudo buscou investigar a influência da exposição materna em períodos cruciais para o desenvolvimento e amadurecimento da glândula prostática, na modulação de mecanismos pós-transcricionais (miRNAs and mRNAs), na atividade funcional do tecido (proteoma), bem como na contribuição destas modulações para a origem do câncer de próstata. Ainda, está investigação focou na transmissão das alterações possivelmente transmitidas para as próximas gerações, buscando estabelecer se a repercussão tardia da exposição é observada na descendência (F1) e na geração (F2). Para atingir tal objetivo, ratas prenhes da linhagem Sprague Dawley foram distribuídas em 4 grupos experimentais: Grupo controle (controle; veículo); 20µg/kg/dia, 200µg/kg/dia e 200mg/kg/dia que receberam o tratamento com a mistura de ftalatos do dia gestacional 10 (DG10) ao dia pós-natal 21 (DPN21), via gavagem. O

grupo exposto 200µg/kg/dia foi avaliado somente para obter o perfil de expressão gênica, para as demais análises e comparação, o grupo foi desconsiderado. Ao final do delineamento experimental, os filhotes foram analisados em 4 checkpoint: no DPN22(F1) para analisar as consequências da exposição materna direta; no DPN120 para avaliar as repercussões tardias; no DPN540 para analisar as consequências da exposição na vida senil, para as análises histopatológicas, e no DPN22, para investigar o efeito transgeracional nos descendentes de pais expostos (F2). A próstata ventral foi coletada ao final de cada checkpoint, para as análises moleculares. Os resultados obtidos nesta investigação estão apresentados no formato de capítulo no decorrer da tese. Cabe ressaltar que dois artigos apresentados nesta tese estao publicados em periódicos científicos da área.

Abstract

Phthalates correspond to a group of plasticizers commercially used in different production environments, with a greater or lesser risk of exposure depending on the material they are used in. On the other hand, phthalates are easily released into the environment because they do not form covalent bonds. Studies evaluating early exposure to phthalates are focused on exposure in isolation or in association with other different substances that act as Endocrine Disruptors (EDs). This study sought to investigate the effect of exposure to a set of phthalates to mimic environmental exposure and evaluate the effect of exposure. In addition, recent studies indicate that phthalate metabolites are detected in urine samples from women of reproductive age, pregnant women, and children at different stages of development, which proves the early exposure to a set of phthalates. At the same time, studies evaluating susceptibility windows show that exposure to phthalates is associated with male and female reproductive disorders, as well as adult and senile diseases. In the prostate, the effect is associated with damage to the morphophysiology of the gland, increased stress, and inflammation, created by a reactive microenvironment and greater vulnerability of the tissue to alterations related to prostatic pathologies, which affect men of reproductive age and senile men. In this sense, this study sought to investigate the influence of maternal exposure in crucial periods for the development and maturation of the prostate gland, in the modulation of post-transcriptional mechanisms (miRNAs and mRNAs), in the functional activity of the tissue (proteome), as well as in the contribution of these modulations to the origin of prostate cancer. In addition, this investigation focused on the transmission of the alterations possibly transmitted to the next generations, seeking to establish whether the late repercussions of exposure are observed in the offspring (F1) and in the generation (F2). To achieve this goal, pregnant rats of the Sprague Dawley strain were divided into 4 experimental groups: Control group (control; vehicle); 20µg/kg/day, 200µg/kg/day and 200mg/kg/day which received treatment with the phthalate mixture from gestational day 10 (GD10) to postnatal day 21 (PND21), via gavage. The group exposed to 200µg/kg/day was evaluated only to obtain the gene

expression profile; for the other analyses and comparisons, the group was disregarded. At the end of the experimental design, the pups were analyzed at 4 checkpoints: at PND22(F1) to analyze the consequences of direct maternal exposure; at PND120 to evaluate the late repercussions; at PND540 to analyze the consequences of exposure in senile life, for the histopathological analyses, and at PND22, to investigate the transgenerational effect on the offspring of exposed parents (F2). The ventral prostate was collected at the end of each checkpoint for molecular analysis. The results obtained in this research are presented in chapter format throughout the thesis. It should be noted that two articles presented in this thesis have been published in scientific journals in the field.

Lista de Abreviações

Introdução

SUG: Seio urogenital

HBP: Hiperplasia Benigna prostática

CaP: Câncer de próstata

DHT: Dihidrotestosterona

KGF: Keratinocyte Growth Factor

EGF: Epidermal Growth Factor receptor

EDCs: Desreguladores Endócrinos Químicos

DOHaD: *Origem Desenvolvimentista da Saúde e da Doença (DOHaD)*

DES: dietilestilbestrol

miRNA: MicroRNA

mRNA: RNA mensageiro/ target

BPA: Bisphenol A

TDS: Disgenesia testicular

MEHP: Mono Etilhexilo phthalate

DEHP: Bis 2-ethilhexil phthalate

DBP: Di-n-butil ftalato phthalate

BBP: Benzil Butil ftalato phthalate

MBP: Mono Butil phthalate

PIN: Neoplasia Intraepitelial Prostática

HPIN: Neoplasia Intraepitelial Prostática de alto grau

DiBP: Diisobutyl Phthalate

BBzP: Butylbenzyl phthalate

DiNP: Diisononyl phthalate

Capítulo I

GD: Gestational exposure

F1: First generation

F2: Second generation

DEHP: Bis 2-ethylhexyl phthalate

DEP: Diethyl phthalate

PND: Postnatal day

DBP: Di-n-butylphthalate

DiBP: Diisobutyl phthalate

BBzP: Butylbenzyl phthalate

DiNP: Diisononyl phthalate

PND22: Postnatal day 22 (F1)

PND120: Postnatal day 120 (F1)

HPA: The Human Protein Atlas

VP: Ventral prostate

TDB: Toxicogenomic database

ECDs: Endocrine Disruptor Chemical

BPA: Bisphenol A

Capítulo II

PCa: Prostate cancer

ECDs: Environmental Chemical Disruptors

BPH: Benign Prostatic Hyperplasia

DOHaD: Origin of Developmental Health and Diseases

GD: Gestational Day

PND: Postnatal day

VP: Ventral prostate

F0: Offspring exposed.

F1: First generation

F2: Second generation

PND22: Postnatal day 22 (F1)

PND120: Postnatal day 120 (F1)

PPI: protein-protein interaction
TFEA: Factor Enrichment Analysis
KEA: Kinase Enrichment Analysis
KTI: Kinase-Transcription Factor Interaction
TCGA: The Genomic Cancer Atlas
HPA: The Human Protein Atlas
PRAD: Prostate Adenocarcinoma
PTPM: Post-translational protein modification

Capítulo III

EDs: Endocrine disruptors
EDCs: Endocrine Disrupting Chemicals (EDCs)
GD: Gestational Day
PND: Postnatal Day
F1-PND22: Postnatal day 22 (F1)
F1-PND120: Postnatal day 120 (F1)
F2-PND22-M: Offspring of mother exposed on postnatal day 22 (F2)
F2-PND22-F: Offspring of father exposed on postnatal day 22 (F2)
DOHaD: Origin of Developmental Health and Diseases
DEHP: Bis (2-ethylhexyl) phthalate)
DEP: Diethyl phthalate
DBP: Di-n-butyl phthalate
DiBP: Diisobutyl Phthalate
BBzP: Butyl benzyl phthalate
DiNP : Diisononyl phthalate
PPI : Protein-protein interaction
CC: Cellular Compartment
BP: Biological Process
MF: Molecular Function
PCa: Prostate cancer

Anexo I

DEP: Diethyl phthalate
DBP: Di-n-butyl phthalate
DiBP: Diisobutyl Phthalate
BBzP: Butylbenzyl phthalate
DiNP: Diisononyl phthalate
MEP: Monoethyl phthalate
MBP: Monobutyl phthalate (
MiBP: Monoiso- butyl phthalate
MBzP: Monobenzyl phthalate (MBzP)
MEHP: Monoetilhexilo phthalate
MiNP: Monoisononil phthalate
VP: Ventral Prostate
GO: Gene Ontology
PPI: Protein-Protein Interaction
INF: Inflammatory Focus (inf)
IRA: Inflammatory Reactive Atypia (IRA)
EA: Epithelial Atypia (EA)
FEH: Focal Epithelial Hyperplasia (FEH)
LGD: Low-Grad Dysplasia (LGD)
CS: Cells into the Stroma (CS)
PIN: Prostatic Intraepithelial Neoplasia (PIN)
ADEN: Adenoma (aden)

Sumário

Capítulo I

Revisão da literatura

Lista de figuras – Introdução

O **capítulo I** foi montado com o intuito de apresentar os principais embasamentos que regem este estudo. Neste capítulo são apresentados os tópicos desde a formação da glândula prostática até as condições patológicas, bem como as relações dos diferentes desreguladores endócrinos e dos ftalatos com as principais doenças que acometem o aparelho reprodutor masculino e a próstata. Este capítulo também aborda a problemática que envolve as consequências da exposição materna para a saúde e desenvolvimento do indivíduo.

1. Introdução

1.1. Próstata: Do desenvolvimento às condições adversas

A organogênese prostática, a morfogênese e os eventos moleculares envolvidos nestes processos são bem similares entre humanos e roedores, por esse motivo, os roedores são frequentemente usados para os diferentes tipos de comparações investigativas associadas ao desenvolvimento dos tecidos ou as condições patológicas que acometem humanos (Cunha and Baskin 2018).

No desenvolvimento intrauterino, o mesoderma intermediário, dará origem ao seio urogenital (SUG) que, após ser estabelecido, se diferencia para originar o sistema urinário e a crista gonodal. A região proximal do SUG, após diferenciação, dará origem a bexiga urinária e a região média dará origem a glândula prostática. Em camundongos, considerando um quadro de condições gestacionais mornais, esse processo de estabelecimento do SUG ocorrerá entre o 14°-18° dia do desenvolvimento gestacional (DG); em ratos esse evento acontecerá por volta do 13°-15° do DG e em humanos o SUG aparece por volta da 8-9 semana de gestação (SG) (Marker et al., 2003; Cunha et al., 2018)(tabela 1). Entre o 16,5° – 18.5° DG, a depender do tempo de cada gestação em roedores e da 13° SG em humanos, por meio de uma cascata de sinalização molecular, as primeiras células já diferenciadas migram da região pélvica do SUG para originar o primórdio prostático (Gail S. Prins and Putz 2008; Scarano et al., 2018; Marker et al., 2003; Francis and Swain 2018).

O desenvolvimento inicial da glândula prostática é dividido em: fase de pré-brotamento do SUG, fase de surgimento dos cordões sólidos epiteliais prostáticos no epitélio do SUG; fase de alongamento e ramificação dos botões; fase de canalização dos cordões epiteliais sólidos; fase de diferenciação das células epiteliais luminais e basais e, por fim, a fase de citodiferenciação secretória ao final deste processo é possível diferenciar o utrículo prostático e o ducto de Wolffian (Cunha et al., 2018) (fig. 1). Neste ponto, cabe destacar que a morfogênese de ramificação prostática só é iniciada após o desenvolvimento dos testículos fetais, que passam a sintetizar e secretar testosterona e andrógenos para ativar os receptores androgênicos no SUG e,

por ação da 5-α-redutase, a testosterona é convertida em Dihidrotestosterona (DHT), hormônio que coordena o processo de ramificação e o amadurecimento da glândula até a puberdade. Neste sentido, o DHT é o hormônio androgênico mais essencial para o desenvolvimento prostático. (Cunha et al., 2018; Gail S. Prins and Korach 2008). Neste sentido, interferências na cascata hormonal ou atraso no estabelecimento e desenvolvimentos dos testículos atrasará a morfogênese prostática, podendo aumentar os riscos para repercussões tardias, as quais podem ser observadas na vida adulta ou envelhecimento.

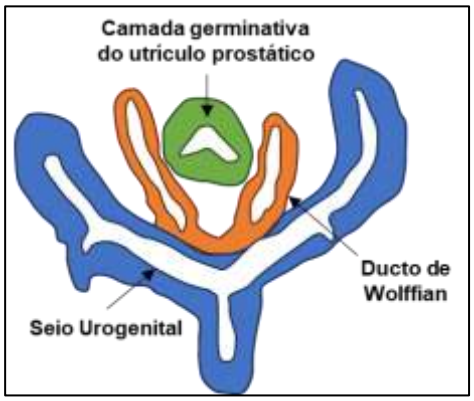

Figura 1: Imagem esquemática mostrando a organização histológica da diferenciação tecidual quando o utrículo prostático está diferenciado.

Tabela 1: Tabela adaptada de Cunha 2018 **(**Development of the human prostate).

Desenvolvimento	Ratos	Camundongo	Humano
Estágio de pré brotamento do seio urogenital	14-18 dias após concepção	13-15 dias após concepção	8-9 semanas
Brotamento inicial	19 dias após concepção	16-18 dias após concepção	10-11 semanas
Alongamento dos brotos e morfogênese de brotamento	1-50 dias de desenvolvimento pós-natal	1-40 dias de desenvolvimento pós-natal	Após a 11 semana
Canalização dos ductos	Aproximadamente 3-50 dias de desenvolvimento pós-natal		Após a 11 semana

Na fase de pré-brotamento do SUG, uma cascata de sinalização que estimula as células do SUG a migrarem formando assim os botões epiteliais que penetram o mesênquima circundante do SUG na base da bexiga urinária (Gail S. Prins and Korach 2008). Na fase de surgimento dos cordões sólidos epiteliais prostáticos no epitélio do SUG, os botões epiteliais são morfologicamente evidentes. Já no desenvolvimento pós-natal, os brotos se alongam e a morfogênese de brotamento passa a ser mais intensa nesta etapa. Nesta fase do desenvolvimento, é possível identificar as ramificações especificas de cada lobo ou zona prostática em ratos e humanos, respectivamente. Entre o 15°- 30° dia de desenvolvimento pós-natal (DPN), ocorrem as fases de alongamento e morfogênese de brotamento e canalização ducal, processos cruciais para a próstata atingir a maturidade completa (Gail S. Prins and Korach 2008; Cunha et al., 2018). Ainda nesta fase, há uma diferenciação de células epiteliais e os ductos passam a ser compostos por células epiteliais basais, componentes da membrana basal formando assim uma camada contínua de células e, as células luminais colunares altas justapostas, organizadas e constituintes da lâmina ducal. Pouco tempo depois, as células luminais sofrem uma citodiferenciação e passam a secretar proteínas específicas da glândula (Marker et al., 2003) (fig. 2).

Em humanos e roedores o desenvolvimento completo da glândula se estende até a pré-puberdade. Na morfogênese prostática tardia, durante o desenvolvimento pós-natal, as células mesenquimais que circundam a membrana basal se condensam e formam a camada periductal de células musculares lisas, por organização das células adjacentes ao ducto e pela diferenciação dos fibroblastos (Gail S. Prins and Putz 2008; Gail S. Prins and Birch 1995; S W Hayward, Cunha, and Dahiya 1996) . Uma rede vascular paralela se forma no estroma do tecido e, por meio de capilares, se estende até a membrana basal, para atender a demanda nutricional de toda a glândula prostática (Gail S. Prins and Putz 2008).

Ao final da morfogênese de ramificação tardia, portanto, período de intensa diferenciação tecidual, canalização e amadurecimento morfofuncional da glândula e com a devida diferenciação nos padrões de ramificação e tempo de desenvolvimento entre a próstata de roedores e humana, a arquitetura das glândulas é completamente

estabelecida, o que permite caracterizar cada um dos lobos e zonas prostáticas, respectivamente (Sugimura, Cunha, and Donjacour 1986; Lung and Cunha 1981). Localizada próxima a base da bexiga urinária, a próstata de roedores é distinguida estruturalmente e morfologicamente em lobo anterior ou glândula coaguladora, lobo ventral, lobo dorsal e lateral ou lobo dorsolateral. A glândula prostática humana, é septada em zona central, envolvida na formação do ducto ejaculatório e projetada sob a base da bexiga urinária; zona de transição, que circunda a uretra proximal no ducto ejaculatório e zona periférica, que corresponde a região apical da próstata (Marker et al., 2003; Verze, Cai, and Lorenzetti 2016; Rebello et al., 2021).

Histologicamente, a glândula madura é composta por um compartimento estromal, região rica em elementos de matriz (metaloproteinases, fibras de sustentação, fibroblastos, células endoteliais), células do sistema imune (Neutrófilos, macrófagos, linfócitos T e B, mastócitos), células musculares, nervosas, adipócitos e outros elementos celulares, que em conjunto, formam uma rede de nutrição e sustentação do tecido. Sendo assim, o estroma é de suma importância na manutenção e controle da homeostasia glandular, na organização estrutural e na sustentação tecidual (Toivanen and Shen 2017; Gail S. Prins and Lindgren 2014). O compartimento epitelial por sua vez é formado por células luminais, células basais e neuroendócrina, que se intercalam, formando um epitélio pseudoestratificado, colunar alto. Os compartimentos epitelial e estromal interagem entre si por diferentes vias de sinalização, o que leva a um microambiente instável quando alterações são desencadeadas por condições adversas, por exemplo, processo inflamatório intenso ou estresse oxidativo (Verze, Cai, and Lorenzetti 2016; Ficarra et al., 2014). Por fim, há ainda o compartimento luminal, local onde toda a secreção glandular é depositada (Fig. 2). Considerando que os compartimentos mencionados anteriormente se tornam mais sensíveis a desregulação frente a um estímulo externo, por consequência, todo o conteúdo secretado e armazenado no compartimento luminal, pode ter qualidade reduzida, o que compromete o volume secretado em cada alvéolo prostático, prejudicando a qualidade do fluido seminal, uma vez que a glândula prostática é responsável por secretar até 30% do volume seminal total.

A glândula prostática é comporta por cinco principais tipos celulares que coordenam o desenvolvimento, a diferenciação e a fisiologia glandular. As células epiteliais secretoras, que expressam receptores de andrógeno e estrógeno, secretam proteínas específicas de cada região glandular e revestem todo o compartimento luminal dos ácinos. As células epiteliais basais e as luminais, que estão aderidas a uma membrana basal, constituem o epitélio e configuram o tipo celular mais proliferativo do tecido. As células neuroendócrinas, mais escassas no indivíduo adulto, porém essencial na organogênese glandular. Estas células expressam proteínas carreadoras, peptídeo hormonais e aminas biogênicas, bem como secretam hormônios e neurotransmissores essenciais para a morfofisiologia da glândula (Gail S. Prins and Lindgren 2014). As células transitórias, células progenitoras não diferenciadas, por isso, transitam entre células basais e secretoras e podem ser encontradas como células progenitoras não diferenciadas. Ainda, a camada basal abriga *stem cells,* que são responsáveis por manter a população de célula do tecido e são constantemente exploradas como alvos terapêuticos (fig. 2).

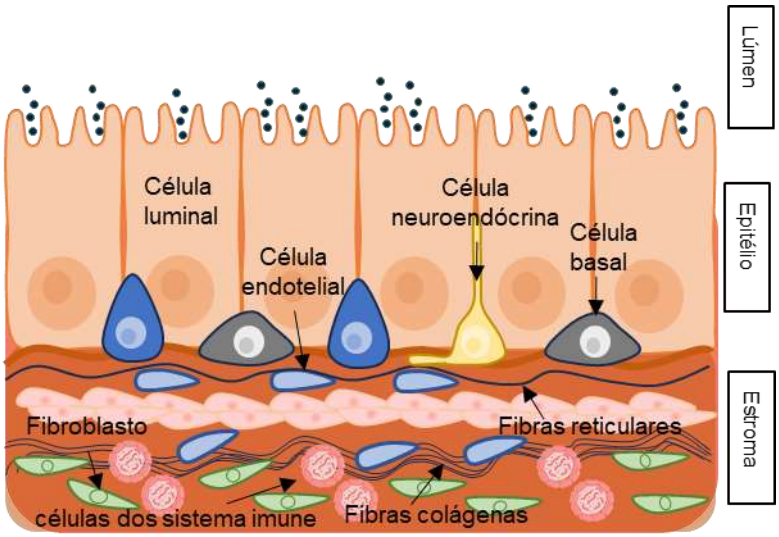

Figura 2: Representação esquemática destacando os tipos celulares que compõem os ductos prostáticos (alvéolos prostáticos). O esquema evidencia a estrutura histológica do ducto prostático e mais internamente a organização histológica dos ductos. Na imagem cada ducto é sustentado por um estroma rico em elementos da matriz extracelular, bem como, em fibroblastos, células endoteliais e células neuroendócrinas. Na camada mais acima é possível observar que

rente as células luminais há outros elementos de sustentação que formam a lâmina basal, composto por células basais, células neuroendócrinas e musculatura lisa. Ainda, o ducto é composto por células epiteliais, colunares altas e justa-postas. Estas células formam o compartimento epitelial e são responsáveis por secretar o conteúdo prostático. No interior da glândula, já no compartimento luminal, é possível observar o lúmen preenchido pela secreção prostática.

Quando atinge a maturidade, a próstata é composta por um corpo firme, glandular e fibromuscular que circunda a uretra prostática. Sua função primária é secretar nutrientes, íons, proteínas (calicreinas (KLKs), fatores de crescimento (insulin like-3) entre outros elementos que serão adicionados ao fluido ejaculatório produzido pela vesícula seminal, pela glândula bulbouretral e liberado após estímulos ejaculatórios. O plasma seminal, rico em diferentes nutrientes, é crucial para garantir a sobrevivência dos espermatozoides durante o percurso até o sistema genital feminino e ativar componentes bioquímicos essenciais para o sucesso reprodutivo (Gail S. Prins and Lindgren 2014; Verze, Cai, and Lorenzetti 2016).

O desequilíbrio na homeostasia da glândula prostática, além de diminuir a viabilidade das células germinativas (espermatozoides), aumenta os riscos para desordens prostáticas como a prostatite, hiperplasia benigna prostática (HBP) e para o câncer de próstata (CaP) (Verze, Cai, and Lorenzetti 2016; Rebello et al., 2021). Prostatite, é uma patologia comum entre homens em idade reprodutiva e, é resultado de um processo inflamatório exacerbado. Essa patologia afeta a fertilidade e está associada a disfunções sexuais, bem como as alterações hormonais. Ainda neste contexto, há vários estudos associando o aumento de prostatite ao desenvolvimento de HBP e câncer de próstata (Ficarra et al., 2014; Verze, Cai, and Lorenzetti 2016; Bostanci et al., 2013). A HBP corresponde a um aumento acentuado da glândula prostática que, quando não tratada, compromete a funções fisiológicas do aparelho reprodutor e urinário, por comprimir órgãos próximos. Apesar desta patologia apresentar perda de controle celular por reguladores como andrógenos ou genes de sinalização como KGF, EGF e insulin-like growth fator e o processo apoptótico ser inibido, o que impede o reparo de dano tecidual, as células não expressam

características de malignidade, portanto, não configura células invasivas (Verze, Cai, and Lorenzetti 2016).

O CaP, no entanto, além de ter características de malignidade, apresenta heterogeneidade devido ao surgimento de um ou mais focos multifocais (neoplasias), que surgem pela diversidade celular específica de cada região da glândula prostática descritas anteriormente e, por consequência de alterações genéticas ou ambientais (Haffner et al., 2020). Apesar de ter uma taxa de cura alta e tratamento eficaz, sem intervenções agressivas, quando diagnosticado precocemente, o CaP ainda é o tipo de câncer mais comum e letal entre homens, ficando atrás apenas do câncer de pele não-melanoma, que é comum entre homens e mulheres (Siegel et al., 2021; Parkin et al., 2002). As doenças que acometem a glândula são negligenciadas devido à falta da cultura de cuidados precoce entre a população masculina, isso contribui de forma significativa para o aumento dos casos letais decorrentes da doença.

Nas últimas décadas, houve crescente interesse das comunidades médico-científicas para entender os mecanismos que resultam no estabelecimento, desenvolvimento e progressão do CaP. Nos dias atuais, sabemos que diversos fatores envolvem a temática câncer e, neste caso especificamente a relação genoma-epigenoma são cruciais para o estabelecimento dos diferentes tipos de câncer (Haffner et al., 2020). Entretanto, a vertente célula-ambiente e ambiente-genoma/epigenoma podem ser determinantes neste contexto patológico, por exemplo, o estilo de vida, os hábitos alimentares, as condições patológicas pré-estabelecidas e o ambiente ao qual o indivíduo é exposto durante a vida pode determinar o surgimento e a gravidade de doenças. (Chiang, Mahalingam, and Flaws 2017; Skinner, Manikkam, and Guerrero-Bosagna 2010a; Monneret, Knez, and Monneret 2017; Sikka and Wang 2008; Yoon et al. 2014). Nesta investigação, o principal foco leva em consideração dois fatores: as condições maternas durante o desenvolvimento, diferenciação e morfogêneses da glândula e a exposição precoce a um conjunto de plastificantes ambientais que atuam como Desreguladores Endócrinos Químicos (DECs), porque ambos os fatores podem aumentar os riscos para a desordem morfofisiológica da glândula e para o CaP.

1.2 Desreguladores endócrinos e Desreguladores Endócrinos Químicos

Os hormônios são essenciais para manter o desenvolvimento, crescimento, metabolismo, imunidade e o comportamento do indivíduo (Monneret 2017). Diferentes estudos mostram que a exposição constante a substâncias que prejudicam o funcionamento do sistema endócrino está relacionada a perturbações sistêmicas a longo prazo. De acordo com Yoon e colaboradores (Yoon et al., 2014), "os Desreguladores Endócrinos Químicos (DECs) são compostos naturais ou sintéticos capazes de prejudicar a biossíntese, o armazenamento, a secreção, o transporte e/ou a ligação dos receptores hormônios endógenos, interferindo assim, em nas funções desses hormônios em responder as cascatas de sinalização celular". Cerca de 800 dos produtos químicos comerciais podem alterar o funcionamento do sistema endócrino, entretanto, os potenciais efeitos adversos destes produtos são pouco explorados (Ying 2012), ou seja, a comunidade médico-científica não consegue acompanhar o crescente número de novos produtos lançados nos mercado constantemente e sem controle das autoridades competentes.

A exposição fetal a produtos químicos ambientais com ação estrogênica e anti-androgênica perturbam a síntese de testosterona e a diferenciação sexual, o que resulta em um processo de disfunção testicular no adulto ou infertilidade, por exemplo (Delbès, Levacher, and Habert 2006; Clark and Cochrum 2007; Wohlfahrt-Veje, Main, and Skakkebaek 2009; Dorostghoal, Moazedi, and Zardkaf 2012). Em fêmeas, a exposição a esses compostos também prejudica a diferenciação sexual e principalmente a viabilidade das células da linhagem germinativa (Monneret 2017).

Os DECs são capazes de mimetizar estrógeno e andrógenos, bloqueando assim as interações com receptores envolvidos nas diversas respostas biológicas controladas pela regulação hormonal. Esse efeito de interação e bloqueio de receptores é conhecido como efeito antiestrogênico e antiandrogênico. Considerando um cenário em que é difícil determinar todas as substâncias que podem ser englobadas nas categorias de DECs e que essas substâncias estão associadas aos efeitos adversos no desenvolvimento, na reprodução, no sistema imune e neurológico (Monneret 2017), é fácil correlacionar o aumento destas patologias, especialmente de tecidos hormônio-

dependentes, com as falha no sistema de saúde pública, pela falta de diretrizes para reduzir ao máximo o uso dessas substâncias nos diferentes meios de produção.

As diferentes substâncias químicas que atuam no sistema endócrino durante a vida fetal interrompem o desenvolvimento do aparelho genital como um todo, porque esta diferenciação é controlada expressamente pela síntese hormonal. Além disso, a exposição altera a expressão de genes que codificam proteínas de sinalização segregadas, essenciais para coordenar o processo de diferenciação tecidual durante o desenvolvimento (L. Ma 2009) e com isso tem potencial efeito na morfogênese e na fisiologia genital em roedores e humanos (Newbold et al., 2004; Baird and Newbold 2005). Por esta razão, a exposição aos DECs ambientais durante a janela crítica de reprogramação, pode induzir mudanças profundas nas vias de sinalização reguladora, com impacto significativo em desordens que afetarão a saúde reprodutiva tardiamente (Wong and Walker 2013).

Avanços cruciais foram alcançados sobre a relação dos hormônios esteroides e outros morfogénos no controle do desenvolvimento, na diferenciação e na maturação do aparelho genital de ambos os sexos. Estes achados reforçam que há um período de sensibilidade na formação dos sistemas e, que esta janela de sensibilidade sofre influência das diferentes substâncias que mimetizam ou interrompem as ações hormonais endógenas. Neste sentido, ainda existem muitas questões acerca das consequências a longo prazo das interações entre os diferentes fatores externos, como a exposição ambiental e as desordens a níveis genômico e epigenético (ambiente-genoma-epigenoma) nos períodos de extrema sensibilidade para o desenvolvimento do aparelho genial. Nesse contexto, um importante aspecto seria entender a influência materna neste processo, uma vez que o desenvolvimento intrauterino é um período determinante para os eventos morfogênicos que coordenam a diferenciação e maturação dos tecidos a curto e longo prazo e, a exposição precoce já é documentada em diferentes registros.

1.3 Desreguladores endócrinos, Influência materna e DOHaD

Há pouco mais de três décadas, David Barker e colaboradores correlacionaram o baixo peso ao nascer com aumento nos riscos de doenças cardiovasculares e metabólicas durante a vida adulta (D. J. Barker et al., 1989). A partir desta investigação, surge o termo programação fetal. O termo define que o ambiente nutricional, hormonal e metabólico proporcionado pela mãe durante o desenvolvimento, pode permanentemente programar a estrutura e a fisiologia da prole gerando repercussões tardias na vida adula, no envelhecimento (D. J. Barker et al., 1989). Após essa publicação, as investigações envolvendo a temática foram ampliadas e, hoje, os estudos são focados estabelecer a influência dos fatores externos ou a condição materna nas janelas críticas temporais de desenvolvimento e da reprogramação dos tecidos para além do desenvolvimento *in* útero, incluindo assim os períodos de desenvolvimento pré-natal, perinatal, neonatal, pós-natal e puberal (Guintivano and Kaminsky 2016; S.-M. Ho et al., 2017a).

Atualmente o conceito da *Origem Desenvolvimentista da Saúde e da Doença (DOHaD, Developmental Origins of Health and Disease)* que, surge desse avanço nas investigações apoiadas pelos achados de Barker, prova que os efeitos das condições maternas adversas como má nutrição, doenças metabólicas crônicas, exposição aos agentes estressores, dentre outros fatores que podem influenciar e a aumentar s doenças crônicas não transmissíveis, reforçam os estudos de Barker e lançam luz as novas investigações para eventos relacionados a programação do desenvolvimento e os processos epigenéticos que podem ser modulados ainda no desenvolvimento *in útero* (Rosenfeld 2015). No mesmo sentido, a popularização do conceito de Origem Fetal das Doenças do Adulto (FOAD), Fetal Origins of adults Disease), ampliou ainda mais a hipótese de que os eventos sofridos durante o desenvolvimento têm relação com as diferentes doenças da atualizada, o que ajudou a aumentar os cuidados com os primeiros anos de vida do indivíduo em desenvolvimento e a identificar os possíveis fatores de riscos (Calkins and Devaskar 2011)

Segundo Ho e colaboradores (2017): “*durante o desenvolvimento, a interação dinâmica entre o genoma, epigenoma e fatores ambientais contribui para o destino de células individuais na construção de sistemas orgânicos funcionais em um estado*

adulto "desenvolvido" com tecidos estáveis diferenciados". Nesse sentido, o grau de plasticidade das células e dos órgãos é variável, já que o fenótipo expresso por um genótipo é consequência das interações ambiente-meio, como os fatores ambientais, por exemplo (S.-M. Ho et al., 2017a). Ainda de acordo com os autores, "*os traços adaptativos decorrentes da exposição precoce são geralmente benéficos para a saúde do indivíduo*". Entretanto, há exceções quando um indivíduo pré-adaptado é exposto a um ambiente contraditório (X. Zhang and Ho 2011). Um bom exemplo é a constante introdução de novos produtos químicos e poluentes ambientais, que alteram a fisiologia sistêmica e, por consequência, aumenta o risco para o desenvolvimento de doenças tardias.

Entre os anos de 1950 e 1970, o dietilestilbestrol (DES) foi utilizado como medicamento para prevenir abortos ou fortalecer os bebês em desenvolvimento, principalmente no norte da América e na Europa (Palmlund 1996). DES foi o primeiro sintético estrogéno não-esteroide (xenoestrogeno) que mimetiza a ação de estrogeno no organismo (Monneret 2017). Anos mais tarde, pesquisadores descobriram que a exposição gestactional aos DES estava associada ao aumento das anomalias estruturais do aparelho genital em ambos os sexos, incluindo uma forma rara de câncer, que é o tumor vaginal, aumento da taxa de infertilidade, obesidade, redução da taxa gestacional na prole feminina, e aumento da incidência de anormalidades genitais urológicas em descendentes do sexo masculino (Palmlund 1996; Blunt 2004; Schrager and Potter 2004; Monneret, Knez, and Monneret 2017). Essas descobertas levaram a *United States Food and Drug Administration* e o governo Frances a proibirem a administração de DES em gestantes (Monneret 2017).

Diferentes fatores ambientais perturbam o controle molecular e fisiológico durante o desenvolvimento de um indivíduo. Os EDCs chamam atenção pela onipresença no ambiente e pelo constante aumento da incidência de distúrbios nos seres humanos e nos diferentes animais, incluindo complicações gestacionais, malformações genitais (como acriptorquidia e hipospádia em crianças do sexo masculino), baixo peso ao nascimento, atrasos no desenvolvimento, doenças metabólicas como o diabetes e a obesidade, bem como doenças mais severas como

câncer de mama, ovário, próstata, testicular (Ying 2012; Skinner, Manikkam, and Guerrero-Bosagna 2010a; Rattan et al., 2017).

Conforme descrito anteriormente, durante o desenvolvimento gestacional o aparelho reprodutor é estimulado por diferentes estímulos moleculares como fatores de crescimento, fatores transcricionais e hormônios esteroides, que controlam todo o processo de estabelecimento e diferenciação dos tecidos (S.-M. Ho et al., 2017b; Gail S. Prins and Korach 2008). Neste sentido, a interferência de fatores externos como os DECs, por exemplo, pode estar relacionada a modificações epigenéticas e pós-transcricionais, por levar a metilação no DNA, modificações de histonas, transcrição de RNA não codificantes (lcnRNA, sRNAs), e as demais eventos de remodelação de cromatina (S.-M. Ho et al., 2017a). Essas alterações são resultantes das diferentes interações que o indivíduo estabelece ao longo da vida. No entanto, o efeito da exposição constante aos DECs nestas modificações pode ser determinante para o desenvolvimento de patologias. No contexto DOHaD e FOAD a exposição materna as diversas substâncias que atuam como DECs, pode estar diretamente relacionada com as modificações epigenéticas mencionadas anteriormente, bem como com o aumento nos riscos de doenças da atualidade.

1.4 Desreguladores Endócrinos Químicos e a herança epigenética transgeracional

O feto e o recém-nascido representam populações particularmente vulneráveis à exposição aos EDCs. O desenvolvimento inicial exige um tempo preciso de ação hormonal para promover o crescimento adequado de tecidos e amadurecimento dos órgãos. Como mencionado anteriormente, os EDCs interferem nesta e em outras janelas de vulnerabilidade devido suas atividades anti-androgênica e estrogênica. Além disso, as enzimas envolvidas na biotransformação de xenobiótico e os processos necessários para eliminar esses compostos do organismo não estão totalmente desenvolvidos no feto e no recém-nascido (de Wildt et al., 1999; Choudhary et al., 2003). Portanto, um composto tóxico pode persistir e acumular-se no organismo, atingindo níveis suficientes para causar efeitos adversos sobre órgãos-alvo entre nessas etapas do desenvolvimento.

Embora os mecanismos precisos responsáveis pelos fenótipos induzidos pela exposição aos EDCs sejam desconhecidos, mecanismos epigenéticos têm sido propostos como mediadores da reprogramação do desenvolvimento e subsequente susceptibilidade à doença que pode ocorrer tardiamente, e nesse sentido, estudos envolvendo o proteoma e o transcriptoma podem elucidar mecanismos genéticos e epigenéticos envolvidos nos distúrbios do desenvolvimento e oncogênese, bem como nos mecanismos transgeracionais, associadas principalmente pela transmissão de características adquiridas após condições adversas maternas.

Modificações epigenéticas são definidas como alterações hereditárias na função gênica que ocorrem sem uma mudança na sequência de nucleotídeos (Bird 2007; Goldberg, Allis, and Bernstein 2007; Berger et al., 2009; X. Zhang and Ho 2011). Segundo Singh e Li (Singh and Li 2012a), no contexto da DOHaD, a epigenética pode ser vista como um importante "termômetro" que permite a um organismo ou a um tecido ativar ou desativar programas de transcrição gênica antecipadamente em resposta a estresse ambientais, levando a alterações fenotípicas adaptativas para aumentar a sobrevivência o que suporta as hipóteses DOHaD/FOAD. Os programas de transcrição e tradução são alterados em um contexto funcional e temporal como respostas imediatas e de longo prazo em reposta aos insultos ambientais (Singh and Li 2012a).

Mecanismos epigenéticos incluem metilação do DNA, modificações das histonas (acetilação, metilação, fosforilação, ubiquitinação, sumoilação e ribosilação de ADP) e expressão de RNAs não codificantes (incluindo microRNA, lcnRNA). Em mamíferos, padrões de metilação do DNA são estabelecidos durante a embriogênese através da cooperação de DNA metiltransferases (DNMTs) e proteínas associadas. O período de desenvolvimento precoce é o mais suscetível a insultos epigenéticos, porque a taxa de síntese de DNA é alta, e a padronização da metilação do DNA elaborado e organização da cromatina necessária para o desenvolvimento normal do tecido é estabelecida neste momento (Dolinoy and Jirtle 2008a).

Os mecanismos reguladores epigenéticos não funcionam isoladamente, mas trabalham juntos em uma rede reguladora extremamente complexa. As perturbações em qualquer um dos mecanismos reguladores epigenéticos acima mencionados estão

associadas ao aumento na susceptibilidade do risco de doença (Portela and Esteller 2010) e a desregulação epigenética, que ocorre em células germinativas fornece um mecanismo para a herança epigenética transgeracional de fenótipos anormais.

Químicos ambientais, como o BPA e os ftalatos, podem desempenhar algumas funções críticas na etiologia de doenças humanas (Dolinoy and Jirtle 2008a; Perera and Herbstman 2011a). Várias linhas de evidências de modelos *in vitro* e *in vivo* estabeleceram que as modificações epigenéticas causadas pela exposição *in útero* a tóxicos ambientais podem induzir alterações na expressão gênica que podem persistir ao longo da vida. Assim, as alterações epigenéticas induzidas pelo ambiente tornam-se cada vez mais relevantes para a saúde humana e compreender o agente potencializador para as doenças da atualidade (L. Liu, Li, and Tollefsbol 2008; Choudhuri, Cui, and Klaassen 2010).

Atualmente, a ideia geral de que os EDCs exercem efeitos a longo prazo através da ação epigenética dos miRNAs/RNAs não codificantes está em construção, especialmente no contexto do desenvolvimento do aparelho genital. Em estudos com ratos, a exposição pré-natal ao vinclozolin, por exemplo, levou à repressão de microRNAs como miR-23b e let-7 no dia embrionário (E)13,5 em células germinativas primordiais (Brieño-Enríquez et al., 2015). Essa interferência na expressão de miRNAs foi observada em três gerações, sem alterações proeminentes na metilação do DNA (Brieño-Enríquez et al., 2015). Recente, Santos e colaboradores (2022) mostrou que o status de má nutrição durante a gestação e lactação, é um fator determinante que acentua a probabilidade de desenvolvimento de câncer de próstata nos desentendes por meio da modulação de miRNA (Santos et al., 2022) e alterações no proteoma (Alcantara Santos et al., 2020). Estes estudos destacam a importância desta temática no contexto atual, olhando principalmente para as futuras gerações. Neste contexto, Skinner e Guerrero descrevem: “A herança epigenética transgeracional induzida pelo ambiente é definida como a transmissão germinativa de informações epigenéticas modificadas entre gerações na ausência de exposições diretas continuadas” (Skinner, Manikkam, and Guerrero-Bosagna 2010a).

Os efeitos transgeracionais dos EDCs sobre sistema genital masculino foram descritos primeira vez por *Skinner e colaboradores* em uma série de estudos envolvendo a exposição de ratas prenhes ao vinclozolin (M. D. Anway et al., 2005; Matthew D. Anway, Leathers, and Skinner 2006; Skinner 2014). Anormalidades na próstata, incluindo hiperplasia epitelial, atrofia glandular e prostatite, foram observadas na próstata ventral de ratos e camundongos senis até a geração F3 e F4 derivados de exposição em F0 ao vinclozolin (Matthew D Anway and Skinner 2008). O fenótipo alterado da próstata foi acompanhado por uma reprogramação transgeracional da expressão das vias de sinalização do cálcio e da via de sinalização WNT (Matthew D Anway and Skinner 2008).

Finalmente, eventos de reprogramação epigenética em grande escala ocorrem em dois momentos críticos do desenvolvimento precoce, o primeiro para estabelecer a potência total no zigoto e o segundo ocorre para especificar a linhagem de células germinativas (Saitou, Kagiwada, and Kurimoto 2012). Os EDCs podem prevenir o apagamento, o restabelecimento ou a manutenção adequada das marcas epigenéticas durante estes períodos, alterar o epigenoma celular e subsequentemente aumentar a susceptibilidade pós-natal a doenças. Se as marcas no epigenoma (metilação do DNA ou modificação de histonas) da linhagem das células germinativa forem interrompidas, o resultado pode ser a transmissão de fenótipos adversos por várias gerações (Xin, Susiarjo, and Bartolomei 2015).

Um crescente interesse em pesquisa nas temáticas DOHaD/FOAD é a herança multi- e transgeracional de um fenótipo anormal. Estes dois fenômenos diferem dependendo se a geração afetada teve exposição direta ao estímulo original. Por exemplo, se uma gestante (designada como F0) estiver exposta a um estímulo adverso, o seu filho (designado F1) é afetado como consequência da exposição direta ao mesmo estímulo *in útero* (Figura 3). Além disso, como as células germinativas da geração F1 estão se desenvolvendo ao longo da gestação, os netos (designados F2) também são expostos diretamente. Neste caso, os efeitos observados na geração F2 seriam considerados multigeracionais. Em contraste, os efeitos observados na geração F3 que não tiveram exposição direta ao estímulo original seriam transgeracionais. Quando uma

exposição ocorre através do pai F0, os efeitos transgeracionais são observados na geração F2, já que a única outra geração diretamente exposta ao estímulo original é a futura prole F1, que é exposta enquanto célula germinativa (Fig. 3); (Xin, Susiarjo, and Bartolomei 2015). No entanto, se observar essa última afirmação por uma perspectiva mais atual, é provável que as células da linhagem germinativas da prole masculina exposta também desencadeiem efeitos transgeracionais, principalmente se os questionamentos foram focados nas alterações a níveis genéticos e epigenéticos originários da linhagem germinativa paterna.

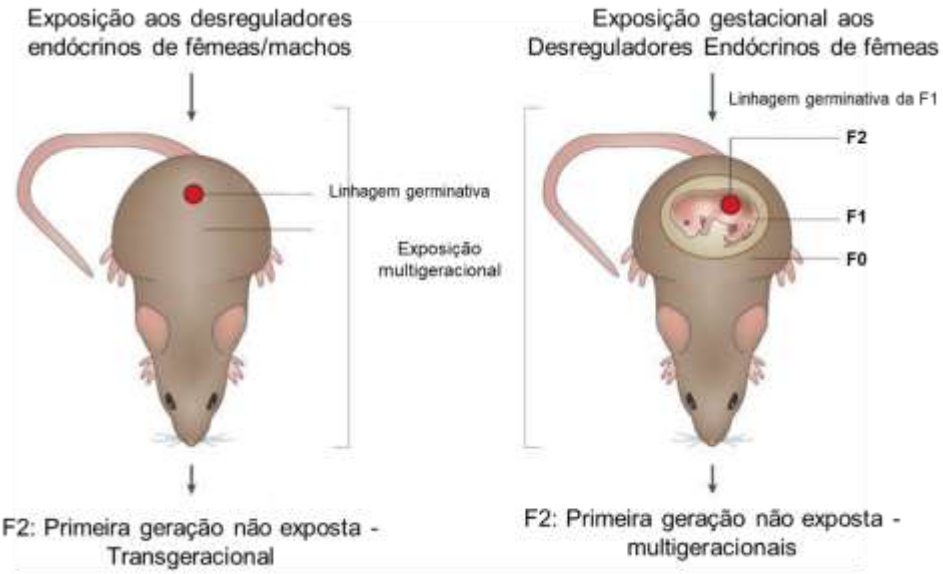

Figura 3: Representação esquemática demonstrando a influência dos desreguladores endócrinos sobre o microambiente uterino e seus efeitos transgeracionais (macho exposto) e multigeracionais (fêmea exposta) (Skinner,2015). Quando a exposição ocorre fora do período gestacional, apenas a linhagem germinativa da mãe é exposta, no entanto, os efeitos podem ser observados na descendência dos filhos (fêmeas). Quando a exposição ocorre durante a gestação, além dos fetos serem expostos, a linhagem germinativa dos fetos também é exposta e, por tanto, a repercussão é observada nas duas gerações (F1 e F2).

Cabe ressaltar que os distúrbios e patologias que acometem a glândula prostática são responsáveis pelos elevados índices de prevalência e mortalidade entre homens no mundo (Siegel et al. 2021; Parkin et al. 2002) e alguns estudos já

relacionam a incidência crescente do CaP com a exposição aos diferentes EDCs onipresentes no ambiente (Gail S. Prins 2008; Diamanti-Kandarakis et al., 2009). Nesse sentido, dados epidemiológicos e experimentais associam a exposição diária aos EDCs, a perturbações no microambiente prostático e ao desenvolvimento do CaP, em indivíduos, expostos durante a gestação ou quando adultos são necessários (Roh et al., 2017). Nesta perspectiva, estudos moleculares mais aprofundados ajudam a esclarecer os reais potenciais riscos destes compostos sobre a reprodução e, principalmente, sobre a oncogênese.

1.4 Os ftalatos, a herança epigenética e a oncogênese prostática

Os ftalatos são uma família de diésteres de ácido ftálico tipicamente utilizados como plastificantes para amaciar e aumentar a flexibilidade dos produtos de plástico de cloreto de polivinilo (Bošnir et al., 2003; Yu Wang, Zhu, and Kannan 2019b; Araki et al., 2017). Uma vez que estes compostos não são ligados covalentemente aos produtos em que são empregados, eles podem lixiviar e contaminar alimentos processados ou armazenados em produtos de plástico e outros produtos aos quais são empregados (Bosnir et al., 2003). Como desreguladores endócrinos, os ftalatos têm sido especialmente estudados por sua atividade antiandrogênico (Fisher 2004).

Após o contato com ftalatos por ingestão, contato ou inalação, os diésteres de ftalatos são rapidamente metabolizados no organismo para as formas mais tóxicas que são os monoésteres (Frederiksen, Skakkebæk, and Andersson 2007b; Schettler et al., 2006b). Brevemente, há duas principais etapas de metabolização do composto: a primeira é a via de ação catalítica das esterases e ligases presente no trato digestório, que hidrolisam os diésteres de ftalatos em metabólitos primários de monoésteres de ftalatos (Fase I da biotransformação) (Fig. 4); a segunda é a principal forma de metabolização e, ocorre no fígado. Após a biotransformação, os monoésteres de ftalatos sofrem hydroxilação ou oxidação, e em seguida são facilmente catalisados pela enzima *uridine 59-diphis-2* para formar o conjugado glucuronídeo hidrofílico que é facilmente excretado pela urina (Fig. 3) (Frederiksen, Skakkebæk, and Andersson 2007a; 2007b).

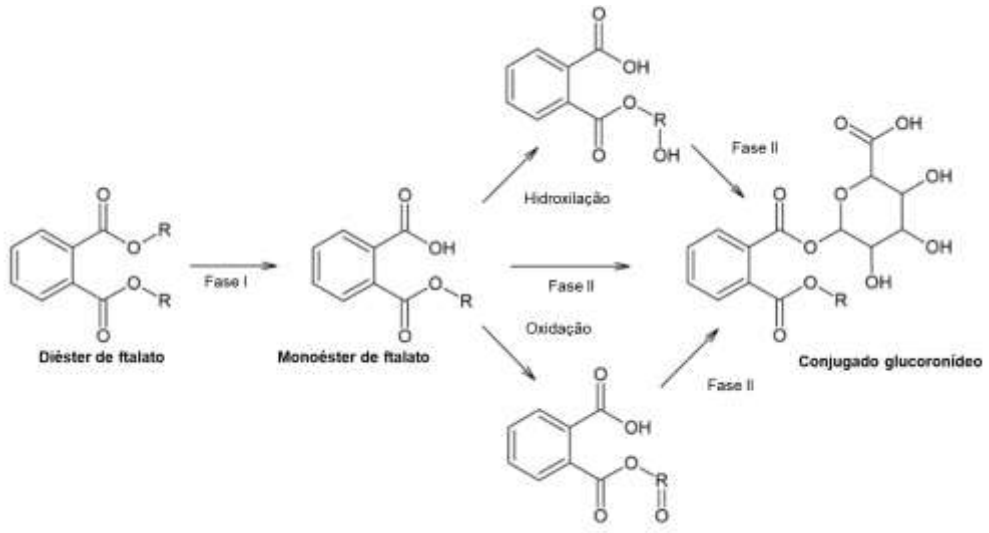

Figura 4: Representação esquemática demostrando o processo de biotransformação dos diésteres de ftalato no organismo. **Fase 1:** Após contato com os diésteres de ftalatos pelas vias dérmicas, inalação ou ingestão, a molécula entra no organismo é rapidamente é metabolizada para a forma de monoésteres. O processo de biotransformação pode ocorrer durante a passagem da molécula pelo organismo ou no fígado, principal via de metabolização. **Fase 2:** Após biotransformação do monoéster pode ocorrer de três formas, pela hidroxilação, oxidação ou ligação direta da molécula ao conjugado gluco-cronídeo hidrofílico, que pode ser eliminado na urina. Apesar do conjugado ser facilmente excretado, as moléculas de monoésteres de ftalatos podem ter uma metabolização lenta a depender da idade ou permanecer no organismo por mais tempo, o que acaba prejudicando e contaminando outros tecidos. Imagem adaptada de (Frederiksen, Skakkebæk, and Andersson 2007a).

Semelhante ao BPA, as investigações avaliando o papel da exposição ao ftalato concentraram-se principalmente nas alterações nos parâmetros reprodutivos, metabólicos e neurológicos. A exposição pré-natal a ftalatos tem sido associada à síndrome da disgenesia testicular (TDS) em roedores e humanos. A TDS engloba um grupo de distúrbios reprodutivos com possíveis causas ambientais subjacentes, incluindo malformações genitais, diminuição da produção hormonal e predisposição ao crescimento hiperplásico (Skakkebæk, Rajpert-De Meyts, and Main 2001).

Os impactos da exposição aos ftalatos na saúde humana também foram extensivamente revistos e relatados pelo Programa Nacional de Toxicologia - Centro

para a Avaliação de Riscos a Reprodução Humana dos Estados Unidos (Shelby 2006). Há provas suficientes em roedores que a exposição aos ftalatos provoca toxicidades no desenvolvimento e na reprodução. Nos seres humanos, os distúrbios dismórficos do trato genital, observados em lactentes do sexo masculino, foram significativamente associados à exposição pré-natal a ftalatos (Shanna H. Swan et al., 2005). Em modelos animais, a distância anogenital é um índice sensível de desmasculinização do trato reprodutivo masculino. Múltiplos estudos epidemiológicos, incluindo estudos de coorte prospectivos, mostraram uma distância anogenital encurtada (sugerindo influência anti-androgênica) em meninos cujas mães apresentaram níveis mais elevados de ftalato urinário durante a gravidez (Huang et al., 2009; S. H. Swan et al., 2015).

Entre os anos de 1999-2000, o National Health and Nutrition Examination Survey examinou amostras de urina de 2540 indivíduos, o resultado desta investigação apontou a presença de metabólitos de ftalatos nas amostras, o que comprovou a contaminação humana pela exposição diária (Silva et al., 2004) . O mapeamento mostrou que o mono-etil-ftalato esteve presente em todas as amostras, o mono-bultil-ftalato em 99% das amostras e o ftalato de mono etilhexilo (MEHP) em 78% das amostras. A mediana de exposição em adultos foi estimada em 8,2 µg/kg/dia e em crianças de 25,8 µg/kg/dia (Shelby 2006). Verificou-se que as mulheres tinham níveis de exposição duas a quatro vezes mais elevados do que os homens (Silva et al., 2004). Crianças têm uma exposição significativamente maior do que adolescentes (Koch and Calafat 2009). Crianças em torno de 1 ano de idade foram consideradas um grupo de alto risco de exposição pelos padrões comportamentais de alimentação e mastigação e por rastejarem em ambientes inalando poeira contaminada (Shelby 2006).

O efeito transgeracional do Bis 2-ethilhexil phthalate (DEHP)e de outros ftalatos em seres humanos ainda não está esclarecido, mas há evidências que ligam os níveis de DEHP e seus metabólitos aos distúrbios reprodutivos em indivíduos diretamente expostos e em seus descendentes (Hauser et al., 2007; Shanna H. Swan et al., 2005). Nesta perspectiva, estudos moleculares mais aprofundados com DEHP e outros diésteres de ftalatos são necessários para entender melhor a interação entre os ftalatos

com o genoma-epigenoma e estabelecer quais os riscos potenciais destes compostos sobre a reprodução e a oncogênese.

O BPA e os cinco ftalatos mais conhecidos (DEHP / MEHP e DBP / BBP / MBP) foram descritos no *Toxicogenomics Database* (Davis et al., 2009) por terem 1232 e 265 interações únicas com gene e proteínas, respectivamente (Singh and Li 2012a; 2011a). Cinco das dez principais redes comuns afetadas pelos ftalatos e pelo BPA estão envolvidas com inflamação (Dietert et al., 2010); e seis delas são preditas pelo *GeneGo* por estarem envolvidas em neoplasias urogenitais, incluindo a neoplasia prostática, a de genitais masculino e feminino, as endometriais e de mama (Singh and Li 2012a).

Estudos experimentais têm associado a exposição fetal e neonatal a EDCs, como o BPA e os ftalatos, a um aumento da susceptibilidade à carcinogênese prostática, da incidência e a gravidade de lesões prostáticas (Wellerson Rodrigo Scarano et al., 2009; Peixoto et al., 2016; X. Wang et al., 2017a). Nesse sentido, a preocupação é crescente em todo o mundo acerca do câncer de próstata, pois trata-se da segunda principal causa de morte por câncer em homens no mundo (Siegel et al., 2021).

1.5 Morfogênese prostática e câncer de próstata

Em humanos, a morfogênese da próstata ocorre a partir do segundo e terceiro trimestre até o momento do nascimento. Em roedores, a morfogênese se inicia na vida fetal tardia e se estende pelo período pós-natal até a pré-puberdade (Gail S. Prins and Putz 2008; Vilamaior, Taboga, and Carvalho 2006). Embora a manifestação do processo carcinogênico na próstata ocorra principalmente em homens mais velhos, a Neoplasia Intraepitelial Prostática (PIN) ou Neoplasia Intraepitelial Prostática de alto grau (HPIN) foram identificadas em autópsias de homens entre 20-30 anos (Saffarini et al., 2015), demonstrando que a carcinogênese da próstata pode iniciar mais cedo. Assim, tem sido proposto que o ambiente hipoandrogênico somado ao excesso do hormônio estrogênico durante o desenvolvimento prostático pode contribuir para a alta

incidência de PIN e carcinoma prostático na população de idosos do sexo masculino (Santti et al., 1994).

A maioria dos estudos que avaliaram modificações epigenéticas após exposições a ftalatos tem como foco a metilação do DNA. Estudos epidemiológicos humanos correlacionaram níveis urinários mais altos de metabólitos de ftalato com genes controlados por *imprinting* como o IGF2/H19 (lcnRNA) alterados e metilação de LINE-1 no tecido placentário (Y. Zhao et al., 2014; LaRocca et al., 2014). Estudos em roedores descrevem alteração da metilação do DNA global e locus-específico após a exposição ao DEHP (Kostka *et al.,* 2010; Martinez-Arguelles *et al.,* 2015).

Estudos *in vitro* recentes revelam informações adicionais sobre mecanismos potenciais de alterações epigenéticas induzidas por ftalato. O tratamento de células de cancro da mama humana MCF-7 com Benzil-Butil-Ftalato (BBP) ou ftalato de di-butilo (DBP) resultou na metilação de DNA no promotor do receptor de estrógeno (ER) (Kang & Lee, 2005), levando a uma ligação entre fatores de transcrição ativados por ligantes, ocasionando aumento do potencial tumoral das células. Recentemente, nosso grupo de pesquisa mostrou que a exposição a um conjunto de monoésteres de ftalatos interfere na expressão de miRNAs (miR-141-3p e miR-184) em células prostáticas normais e tumorais, o que afeta diretamente a expressão génica e acentua as alterações em vias de proliferação, morte e dano de DNA (Cavalca et al., 2022).

Um estudo realizado em mulheres grávidas, determinou a concentração dos 6 principais metabólitos de ftalatos na urina: DEHP (Bis(2-ethylhexyl) phthalate), DEP (Diethyl phthalate), DBP (Di-n-butil phthalate), DiBP (Diisobutyl phthalate), BBzP (Butylbenzyl phthalate) e DiNP (Diisononyl phthalate), apontando para as proporções dos diferentes ftalatos aos quais os fetos estão indiretamente expostos durante a gestação (Zhou & Flaws, 2017). Um estudo epidemiológico mais recente reforça estes dados e mostra a proporção de diferentes metabólitos de ftalatos (DEHP, DBP, DiBP, BBzP, DiNP and DPHP) na urina, no sangue e em outros fluídos corporais de humanos e demais animais nos diferentes continentes (Y. J. Zhang et al., 2021). A maioria dos estudos toxicológicos que investigam ftalatos exploram a exposição isolada do ftalato, no entanto, há um concesso de que os seres humanos e demais animais são expostos

diariamente a misturas de diferentes ftalatos (Ferguson *et al.,*2014; Meeker & Ferguson, 2014; Watkins *et al.,*2014).

Estudos que descrevem os efeitos epigenéticos transgeracionais e multigeracionais dos ftalatos são limitados, e esse número é menor ainda quando se refere a alterações pós-transcricionais por miRNAs, transcriptoma, proteoma e oncogênese. Assim, independente do mecanismo epigenético associado, a alteração no perfil da expressão de proteínas segue como elemento condutor na avaliação dos efeitos tóxicos transgeracionais e multigeracionais. Ainda, estudos com foco transgeracionais, buscam avaliar a influência materna na transmissão das alterações especialmente na prole feminina, após a exposição a formas isoladas dos ftalatos e outros DECs (BPA, microplásticos, Poluentes ambientais e outros). Neste estudo, além de mimetizar uma exposição gestacional (F0) aos principais ftalatos normalmente presentes no ambiente, também foi possível investigar a transmissão das alterações transmitidas para a descendência (F1 e F2), sendo que para a geração F2, pais e mães expostos (F1) foram analisados. A hipótese inicial de que descendentes expostos durante a gestação e lactação pudessem ter o desenvolvimento prostático alterado e desenvolvessem alterações histopatológicas e moleculares durante o envelhecimento foi confirmada, reforçando a teoria de Gardner (1995) que postulou que o câncer de próstata poderia ter origem na vida pré-natal a partir de hipótese DOHaD (Gardner 1995).

As próximas páginas desta tese estão organizadas em capítulos e, para facilitar a compreensão do delineamento experimental, será apresentado uma ilustração gráfica em cada um dos capítulos.

Breve descrição destacando o conteúdo apresentado nesta tese

Os ftalatos correspondem a um grupo de plastificantes comercialmente utilizados em diferentes meios de produção com maior ou menor risco de exposição a depender do material ao qual estão empregados. Por outro lado, os ftalatos são facilmente liberadas

para o meio ambiente, porque não formam ligação covalente. Os estudos que avaliam exposição precoce aos ftalatos, são focados na exposição isolada ou associada a outras diferentes substâncias que atuam como Desreguladores Endócrinos (DEs). Este estudo buscou investigar o efeito da exposição a um conjunto de ftalatos para mimetizar a exposição ambiental e avaliar o efeito da exposição. Além disso, estudos recentes apontam que metabólitos de ftalatos são detectados em amostras de urina de mulheres em idade reprodutiva, gestantes e crianças nas diferentes fases do desenvolvimento, o que comprova a exposição precoce a um conjunto de ftalatos. Paralelamente, estudos avaliando as janelas de susceptibilidade apontam que a exposição aos ftalatos está associada as desordens reprodutivas masculina e feminina, bem como as doenças do adulto e do envelhecimento. Na próstata, o efeito está associado a prejuízos na morfofisiologia da glândula, aumento de stress e inflamação, criado por um microambiente reativo e, maior vulnerabilidade do tecido para alterações relacionadas as patologias prostáticas, que acometem homens em idade reprodutivas e homens senis. Neste sentido, este estudo buscou investigar a influência da exposição materna em períodos cruciais para o desenvolvimento e amadurecimento da glândula prostática, na modulação de mecanismos pós-transcricionais (miRNAs and mRNAs), na atividade funcional do tecido (proteoma), bem como na contribuição destas modulações para a origem do câncer de próstata. Ainda, está investigação focou na transmissão das alterações possivelmente transmitidas para as próximas gerações, buscando estabelecer se a repercussão tardia da exposição é observada na descendência (F1) e na geração (F2). Para atingir tal objetivo, ratas prenhes da linhagem Sprague Dawley foram distribuídas em 4 grupos experimentais: Grupo controle (controle; veículo); 20µg/kg/dia, 200µg/kg/dia e 200mg/kg/dia que receberam o tratamento com a mistura de ftalatos do dia gestacional 10 (DG10) ao dia pós-natal 21 (DPN21), via gavagem. O grupo exposto 200µg/kg/dia foi avaliado somente para obter o perfil de expressão gênica, para as demais análises e comparação, o grupo foi desconsiderado. Ao final do delineamento experimental, os filhotes foram analisados em 4 checkpoint: no DPN22(F1) para analisar as consequências da exposição materna direta; no DPN120 para avaliar as repercussões tardias; no DPN540 para analisar as consequências da

exposição na vida senil, para as análises histopatológicas, e no DPN22, para investigar o efeito transgeracional nos descendentes de pais expostos (F2). A próstata ventral foi coletada ao final de cada checkpoint, para as análises moleculares.

Os resultados obtidos nesta investigação estão apresentados no formato de capítulo no decorrer da tese. Cabe ressaltar que dois artigos apresentados nesta tese estao publicados em periódicos científicos da área.

Brevemente, o primeiro capítulo desta tese faz uma apresentação introdutória geral das principais problemáticas que envolvem este estudo. Os demais capítulos são apresentados no formado de artigo e anexos. O segundo capítulo apresenta os dados do artigo científico denominado "*In silico investigation of the potential interference of plasticizers in the origin of prostate cancer in offspring after maternal exposed by modulation of miRNA expression*", este mostra que o tratamento aumento a expressão do rno-miR-141-3p em todos os grupos de tratamento no DPN22 (F1), no entanto, esta desregulação se mantém alterada apenas no grupo exposto a 20µg/kg/dia nos animais do DPN120 (F1) e, não houve alterações significativas na geração F2. Por outro lado, a expressão do rno-miR-184 se mantém aumentada nos grupos de tratamento 20µg/kg/dia e 200mg/kg/dia no DPN22 (F1), se mantém no grupo exposto a 20µg/kg/dia, no DPN120 (F1) e nos filhos de pai exposto no DPN22 (F2). Os bancos de dados MirWalk e MirNet foram usados para fazer uma análise de predição dos alvos (mRNA) dos miRNAs rno-184 e rno-141-3p. Foram identificados 510 alvos preditos (mRNA) que podem ser comumente regulados pelos dois microRNA. Na sequência uma análise de enriquecimento foi realizada e o resultado mostrou que ao menos 28 destes alvos atuam em vias de síntese e secreção hormonal ou com o desenvolvimento do sistema reprodutor. Esses alvos mostram uma rede de interação interessante, o que aponta uma possível interferência do tratamento na modulação de eventos pós-transcricionais. Além disto, esses alvos estão envolvidos em vias como regulação de processos apoptóticos, respostas hormonais, desenvolvimento do sistema urogenital, tradução de sinal, entre outros. Por fim, foi possível identificar a relação dos alvos com diferentes metabolitos de ftalatos e doenças associadas a condições patológicas do sistema reprodutor masculino e com o câncer. O banco de dados *The Human Protein*

Atlas foi usado para identificar a expressão de 15 alvos no adenocarcinoma prostático e os alvos Wnt, Stat3, TP53 and p63 foram validados por WB no DPN22 e DPN120 (F1), nos diferentes grupos experimentais grupo controle; 20µg/kg/dia, 200µg/kg/dia e 200mg/kg/dia da mistura de ftalato. Os resultados apontam que o tratamento desencadeia alterações tardias na glândula prostática, bem como pode interferir em eventos pós-transcricionais pela modulação na expressão dos miRNAs, as quais podem elevar os riscos para uma condição patológica na vida adulta e no envelhecimento.

O terceiro capítulo apresenta os dados do artigo científico denominado "*Integrated transcriptome and proteome analysis indicates potential biomarkers of prostate cancer in offspring of pregnant rats exposed to a phthalate mixture during gestation and lactation*" (https://doi.org/10.1016/j.chemosphere.2023.140020). Neste artigo, os dados publicados sobre o perfil de transcritos (transcriptoma) dos animais expostos ao mesmo protocolo experimental foram cruzados com os dados obtidos no perfil proteômico a fim de identificar potenciais alvos comumente desregulados nas duas análises no DPN22 e comparar se as alterações permanecem alteradas no DPN120 (F1), nos grupos experimentais ctrl: controle; veículo; 20µg/kg/dia e 200mg/kg/dia. Os resultados apontam que há 8 targets (gene/proteínas) comumente desregulados nas duas análises, nas duas idades e nos dois grupos de tratamento em relação ao grupo controle. A análise de enriquecimento usado o banco de dado KOBAS 3.0 demonstra que os alvos estão associados com vias de modificações post-transcricionais, processamento de proteínas, homeostasia, HSP90 chaperonas, gap-junções e proteínas quinases. Todas essas vias são importantes para manter a interação célula-célula, essencial para a morfofisiologia da glândula prostática, comunicação intracelular, bem como diferenciação tecidual ou até mesmo para mediar as diferentes respostas celular aos agentes estressores externos. Após estabelecer a natureza da interação destes alvos, usando a plataforma String, os dados foram cruzados com dados disponíveis na plataforma K2kWeb para estabelecer a interação destes alvos com proteínas quinases e fatores transcricionais. Após, uma nova análise de enriquecimento foi gerada para entender a possíveis consequências das interações mRNA - proteínas quinases - fatores transcricionais, associado as desordens que

podem acometer a glândula prostática. Por fim, os bancos de dados "*The human protein Atlas*" - HPA e "*The Cancer Genômic Atlas"* - TCGA) oram usados para verificar dos desregulados identificados nesta investigação com o adenocarcinoma prostático em humanos.

O quarto capítulo desta tese é intitulado "Prenatal exposure to a phthalate mixture alters prostatic proteome profile in F1 and F2 generations of rats", neste artigo os dados foram analisados considerando as proteínas comumente desreguladas nos grupos de tratamento 20µg/kg/dia and 200mg/kg/dia e nas diferentes idades, ou seja, a análise não considera a dose dependência dos grupos de tratamento, nem as fases do desenvolvimento (DPN22 e DPN120 (F1)) e DPN22 (F2).

Os dados apontam que a mistura down-regula um número significativo de proteínas em todas as idades. Quando os dados foram analisados em conjunto, observou-se que 54 proteínas estão desreguladas nos dois grupos de tratamento e se mantiveram desreguladas em todas as idades. Há uma forte rede de interação entre essas proteínas, que evidência ao menos 5 clusters de proteínas da mesma família como é o caso das Chaperonas, histonas e proteínas Rabs ou de proteínas que desempenham funções conjuntas, caso das Tubulinas e Enolases. Para entender melhor a influência destas proteínas dentro de uma função biológica mais complexa, os dados foram analisados por cluster. Os resultados apontam que o tratamento está influenciando em vias envolvidas com divisão celular, cascata de sinalização para metabolização de xenobiótico pelas enzimas do citocromo P450, hipoxia, vias hormonais, bem como em vias de sinalização que podem prejudicar o desenvolvimento da glândula prostática e aumentar os riscos para condições patológicas. Além disso, foi possível estabelecer um conjunto de proteínas alteradas entre os descendentes de pais expostos, o que permitiu identificar um conjunto de alterações que podem estar relacionadas ao sexo do pai exposto e são transpassadas de geração para geração. Ainda, os resultados apontam que há forte interação entre as proteínas desreguladas entre os filhos de pai exposto do que entre os filhos de mãe exposta. Por outro lado, há uma interessante interação entre as proteínas que permanecem desreguladas independentes dos sexos dos pais expostos, o que leva a hipótese de que a modulação

do tratamento pode ter relação com o tipo de tecido analisado e com as alterações que são mantidas quando as gerações são analisadas.

O quinto capítulo desta tese apresenta os resultados preliminares do projeto de pesquisa intitulado “Investigative analysis of the relationship between development of prostate cancer with modifications in the proteome of senile rats exposed to a plasticizer during gestational and lactational development”. O foco desta investigação foi identificar as principais vias moleculares alteradas no proteoma dos filhos senis de mãe exposta a mistura de ftalatos no período gestacional e lactacional, e relacionadas as características histopatológicas que foram diagnosticadas nestes animais. Além disso, com o intuído de tentar estabelecer os processos iniciais de respostas celular frente a exposição aos ftalatos e as consequência desta interferência, o modelo *in vitro* também foi avaliado nesta investigação. Diferente do parâmetro adotado para os resultados obtidos no proteoma dos animais jovens (DPN22) e adultos (DPN120) e de os resultados obtidos para os animais senis (DPN540), aqui, optou-se por analisar o efeito do tratamento dose dependente, porque houve variação na natureza dos dados de acordo com o grupo experimental em relação ao grupo controle e porque os animais senis já apresentavam condições patológicas diagnosticadas na histopatologia. Os resultados mostram que os animais expostos a maior dose do tratamento (200mg/kg/dia), os quais também apresentaram lesões histopatológicas mais acentuadas, tem um perfil proteômico que apresenta alterações interessantes relacionadas ao estresse de reticulo endoplasmático, o que pode estar diretamente relacionado a uma deficiência na resposta biológicas a condição patológica estabelecida, e alteração na matriz extracelular, extremamente importante para manter o controle da homeostase tecidual, bem como a sustentação do tecido. Por outro lado, os animais do grupo exposto a menor dose de tratamento apontam um quadro de estresse intenso, o que resulta na ineficiência de vias como migração e diferenciação de células epiteliais e vias hormonais e de resposta ao estresse.

No modelo *in vitro* o perfil proteômico muda principalmente porque as células foram expostas a mistura dos monoésteres de ftalatos e porque as células prostáticas são tumorais (DU145). Assim como no modelo *in vivo* há muitas proteínas desreguladas

relacionadas a vias metabólicas, o que reforça a necessidade de entender a interferência dos ftalatos no metabolismo e as consequências principalmente para doenças metabólicas, especialmente no microambiente tumoral. Outro resultado importante e, pouco explorado na literatura, foi a relação dos ftalatos com o processo autofágico, o que pode ajudar a elucidar as interferências já descritas sobre a influência dos ftalatos isolados ou em combinação em vias de regulação, estresse e comunicação celular.

Os anexos desta tese estão organizados da seguinte forma: o primeiro anexo é referente ao protocolo do comitê de ética aprovado para o desenvolvimento deste estudo. O segundo anexo é referente ao artigo publicado em colaboração com a parceria internacional do grupo de pesquisa, o qual compartilho a primeira autoria do trabalho. Além disso, este artigo é referente as alterações identificadas nas fêmeas expostas ao mesmo delineamento experimental apresentado nesta tese. O anexo III apresenta as atividades complementares que foram desenvolvidas durante o período do doutorado.

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Capítulo II

Artigo científico

Lista de figuras – Capítulo II

Lista de tabelas – Capítulo II

Os dados deste capítulo estão submetidos na revista cientifica "*Toxicological Science*", com fator de impacto de 4.109, portanto, o manuscrito está apresentado no padrão da revista.

Delineamento e metodologia – Capítulo II

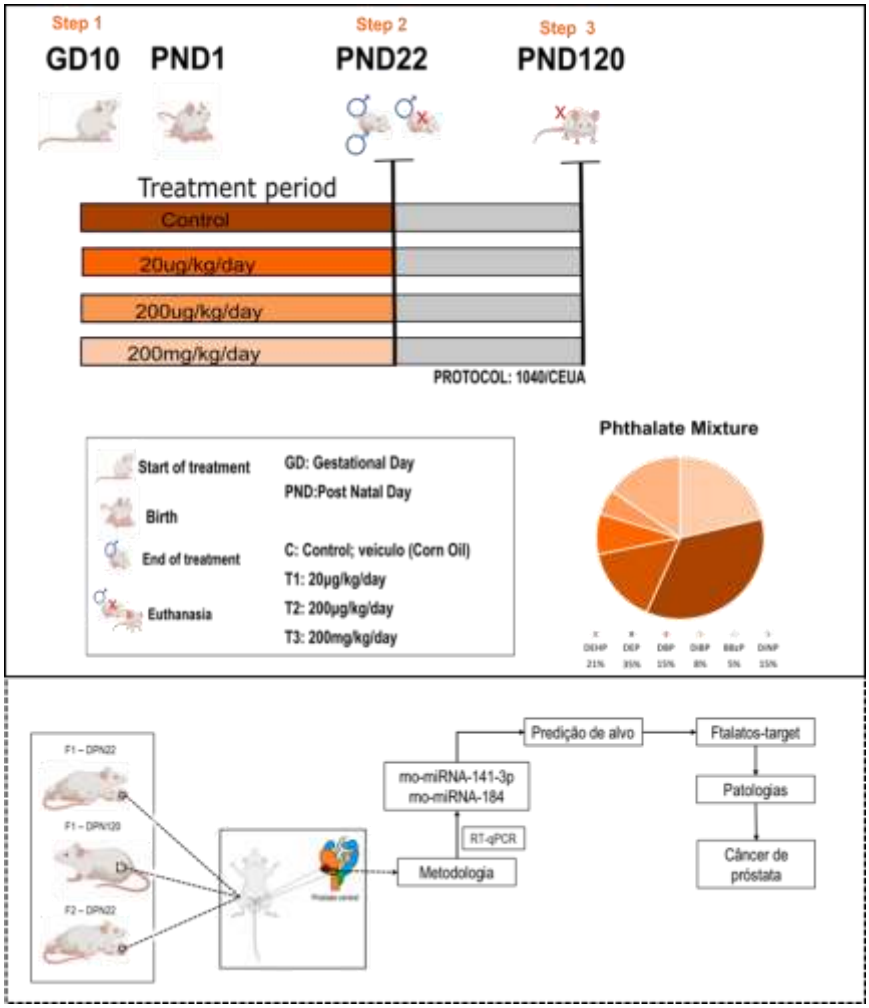

Figura representativa do delineamento experimental e da metodologia utilizada neste capítulo. Detalhes mais específicos deste delineamento experimental serão apresentados na metodologia de cada capítulo.

In silico investigation of the role of miRNAs in a possible developmental origin of prostate cancer in F1 and F2 offspring of mothers exposed to a phthalate mixture

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Abstract

Phthalates are plasticizers that are detected at different concentrations in breast milk, urine of pregnant women, and maternal fetal tissue samples from humans and rodents. Previous studies revealed that chronic and early exposure to single phthalate compounds increased the susceptibility to prostatic disorders development in adulthood. Although the mechanisms underlying the developmental effects of phthalates on adult diseases are unknown, it is possible that epigenetic changes are responsible for late repercussions in life, increasing reproductive disorders and cancer incidence. A previous study from our group showed that the exposure to a phthalate mixture during gestation and lactation (F0) deregulates rno-miR-184 and rno-miR-141-3p in the prostate of F1 male rats. Thus, this investigation aimed to validate the expression of previously investigated miRNAs and enable the prediction of targets related to prostate development and oncogenesis through in silico analyses. Prostate samples from the F1 and F2 generations of male exposed to the phthalate mixture were used to perform miRNAs expression (qRT-PCR) and western blot (WB) to validate specific targets. Up-regulation of rno-miR-184 and rno-miR-141-3p was observed by qRT-PCR in the offspring of mothers exposed to the phthalate mixture (21% DEHP, 35% DEP, 15% DBP, 8% DiBP, 5% BBzP, and 15% DiNP). Target prediction revealed 40 common targets for both miRNAs, 28 of them were enriched for biological processes such as estrous cycle, reproductive process, reproductive structure, developmental process, and response to estradiol. Furthermore, a phthalate-target interaction analysis showed that some of the targets were associated with different male disorders including prostate diseases. The targets NOTCH2, NCOA1, RET, MED1, ATM, JAG1, NOTCH1, STAT3, KEAP1, TP53, ETS1, LRP6, SP1, ERBB2 and PPARD were correlated with prostate adenocarcinoma. Further, enrichment analyses

pointed to an association among the possible deregulated targets with tumor progression, initiation and angiogenesis, carcinogenesis, prostate adenocarcinoma, prostatic and neoplasms. WB showed deregulation of targets associated with cell cycle regulation and prostate differentiation in both generations of exposed animals. In conclusion, this investigation reinforces the relationship of phthalates with the modulation of epigenetic mechanisms and tumor suppressor targets through maternal exposure, leading to increased susceptibility to oncogenesis in the F2 generation.

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key words: Phthalate mixture, transgenerational effects, epigenetic modifications, and miRNA expression

Abbreviation: PCa, Prostate Cancer; EDCs, Endocrine Disrupting Chemicals; DOHaD, Developmental Origin of Health and Disease; GD, Gestational day; no-coding RNA molecules (sncRNAs), PND, Post-Natal day; DEHP, , DEP, Biethyl phthalate; DBP Di-n-butyl phthalate, DiBP, Diisobutyl phthalate; BBzP, Butyl benzyl phthalate; DiNP, Diisononyl phthalate, BPA, Bisphenol A, MEHP mono (2-ethylhexyl) phthalate, VP, Ventral prostate, IPP Protein-Protein Interaction network (IPP), HPA, The Human Protein Atlas, CTB, Comparative Toxicogenomic database, WB, Western Blotting.

Introduction

Phthalates are a plasticizers that impair reproductive quality by interfering with the synthesis and secretion of endogenous hormones [1]. Phthalates are used in the production of many types of plastics and therefore, they are easily found in the environment at different concentration levels [2]. An interesting feature of phthalates is their inability to tightly bind to the material to which they are used, and this characteristic leads to them being easily released from the different products [3]. Epidemiological studies have shown that phthalate metabolites are present in maternal and fetal samples such as the placenta, blood, urine, breast milk, and umbilical cord [2,4,5].

The effects of phthalates exposure on reproductive health include dysregulation of hormone levels, decreased anogenital distance, and interference in the quality of germ cells and fertility parameters in both sexes [6–8]. Repoususkou et al. (2019) showed that gestational exposure (GE) to a four-phthalate mixture increased the incidence of aberrations in gonads and harmed steroidogenesis in male and female offspring in pre-puberty and adulthood. In addition, the same investigation pointed that the interference can be

toughest in male offspring [9]. Moreover, pre- and postnatal exposure to phthalates has been identified as a strong interference in the development of the genital system [8,10]. Previously, our group showed that phthalate mixture exposure during gestation and lactation caused dysfunction in ovarian folliculogenesis and steroidogenesis in F1 and F2 females [11]; and increased the breast cancer susceptibility in adult female rats after a chemical-induced carcinogenesis protocol [12]. In male offspring, in addition to altering phenotypic parameters, phthalate mixture exposure interfered with epigenetic mechanisms in the prostate [13]. Data from this study showed influence of phthalate mixture exposure on the rno-miR-184 and rno-miR-141-3p expression [13]. Other investigations have demonstrated that prenatal phthalate exposure can deregulate biomarkers of reproductive aging [14] and prostate oncogenic process in offspring [13,15].

Epigenetics has an important function in gene expression and transcriptome profile in all tissue types [16]. Furthermore, epigenetic alteration is a great way to measure environmental chemical exposure under the quality of health and origin of diseases [17]. MicroRNAs are small sequences (~22 nucleotides) of non-coding RNA molecules (sncRNAs), which control the expression of mRNA and in a transient and permanent manner [18,19]. Both phthalate and BPA exposure have been shown to influence epigenetic mechanisms, and thus, are considered as epigenetically toxic [16]. Interestingly, exposure to a mixture of BPA, DEHP and DBP impacted epigenetic programming up to the F3 generation [20].

In a detailed review, Vrijen and colleagues (2015) showed that environmental exposure can be monitored by microRNA signature [21]. The mir-184 is thought to be a tumor suppressor and its up-regulation is able to inhibit migration and invasion of prostate cancer cells by decreasing DLX1 [22], which stimulates cancer cell viability and migration through β-catenin activation [22,23]. Furthermore, DLX1 can be involved in tumor initiation and in the epithelial-mesenchymal transition in prostate cancer cells [23]. The upregulation of rno-miR-184 has been pointed to as a great biomarker of benign prostatic hyperplasia in rats [24]. In humans, hsa-miR-184 expression was identified in prostate cancer with high aggressivity [25]. On another hand, the downregulation of rno-miR-141-3p has been related to increased apoptosis in intestinal epithelial cells and incremental changes in USP5 expression and β-catenin signaling in embryos [26]. According to Fang et al. (2021), miR-141-3p is involved in inflammatory diseases [27] and rno-miR-141-3p expression is associated with downregulation of targets as TNF-α, IL-1β, and IL-6. Moreover, miR-141-3p expression in plasma from of patients with castration resistant prostate cancer may be a biomarker for treatment [28].

Our previous results demonstrated that a mixture of phthalates altered the miRNAome, particularly rno-miR-184 and rno-miR-141-3p in the offspring (Scarano et al., 2029; Gonsioroski et al., 2022. Here, the investigation was focused on identifying the potential targets for both miRNAs as well as the possible consequences for F1 and F2 offspring. Additionally, some protein targets were validated in descendants from both generations.

Material and Methods

Animals

Male and female Sprague-Dawley rats were supplied by Multidisciplinary Center for Biological Research (CEMIB/UNICAMP), and they were kept during the experimental period in the biotherium of Small Mammals at Department of Structural and Functional Biology – Morphology Sector, Institute of Biosciences - São Paulo State University (UNESP). The rats were kept under controlled temperature (23 ± 2°C) and regulated humidity conditions, with periods of 12 hours light/dark cycles and food and water *ad libitum*. This study was carried out in accordance with the Brazilian Council on Animal Experimentation Control (CONCEA) and the protocol was approved by the Ethical Committee on Animal Use of the Institute of Biosciences, São Paulo State University (Protocol: 1040/CEUA).

Experimental Design

The experimental design was the same as described by Scarano and collaborators (2019). Briefly, pregnant female rats (F0 generation) were exposed daily to a phthalate mixture (oral route). Animals were divided in four groups: Control group (exposed to vehicle; corn oil); 20µg/kg/day; 200µg/kg/day; and 200 mg/kg/day of phthalate mixture. The phthalate mixture was diluted in tocopherol-stripped corn oil (vehicle control). Animals from the 200 µg/kg/day, 200 µg/kg/day, and 200 mg/kg/day groups received the respective dose of the mixture in the proportion: 21% DEHP, 35% DEP, 15% DBP, 8% DiBP, 5% BBzP, and 15% DiNP (Zhou, Gao, and Flaws 2017a; Scarano et al. 2019). The lowest two doses were selected to mimic daily human exposure levels based on the amount of DEHP, and the higher dose was selected to compare our results with available single phthalate studies [30]. To ensure the establishment of normal reproductive tissues for the prediction of embryological development, the pregnant rats received treatment from gestational day (GD) 10 to postnatal day (PND) 21. At PND22 and PND120, F1 male rats (n=4-

6/group) were euthanized by anesthetic saturation (sodic pentobarbital) followed by cardiac puncture, and then ventral prostates were collected.

A set of males and females (n=4-6) from F1 generation (all groups) were kept under normal housing and untreated conditions until PND120, when they were mated with unexposed animals to produce the F2 generation. F2 male descendants were euthanized on PND22and ventral prostates were collected for RT-qPCR analysis. To labelthe offspring generated in F2, the male offspring from exposed F1 dams were named PND22-M, whereas the male offspring from exposed F1 fathers were named PND22-F.

MicroRNA expression

Total RNA extraction of ventral prostates was performed using the Trizol® reagent (Ambion, USA) according to the manufacturer's instructions. RNA was quantified by spectrophotometry using the NanoVue equipment (GE Healthcare Life Sciences, USA). The analysis of RNA quality was made by the RNA integrity number (RNA Integrity Number, RIN) from the analysis of ribosomal RNAs based on microfluids using the 2100 Bioanalyzer system (Agilent, USA).

MicroRNA (miRNA) cDNA was synthesized from the total RNA of 1µg using the primers of specific miRNA acceleration and the TaqMan MicroRNA reverse transcription kit (Thermo Fisher Scientific, USA). Total RNA was reverse transcribed into cDNA using the Master RNA Mixture for high-capacity cDNA (Thermo Fisher Scientific, USA). Analysis miRNA was performed with 10µl reaction in duplicate (TaqMan ™ Gene Expression Master Mix for miRNAs; Thermo Fisher Scientific, USA) as described by the manufacturer, and performed using the QuantStudio™ 12K Flex System (Thermo Fisher Scientific, USA) with the following cycle conditions: 95°C for 10 minutes followed by 40-95°C cycles for 15 seconds and 60°C for 1 minute. Oligonucleotides TaqMan assays for miRNAs (Thermo Fisher Scientific, USA) used were: rno-rno-miR-184 (Assay ID #000485), rno-rno-miR-141-3p (Assay ID #000463) and rno-mir-U6 (Assay ID #001973), in a 96-well plate according to the manufacturer's specifications. miR-184 and mir-141-3p were selected based on our previous sequencing results [13]. The MammU6 was used as reference gene to normalize the data of miRNA expression. These reference genes were selected based on geNorm calculations, and the levels of relative expression in these reference genes were similar among the control treated rno-miR-184 and rno-miR-141-3p as showed by the CT value reported after the analysis of the raw RT-qPCR data. The relative quantification of miRNA was evaluated using the 2-ΔΔCT method. Significant changes were set at p-value ≤ 0.05 [31].

Target prediction analysis

Target prediction of miRNA targets was performed in the MirWalk 3.0 database (http://mirwalk.umm.uni-heidelberg.de/) [32]. After filtering the data by energy (> -20), 4431 predicted targets were obtained for rno-mir-184 and 1428 predicted targets for rno-mir-141-3p (Rattus norvegicus). The Venny 2.1.0. tool was used to obtain the common targets for both miRNAs (https://bioinfogp.cnb.csic.es/tools/venny/). After setting up the common targets, the Gene Ontology enrichment analysis and visuaLizAtion (GORILLA) database (http://cbl-gorilla.cs.technion.ac.il/) was used to investigate the influence of the selected targets on the pathways of interest.

Enrichment Analysis

After finding the potential targets modulated by the deregulation of miRNAs, the network interaction between these targets was performed. Data were analyzed using the String tool (string-db.org/). In addition to showing the target-target interaction, the tool performs enrichment analysis of cellular pathways and ontological terms, collects data from different databases such as experimental validation data, computational prediction methods and public text collections. Here, we used data from enrichment analysis for biological processes to show different biological pathways potentially deregulated by selected targets. Cellular pathways were selected by FDR value and data were represented by -log10 (FDR). Data were extracted from the KOBAS 3.0 tool (kobas.cbi.pku.edu.cn/).

The Comparative Toxicogenomic database (CTB) was used to investigate the association of the predicted targets with different phthalate types. Then, it was performed an investigative analysis to see the relation of the predicted targets with different prostate and reproductive diseases in the Enrichr database (https://maayanlab.cloud/Enrichr/) using data from DisGeNet database.

Data representation

GraphPad Prism (GraphPad Software) (https://www.graphpad.com/scientific-software/prism/) was used to perform statistical analysis and construct bar graphs. The GORILLA database (http://cbl-gorilla.cs.technion.ac.il/) was used to create schematic graphs showing biological processes dysregulated by predicted targets. The Venny 2.1.0. tool was used to create the intersection graphs (https://bioinfogp.cnb.csic.es/tools/venny/). The “circlize” package in the RStudio environment software to create circus plots. The String tool (https://string-db.org/) to obtain the target-target interaction and the

Sankey tool (https://sankeymatic.com/build/) to show the association between selected predicted targets with different diseases.

Data Validation

The Human Protein Atlas (HPA) database was used to investigate whether the expression of targets modulated by early exposure to a phthalate mixture was presented in prostate adenocarcinoma. The targets available in HPA database were represented by photomicrograph to the positive marking to selected targets.

Western blotting

Aliquots containing 70µg of proteins samples (n=3) were separated on SDS-PAGE. Following the electrophoresis, the proteins were transferred to nitrocellulose membranes. The nonspecific binding of proteins was blocked by incubating the membrane in 5% non-fat milk in TBST buffer for 90 min at room temperature. The membranes were incubated with the respective primary antibody in 1% non-fat milk (1:1.000) overnight at 4 °C in primary antibody: Wnt5 (M-58, sc-30224, Santa Cruz), Stat5 (ab97734, Abcan), p53 (GTX102425, GENETEX) and p53 (2524S, Cell Signaling).

The membranes were then incubated with a specific secondary antibody conjugated with peroxidase, which was diluted (1:10.000) in TBST for 1h at room temperature (IgG goat-antirabbit, ab97051 and IgG goat-anti-mouse, ab97023, Abcam® Inc., USA). The immunoreactive components were revealed by GE Amersham ECL chemiluminescent substrate (GE Healthcare). Analyses were done using different biological samples per group. To calculate the mean and SEM, the optic densities of bands were used as the unit of measure with software Image J (version 1.33u—National Institutes of Health, USA), and normalized by β-actin values and the results were normalized from fold change and expressed as means ± SD.

Statistical analysis

All statistical analyzes were performed using GraphPad Prism® Software (version 8.0, Graph Pad, Inc., San Diego, CA). First, the data were analyzed using the “Shapiro-Walk” test to perform the normality test. All data were considered non-parametric. Data were compared using the “Mann-Whitney” test, comparing groups two by two. Statistical significance was considered when p-Value *$p<0.05$, **$p<0.01$.

Results

miRNAs expression in ventral prostate

Prenatal phthalate mixture exposure (20µg/kg/day; 200µg/kg/day; 200mg/kg/day) increased in rno-miR-141-3p at PND22 compared to control in the F1 generation. In adult animals (PND120), the group exposed to 20µg/kg/day maintained significantly increased expression compared to the control group, while the other groups did not show significant changes compared to the control. No changes were observed in the different groups of the F2 generation (Fig. 1a).

Prenatal phthalate mixture exposure significantly increasedrno-miR-184 expression at all three exposure doses at PND22 in the F1 generation. In the long term, this increase in the expression was maintained only in the group exposed to 20µg/kg/day. Contrary to the results obtained for the expression of rno-miR-141-3p, this alteration was also observed in the offspring from fathers exposed to the phthalate mixture lowest dose (PND22F) (Fig. 1b).

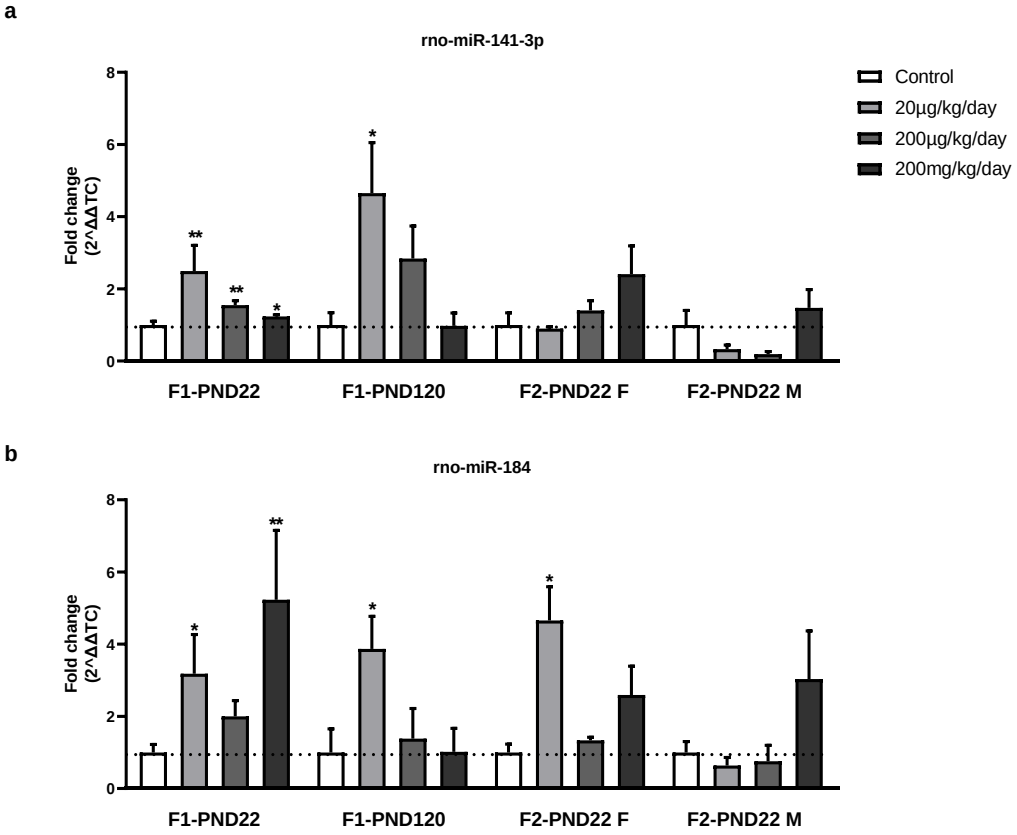

Figure 1: Representative bar graph showing the expression of rno-miR-141-3p (a) and rno-miR-184 (b) in ventral prostates of animals exposed to different concentrations of the phthalate mixture (20µg/kg/day; 200µg/kg/day; 200mg/kg/day) during gestational and lactational development. Statistical analysis: Mann-Whitney test. *p<0.05, **p<0.01.

By analyzing the common target interaction for both miRNAs, 514 common targets were identified (Fig1a). These targets were enriched for numerous biological processes. The enrichment analysis for biological processes considering the 514 proteins obtained on the *GORILLA* platform, showed the influence of 28 targets on pathways associated with reproductive and hormonal processes (fig.2b and S-table 1).

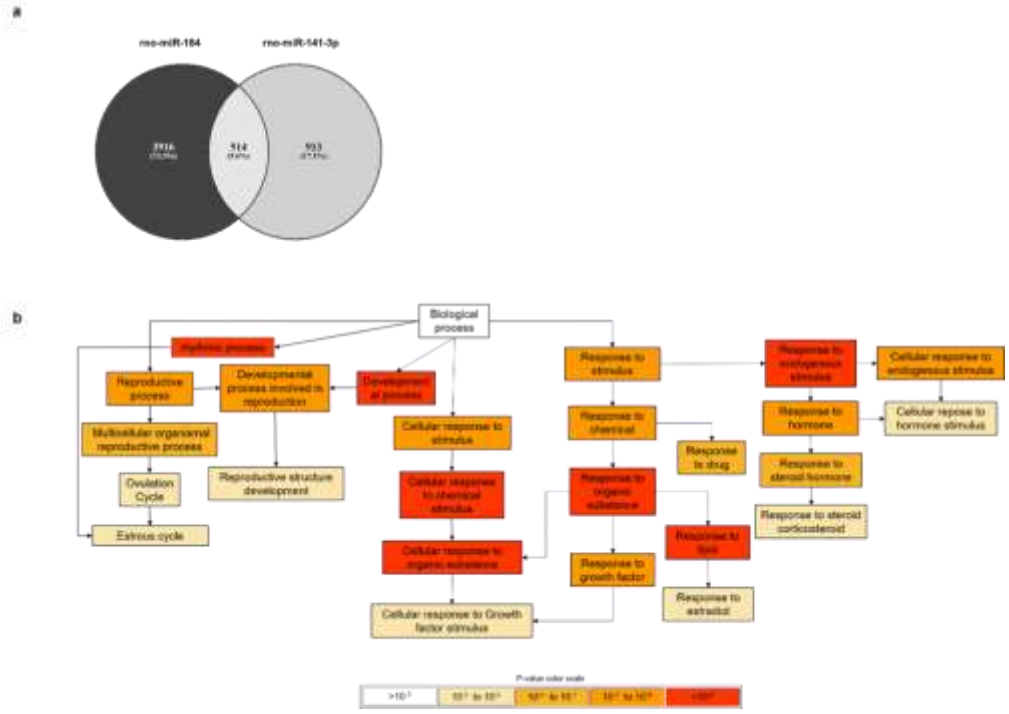

Figure 2: a. Venny plot evidencing the common and unique targets of rno-miR-141-3p and rno-miR-184 **b**. Schematic graph highlighting the main biological processes influenced by the common target genes regulated by both microRNAs. The light-yellow boxes represent target-enriched terms with significant values between 10^{-3} to 10^{-5}. Dark-yellow boxes represent terms enriched by targets with significance values between 10^{-7} to 10^{-9}. Orange boxes are terms enriched by targets with significant values between $<10^{-9}$.

The 28 targets related to potentially altered pathways in prostate tissue due to upregulation of rno-miR-141-3p and rno-miR-184 make up a strong interaction network. The protein-protein interaction network (IPP) highlighted targets with multiple interactions such as: TP53, ERBB2, NOTCH1, JAG1, STAT3, WNT5, STAT3, SP1, RET and MAPK9 (Figs.3 a-b). Furthermore, the enrichment analysis pointed out that these targets are involved in pathways such as regulation of growth, cell proliferation, cell adhesion, cell migration and developmental process, as well as response to hormone, urogenital system development, signal transduction (Fig. 3c). All these pathways are of primary importance in the early and late development of reproductive tissues, such as the prostate, for example.

The comparative toxicogenomic database (CTB) showed that 17 targets (ATM, ERBB2, ETS1, JAG1, KEAP1, LRP6, MED1, NOTCH1, NOTCH2, SP1, STAT3, TP53, WNT5A, WNT9B, NCOA1, PPARD, RET) were modulated for different types of phthalates (di-ethyl-phthalate, di-butyl-phthalate, mono-benzyl-phthalate, di-ethyl-hexyl-phthalate, phthalic acid) (Table 1). The enrichment analysis for diseases showed that 15 targets (ATM, ERBB2, KEAP1, TP53, ETS1, JAG1, NOTCH1, NOTCH2, RET, STAT3, WNT5A, MED1, NCOA1, LRP6 and SP1) are associated with different male disorders (S-table2).

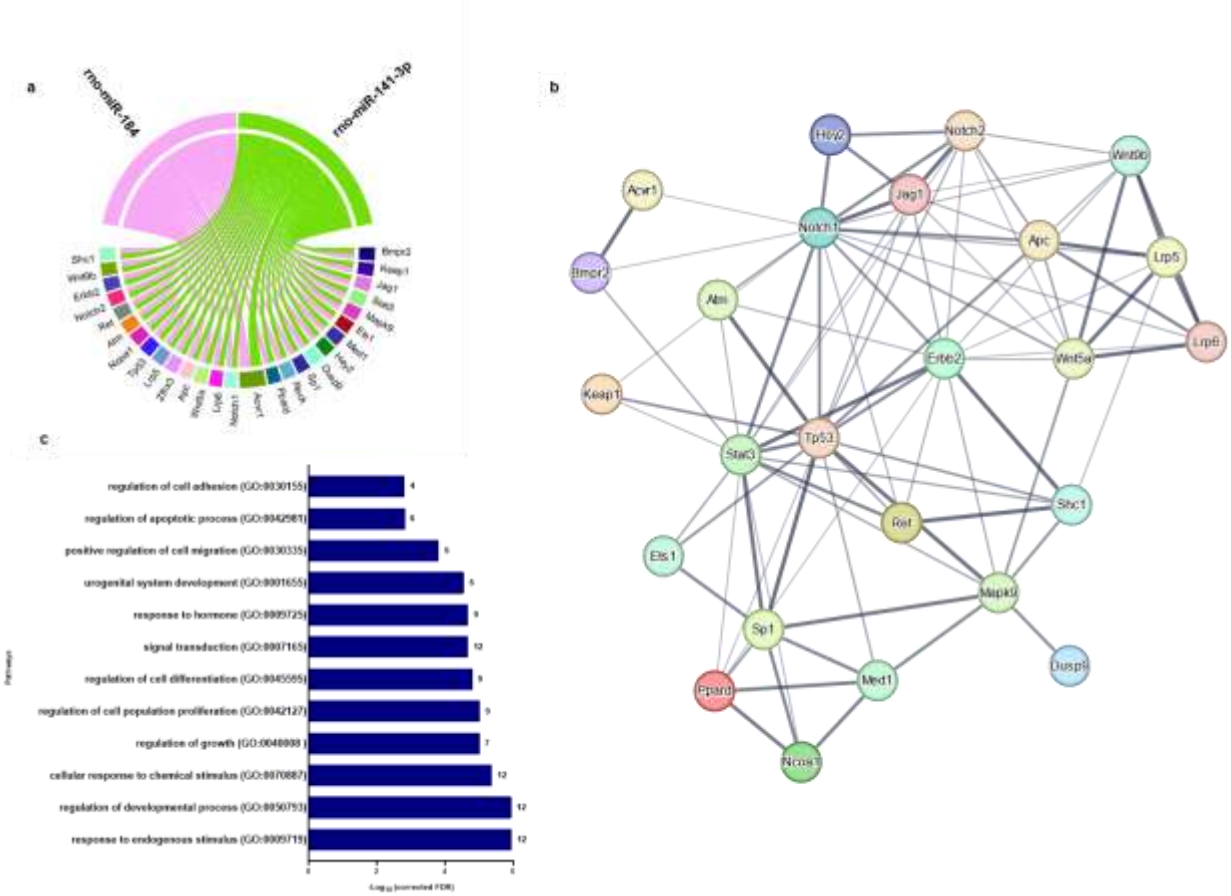

Figure 3: **a.** Circus plot showing the common targets of rno-miR-141-3p and rno-miR-184. **b.** Network showing the interaction of the targets. The network was obtained from string tool. **c.** Bar graph showing the biological processes influenced by the main common targets of rno-miR-141-3p and rno-miR-184.

Table 1: Table showing the targets of phthalates. The data obtained from the toxicogenomic database (accessed on 13th Dezember 2023).

Phthalates	Count in toxicogenomic database	Protein
Diethyl phthalate	1/220	WNT9B
Dibutyl phthalate	15/37489	APC, WNT9B, ATM, ERBB2, ETS1, JAG1, KEAP1, LRP6, MED1, NOTCH1, NOTCH2, SP1, STAT3, TP53, WNT5A, WNT9B.
Diethylhexyl phthalate	10/3847	ATM, ERBB2, NCOA1, NOTCH1, NOTCH2, PPARD, RET, SP1, TP53, WNT9B.
Monobenzyl phthalate	2/76	PPARD, TP53.
Phthalic acid	02/2022	MED1, NCOA1.

The investigative analysis of the association with the targets obtained after filtering in the CTB showed that 17 targets are involved with 1 or more pathways directly associated with tumor progression, tumor angiogenesis, carcinogenesis, adenocarcinoma, and adenoma. Furthermore, these targets have a direct relationship with the different pathologies that affect the prostate gland, as well as the reproductive quality such as: prostatic neoplasms, prostatic adenocarcinoma, benign prostatic hyperplasia, and infertility (Fig. 4a). According to data obtained into The Human Protein Atlas (HPA), except for the WNT5A, all targets are pointed to by CTB expressed positive tissue reactivity in prostate adenocarcinoma in men between 52 and 84 years of age (Fig. 4b).

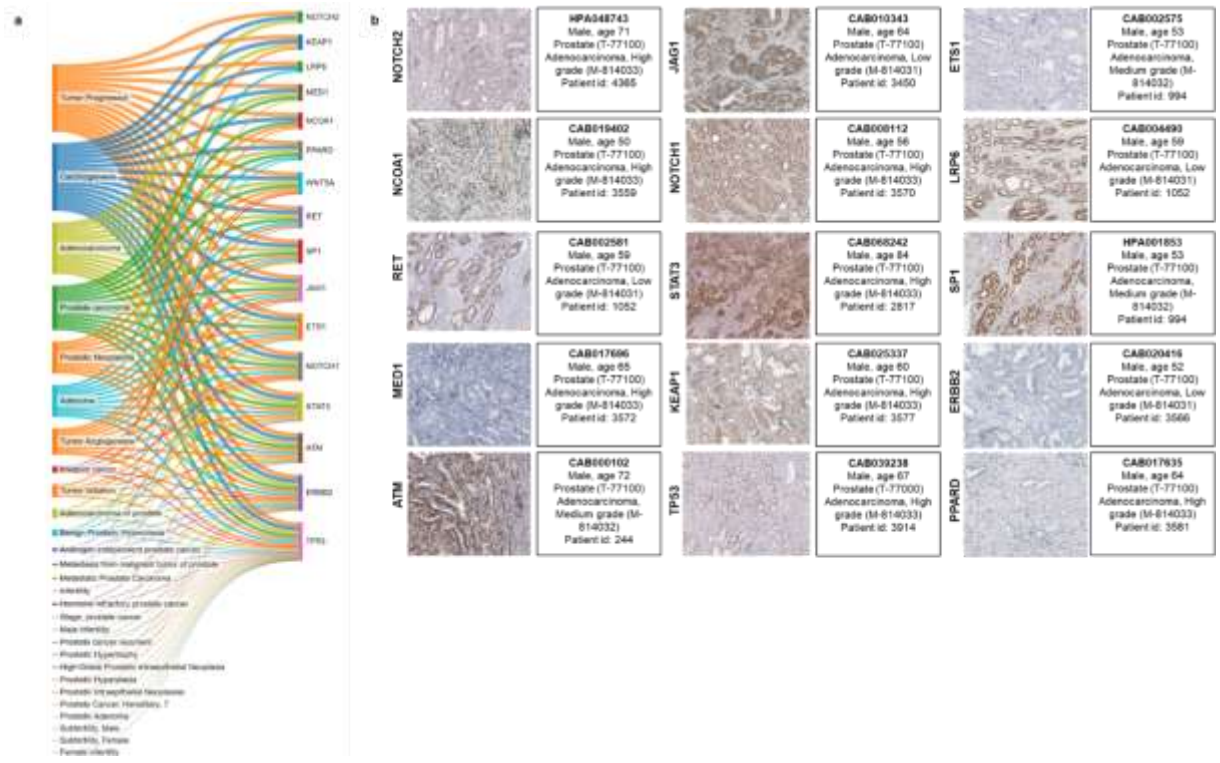

Figure 4: **a.** Sankey graph correlating the different diseases that affect the prostate gland with the target of rno-miR-141-3p e rno-miR-184. **b.** Histological plate with representative images showing the positive reactions to NOTCH2, NCOA1, RET, MED1, ATM, JAG1, NOTCH1, STAT3, KEAP1, TP53, ETS1, LRP6, SP1, ERBB2 and PPARD targets in prostate adenocarcinoma of men aged 52 to 84 years. There are no data on positive histological reaction for WNT5A in prostate adenocarcinoma. Histological images obtained from the HPA tool.

The western blotting (WB) analysis showed no significant variation in P53 at PND22 in all experimental groups. However, P63 abundance was exclusive in the 200mg/kg/day group, whereas WNT5 abundance was higher in the same group compared to the control group (Fig. 5a). InPND120 animals, the 20ug/kg/day phthalate mixture slightly increased P53 and the 200mg/kg/day markedly increased i STAT3 compared to the control group. Similarly, in adult animals P63 was expressed only in the group exposed to 200mg/kg/day (fig.5b). No expression of STAT3 and WNT5 was identified in the samples from young (PND22) and adult animals (PND120), respectively.

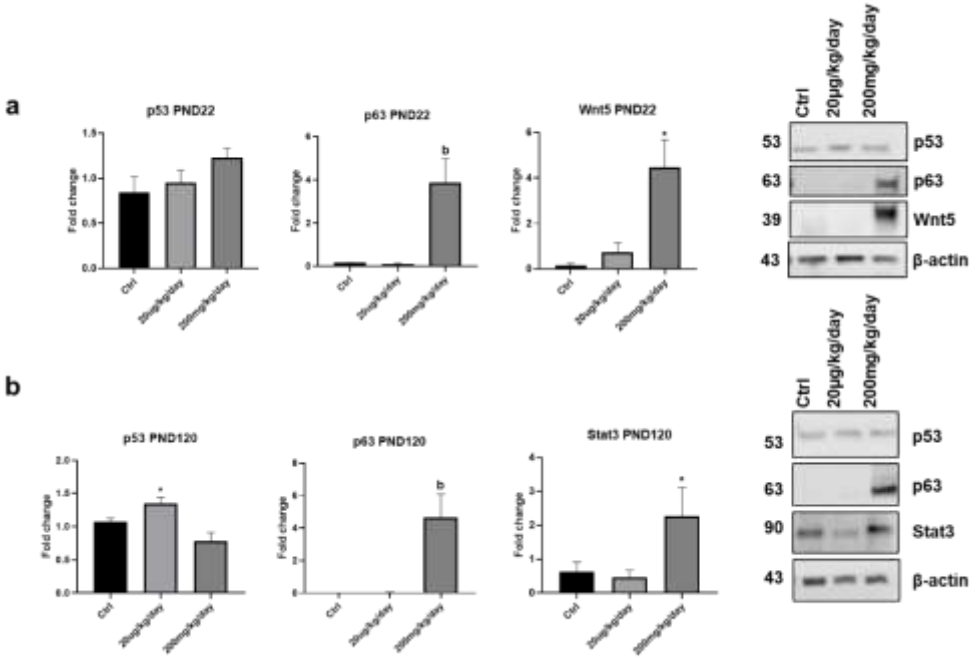

Figure 5: a. Representative graphs showing the abundance of p53, p63, and Wnt5 proteins in ventral prostates of offspring (PND22) from mothers exposed to vehicle control and phthalate mixture. **b.** Representative graphs showing the abundance of p53, p63, and Stat3 proteins in ventral prostates of adult offspring (PND120). Abundance analyzed by mean ± SEM in the comparation two by two. Asterisks represent statistical difference by Many-Whitney test: *p <0,05 and b expression in only one experimental group.

In the F2 generation, there was no change in STAT5 in either offspring of exposed father (PND22 F) or mother (PND22 M) (Fig. 6a-b). However, the prostate of offspring from both exposed father and mother pointed for an inhibition of P53 (Fig. 6b), which was not observed in the F1 males at PND22 or at PND120 (Fig. 6a-b).

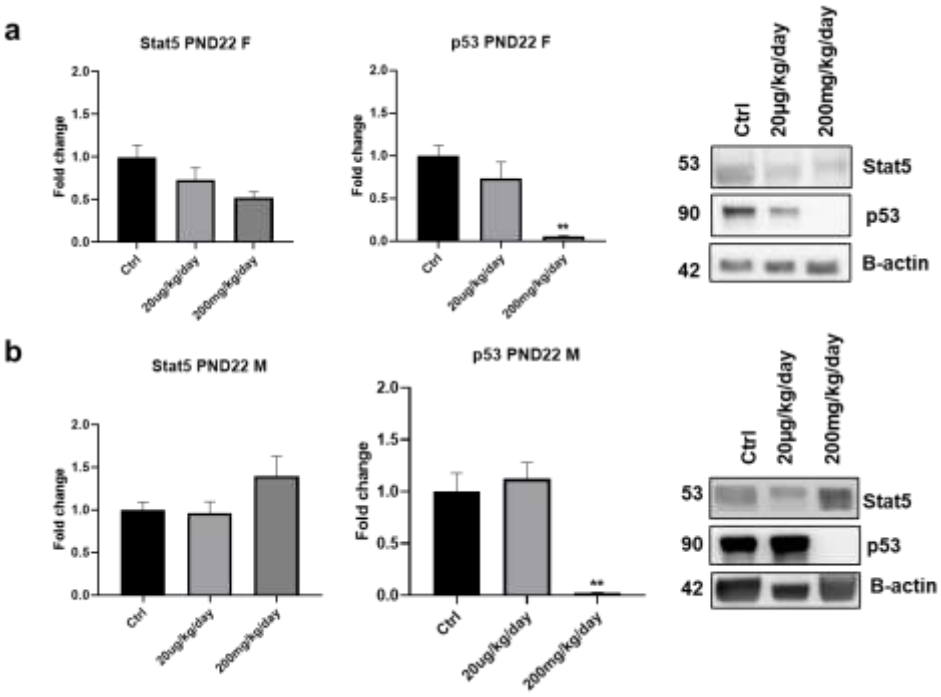

Figure 6: a. Representative graphs showing the abundance of Stat5 and p53 proteins in ventral prostates of offspring (F2-PND22 F and F2-PND M) from mothers exposed to vehicle control or phthalate mixture. **b.** Representative graphs showing the abundance of p53, p63, and Stat3 proteins in ventral prostates of adult offspring (PND120) Abundance was analyzed by mean ± SEM in the comparation two by two. Asterisks represent statistical difference by Many-Whitney test: *p <0,05 and b expression in only one experimental group.

Discussion

Endocrine disrupting chemicals (EDCs) such as phthalates modulate post-transcriptional events, especially microRNA interaction and mRNA expression [33,34]. Our previous results showed that maternal exposure to a phthalate mixture altered the expression of microRNAs in the ovary and ventral prostate of rat offspring [11,13]. Likewise, phthalate monoester mixture exposure modulated benign and tumor human prostate cells and increased the expression of the miR-184 and miR-141-3p *in vitro* [35]. The results presented here partially agreed with the microRNA sequencing profile presented by Scarano et al. (2019). Using sequencing, Scarano et al (2019) identified the miRNAs modulated by maternal exposure to

phthalates in the offsprings prostate. The two main miRNAs rno-miR-184 and rno-miR141-3p. The mRNAs identified here were not identified in the investigations published by Scarano. It is believed that this divergence may be related to the fact that the analyses carried out in the previous study were focused on the general mapping of the transcriptome, i.e., the authors were not focused on a modulation pathway. Here, the study is directed at the predicted targets of miRNAs. Furthermore, our results showed that the perinatally phthalate exposure was able to modify the rno-miR-184 expression in the prostate up to the F2 generation. This is another divergence in the studies. Scarano only evaluated the animal in PND22 (F1).

According to our previous results, the expression profile of rno-miR184 and rno-miR-141-3p in the ovary of directly exposed females (F1-PND22) showed no difference in expression pattern as was observed in the ventral prostate of male offspring subjected to the same experimental protocol at the same age [11,13], showing that the epigenetic changes observed can be tissue, sex or phthalate mixture dependent [36].

Different EDCs triggeran epigenetic programming by miRNA modulation [16] or DNA methylation [17] during the development. Here, we showed that the early exposure to a phthalate mixture changed the miRNA expression pattern and consequently, interfered with proteins synthesis in the prostate during perinatal development, leading to late repercussions and transgenerational effects. Similarly, maternal exposure to another EDC (vinclozolin) has been associated with promoting epigenetic reprogramming by methylation in the male germline in the offspring with transgenerational repercussions [17].

Several EDCs can alter the expression of different miRNAs by different exposure routes. This modulation can be classified from two different points of view, acting as an important biomarker of diseases or as a biomarker of environmental exposure [21]. Furthermore, the influence of EDCs on the miRNA expression could be a potential way to predict reproductive disorders in male and females [37]. The data presented here showed that many of the predicted targets are associated with various prostatic diseases and reproductive disorders. Additionally, exposure to isolated or mixed phthalates was associated with predicted targets for both miRNAs, which reinforces the importance of investigating the epigenetic influence of EDCs on human and animal health.

As described for Singh and Li (2011a), the top 20 diseases influenced by targets that interacted with different phthalates were enriched for urogenital system and urological diseases, adenocarcinoma, endocrine glands and urogenital neoplasms, urological and male genital diseases, prostatic diseases ,and male genital diseases [38]. Our results showed that 15 of the miRNAs targets interact with different

phthalates (phthalate-target interaction), and all of them are associated with the common diseases described by Singh and Li (2011a). However, only two targets (PPARD and NOTCH2) were common between the Singh and Li investigation [39] and ours. Additionally, the HPA database showed that 14 of the identified phthalate-targets had positive reactivity in the database for prostate adenocarcinoma. The difference observed among the investigations could be explained by the differences in the timing of exposure or tissue collection, but interestingly, both studies pointed to some potential biomarkers being modulated by phthalate exposure. In addition, as the phthalates in the mixture have one or more targets in common, these data may contribute to establishing the preferred targets of the phthalate molecules.

The bioinformatics approach used in this investigation identified phthalate targets that are potentially modulated by rno-miR184 and rno-miR-141-3p. Some of them have been widely studied due to their high biological value for prostate development and oncogenesis. Notch family proteins play a key role in a variety of developmental processes by controlling cell fate decisions and operating in a great number of biological processes (Vlachakis et al., 2020). Notch-1 is an aberrant tumor suppressor in prostate tumor cells, and expression of Notch-1, in turn, can control PTEN and p53 expression [40,41]. The Notch pathway is important for maintaining prostate function as well as modulating prostatic adenocarcinoma in adult mice. However, in prostate adenocarcinoma, the expression of Notch ligands (jagged-1 and 2) and Notch receptors (Notch-1 and 2) significantly increase [42] and therefore, the dysregulation of the Notch pathway is directly related to the increase in the risk of carcinogenesis [41]. Exposure to the MEHP was able to affect Notch pathways by increasing levels of the transmembrane ligand NOTCH-1, 2, 3,4 and jagged-1, 2 [43] in 3T3-L1 cells. Additionally, DEHP impaired folliculogenesis by decreasing NOTCH2 and JAG2 expression in mouse ovary [44]. Furthermore, exposure to DEHP inhibited the activation of Notch pathways by reducing the levels of essential targets [44]. In this sense, the three targets modulated by the overexpression of both miRNAs presented in this investigation point to a potential form of interference of phthalates in Notch signaling and prostate oncogenesis.

NCOA1 is a steroid and nuclear coactivator of hormone receptors. Increased NCOA1 expression interferes in prostate cell migration and invasion and NCOA1 may deregulateandrogen receptor (AR) expression by interaction with secondary targets [45]. MED1 is involved in the activation of gene transcription in targets such as ERα/β, PPAR, GR and AR [46] and plays important role in the AR-transcriptional complex [47]. In prostate cancer, downregulation of the MED1-AR complex has been linked as a potential way to block the functional expression of oncogenic targets [47].

Another interesting transcriptional activating target is ETS1, which is directly involved in the regulation of the RAS/RAF/MEK/ERK1/2 pathway and has an important contribution to cancer promotion by association with dysregulation in the process of invasion, proliferation, differentiation and angiogenesis [48]. Phthalates triggered invasion of hepatocellular carcinoma cells by activating PXR/EST1 expression [49]. Patients with prostate adenocarcinoma showed overexpression of EST1, which was associated with rapid acquisition of progression of castration resistance and biological characteristics of high aggressiveness [50]. SP1 is also a transcription factor involved in several cellular functions such as differentiation, growth, response to DNA damage and others. Breast cancer cells exposed to DEHP had increased expression of the SP1/E2F1 complex, which triggered high DNMT1 (DNA methyltransferase 1) activity and consequently, increased DNA methylation [51]. Additionally, male rats exposed to DEHP during the gestational period exhibited decreased SP1expression in Sertoli cells and the authors hypothesized that this effect would be responsible for increasing methylation by increasing DMNT1 and DNMT3 [52]. In prostate cancer cells, SP1 expression was associated with increased autophagy process by active expression of PK2 [53].

PPARD is a nuclear hormone receptor that controls target expression after endogenous or exogenous stimulation, and is involved in important functions such as gluconeogenesis, lipogenesis, and anti-inflammatory response [54,55]. Reproductive organs are constantly affected by dysregulation in the PPARα/γ receptor because some EDCs, such as DEHP metabolites, can bind to the receptors and interfere with different cellular responses [3]. PPARD plays a key role in the WNT1 pathway in normal and tumor tissue. In prostate cancer, alteration in PPARD expression has been associated with interference with cancer stem cell behavior and transformation by interfering with the WNT1 pathway [56]. In addition, PPARD acts as tumor suppressor and the downregulation implies an increase in the aggressiveness of prostate cancer [54].

Most intracellular processes are controlled by one or more protein kinases. Despite the targets presented above being regulated by kinases, RET, ERBB2, STAT3, ATM and TP53 may point to an interesting approach to establish the effect of the mixture on pathways commonly altered in prostate diseases. RET and ERBB2 are protein tyrosine kinase receptors that control cell proliferation and survival. The prostate is derived from the urogenital sinus, which develops under the action of androgens produced by the fetal testis [57,58]. In the testis, RET signaling is essential for controlling germ cell differentiation and for the spermatogonial stem cell population. RET also has an important function in human pathologies when

there is the loss of its expression or reduction (Mulligan 2014). Both receptors (ERBB2 and RET) interact with STAT3, a transcription factor responsible for controlling cell growth, differentiation, and survival by active signaling of cytokines and growth factors (Grasso et al. 1997) and JAK-STAT and FGFR activity have been described to increase prostate tumor cell plasticity (Chan et al. 2022).

The ATM is an important target involved in activation of G1/S and intra-S phase cell check-point by control the expression of several proteins such as the tumor suppressor proteins P53, CHK2 and BRCA1 and stimulate the activation of apoptotic genes [59]. Moreover, the TP53 is involved in cell cycle and apoptosis regulation and DNA repair. In prostate cancer, mutations in targets such as TP53, PTEN, AR, MYC, BRCA and ATM have been detected [59,60]. Here, the western blots results showed that there was an increase in P53 only in 20µg/kg group at PND120, which may point to a late effect of treatment on the target and cell cycle. Additionally, P53 is capable of regulating positively miR-184 expression in lung cancer cells[61]. Thus, it is possible that the increase in P53 protein induces an increase in miR-184 expression in the 20µg/kg group, at PND120. On the other hand, the inhibition of P53 expression in the prostate of F2 descendants of fathers and mothers perinatally exposed to the higher dose of the mixture can be related to a heritable mechanism independent of the activation of miR-184 because its expression was not altered in these animals. Additionally, the decrease of P53 expression in F2 may increase the instability in DNA repair and in the spreading of genetic errors.

Our results showed that the phthalate mixture causes overexpression of the P63 protein, another important cell cycle regulating protein, in the F1 offspring exposed to the highest dose at both ages. P63 has been shown to play a critical role in prostate development and is essential for the normal function of stem cells in the prostate [62]. Additionally, P63-positive basal cells of the fully developed prostate include stem cells responsible for the maintenance and repair of the adult prostate epithelium [62]. Recently, Scarano et al., (2019) showed that maternal exposure increases the cell proliferation index in the prostate of adult offspring at all experimental doses analyzed (20µg/kg/day, 200µg/kg/day, 200mg/kg/day). Thus, the increase in P63 expression in the adult epithelium seems to point to an increase in the population of undifferentiated cells, and possibly may be related to an increase in cell turnover. Together, these data reinforce that there was a lack of control in the cell cycle, which can be associated with an increasing risk for prostatic disorders and pathologies.

Finally, KEAP1 is a related target of ROS stress and xenobiotic response by controlling NRF2 expression. Furthermore, the KEAP1/NRF2 complex is responsible for inducing cytoprotection in response

to environmental stress [63]. In prostate cancer, overexpression of P62/keap1/Nrf2 pathways triggers transcriptional blockade of targets associated with reactive oxygen species response, cell proliferation, and invasion [64]. The KEAP1/NRF2 complex has been identified as an important way to stimulate the autophagy process in immune cells and in prostate cancer [64,65]. Interestingly, according to Wang (2016), post-transcriptional levels of miRNA 141-3p are responsible for controlling KEAP1/NRF2 signaling in patients with prostatitis [66].

At least 6-12 targets identified in this investigation are associated with cell population regulation, cell growth hormone response, cell adhesion, signal transduction and other pathways. Considering that all targets interact with phthalates, it is possible to hypothesize that early exposure to phthalates interferes with the main pathways related to abnormal prostate development and changes associated with the pathological condition throughout life. Furthermore, it is possible to point out that the targets found in this study can interfere in essential signaling pathways through the negative regulation of protein kinases, transcription factors, and targets commonly involved in prostatic disorders.

These data draw attention to early environmental exposure, which reflects the global reality, as well as the maternal influence on this exposure during early development and the long-term repercussions. The maternal condition during the individual's development can be determinant in the long term, mainly for the development of diseases [33,67–69]. Based on the DOHaD hypothesis, this and other studies highlight the importance of environmental factors that impair key developmental susceptibility windows.

Animal and human studies show that maternal exposure to phthalates influences prostatic disorders in offspring (Wang et al., 2017b; Peixoto et al. 2016; Yong et al. 2016). The increased risk of developing prostatic diseases has been related to the genetic and epigenetic [13,15,72], reinforcing the hypothesis that maternal phthalates exposure increase the susceptibility of the offspring to prostate cancer by post-transcriptional and phenotypic alterations (Wang et al. 2017b; Scarano et al. 2019). Here, we showed new potential phthalate-target biomarkers that can be modulated by dysregulation of miR-184 and miR-141-3p in the F1 and F2 generations. Together, these data point out the importance of investigating the consequences of early environmental exposure to understand and discuss the developmental origin of prostatic diseases.

Confidential interest

The authors declare that there are no conflicts of interest.

Funding sources

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Data availability

The data underlying this article will be shared on reasonable request to the corresponding.

Supplementary material

Table 1: The table shows the main ontology terms inflected by the 28 common targets of rno-miR-184 and rno-miR-141-3p.

GO term	Description	P-value	FDR q-value	Enrichment (N, B, n, b)	Genes
GO:0022414	Reproductive process	9.24E-9	6.89E-7	2.28 (465,178,31,27) 6.14	Bmpr2, Keap1, Jag1, Stat Ets1, Med, Hey2, Dusp9, S Ppard, Acvr1, Notch1, Lrp Apc, Zfhx3, Lrp5, Tp53, N Ret, Notch2, Erbb2, Wnt
GO:0048609	Multicellular organismal reproductive process	2.16E-6	7.49E-5	6.14 (465,22,31,9)	Med1, Wnt5a, Bmpr2, Ncoa Ets1, Erbb2, Notch
GO:0042698	Ovulation cycle	1.64E-5	4.38E-4	15.00 (465,4,31,4)	Wnt5a, Ncoa1, Ets1, I
GO:0044849	Estrous cycle	1.64E-5	4.4E-4	15.00 (465,4,31,4)	Wnt5a, Ncoa1, Ets1, I
GO:0003006	Developmental process	8.52E-9	6.52E-7	5.91(465,33,31,13)	Bmpr2, Wnt5a, Lrp6, Mec Atm, Hey2, Notch2, Sp1, W Notch1, Acvr1.
GO:0048608	Reproductive structure	3.28E-5	7.99E-4	3.75 (465,44,31,11)	Wnt5a, Bmpr2, Ret, Atm Mapk9, Erbb2, Tp53, Shc
go:0050896	Response to stimulus	1.39E-8	9.85e-7	2.36 (465,165,31,26)	Bmpr2, Keap1, Jag1, Stat Est1, Med1, Hey2, Sp1, Ppa Acvr1, Notch1, Wnt5a, Apc, Lrp5, Tp53, Ncoa1, Atm, R Erbb2, Wnt8b, Shc
GO:0042221	Response to chemical	1.05E-9	1.2E-7	3.11 (465, 111, 31, 23)	Bmpr2, Apc, Wnt5a, Lrp6, J Zfhx3, Mapk9, Stat3, Lrp5, Med1, Ncoa1, Ret, Atm, S Acvr1, Mapk11.
GO:0051716	Cellular response to stimulus	5.26E-8	3.08E-6	2.92 (465, 108, 31,21)	Bmpr2, Wnt5a, Apc, Lrp Mapk9, Stat3, Est1, Tp5 Ncoa1, Atm, Ret, Sp1, Erb Ppard, Shc1, Notch1, Acvr
GO:0070887	Cellular response to chemical stimulus	1.17E-10	1.77E-8	4.17 (465, 72, 31, 20)	Lrp6, Bmpr2, Apc, Wnt5a, K Mapk9, Est1, Tp53, Med1, Atm, Sp1, Erbb2, Ppard, Sh Acvr1, Notch1.
GO:0071363	Response to organic substance	4.04E-12	1.22E-9	3.92 (465, 88, 31, 23)	Lrp6, Bmpr2, Wnt5a, Apc, K Zfhx3, Stat3, Mapk9, Lrp5, Med1, Ncoa1, Ret, Atm, S Ppard, Shc1, Mapk11,
Go:0070848	Response to growth factor	2.35E-8	1.5E-6	7.89 (465, 19, 31, 10)	Med, Bmpr2, Apc, Wnt5 Mapk9, Shc1, Acvr1, N
GO:0033993	Response to lipid	7.82E-11	1.21E-8	6.43 (465,35,31,15)	Wnt5a, Lrp6, Stat3, Mapk9, Med1, Ncoa1, Atm, Ret, Er Shc1, Notch1, Acv
GO:0032355	Response to estradiol	4.72E-4	7.76E-3	2.39 (465,88,31,14)	Bmpr2, Wnt5a, Keap1, Map Med1, Ncoa1, Atm, Ret, Du Erbb2, Shc1, Mapk11,
Go:0042493	Response to drug	1.37E-6	5.04E-5	4.50 (465, 40,031,12)	Lrp6, Apc, Jag1, Ncoa1, F Mapk9, Est1, Erbb2, Tp5 Notch1.

Table 2: The table shows the relation of the phthalate-target interaction with different male disorders.

Disease Name	Disease Categories	Corrected P-value	Annotated Genes
Urologic Neoplasms (MESH:D014571)	Cancer-Urogenital disease (female)-Urogenital disease (male)	0,00281	ATM,ERBB2,KEAP1,TP53
Endocrine System Diseases (MESH:D004700)	Endocrine system disease	1,17e-9	ATM,ERBB2,ETS1,JAG1,NOTCH1,NOTCH2,RET,STAT3,TP53,WNT5A
Urogenital Diseases (MESH:D000091642)		1,28e-8	ATM,ERBB2,KEAP1,MED1,NCOA1,NOTCH1,NOTCH2,RET,STAT3,TP53,WNT5A
Cell Transformation, Neoplastic (MESH:D002471)	Cancer-Pathology (process)	1,35e-6	NOTCH1,SP1,STAT3,TP53,WNT5A
Carcinogenesis(MESH:D063646)	Cancer-Pathology (process)	1,64e-8	ERBB2,NOTCH1,SP1,STAT3,TP53,WNT5A
Metabolic Diseases (MESH:D008659)	Metabolic disease	1,68e-5	ATM,ETS1,NOTCH1,NOTCH2,RET,SP1,STAT3,TP53
Adenocarcinoma (MESH:D000230)	Cancer	1,70e-8	ATM,ERBB2,JAG1,KEAP1,MED1,NOTCH1,RET,STAT3,TP53
Urologic Diseases (MESH:D014570)	Urogenital disease (female)-Urogenital disease (male)	2,32e-4	ATM,ERBB2,KEAP1,NOTCH2,RET,TP53
Urogenital Neoplasms (MESH:D014565)	Cancer-Urogenital disease (female)-Urogenital disease (male)	2,49e-8	ATM,ERBB2,KEAP1,NCOA1,NOTCH1,NOTCH2,STAT3,TP53,WNT5A
Endocrine Gland Neoplasms (MESH:D004701)	Cancer-Endocrine system disease	3,72e-10	ATM,ERBB2,JAG1,NOTCH1,NOTCH2,RET,STAT3,TP53
Carcinoma(MESH:D002277)	Cancer	4,36e-9	ATM,ERBB2,JAG1,KEAP1,MED1,NOTCH1,NOTCH2,RET,STAT3,TP53
Genital Diseases(MESH:D000091662)		4,93e-9	ATM,ERBB2,KEAP1,MED1,NCOA1,NOTCH1,NOTCH2,STAT3,TP53,WNT5A
Pathologic Processes (MESH:D010335)	Pathology (process)	4,97e-11	ATM,ERBB2,ETS1,JAG1,KEAP1,LRP6,NCOA1,NOTCH1,NOTCH2,SP1,STAT3,TP53,WNT5A
Male Urogenital Diseases (MESH:D052801)	Urogenital disease (male)	5,53e-7	ATM,ERBB2,KEAP1,NCOA1,NOTCH2,RET,STAT3,TP53,WNT5A
Prostatic Neoplasms (MESH:D011471)	Cancer-Urogenital disease (male)	5,91e-5	ATM,ERBB2,KEAP1,NCOA1,STAT3,TP53
Prostatic Diseases (MESH:D011469)	Urogenital disease (male)	6,07e-5	ATM,ERBB2,KEAP1,NCOA1,STAT3,TP53

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Capítulo III

Artigo científico - published.

Lista de figuras – Capítulo III

Lista de tabela

The article has been published.

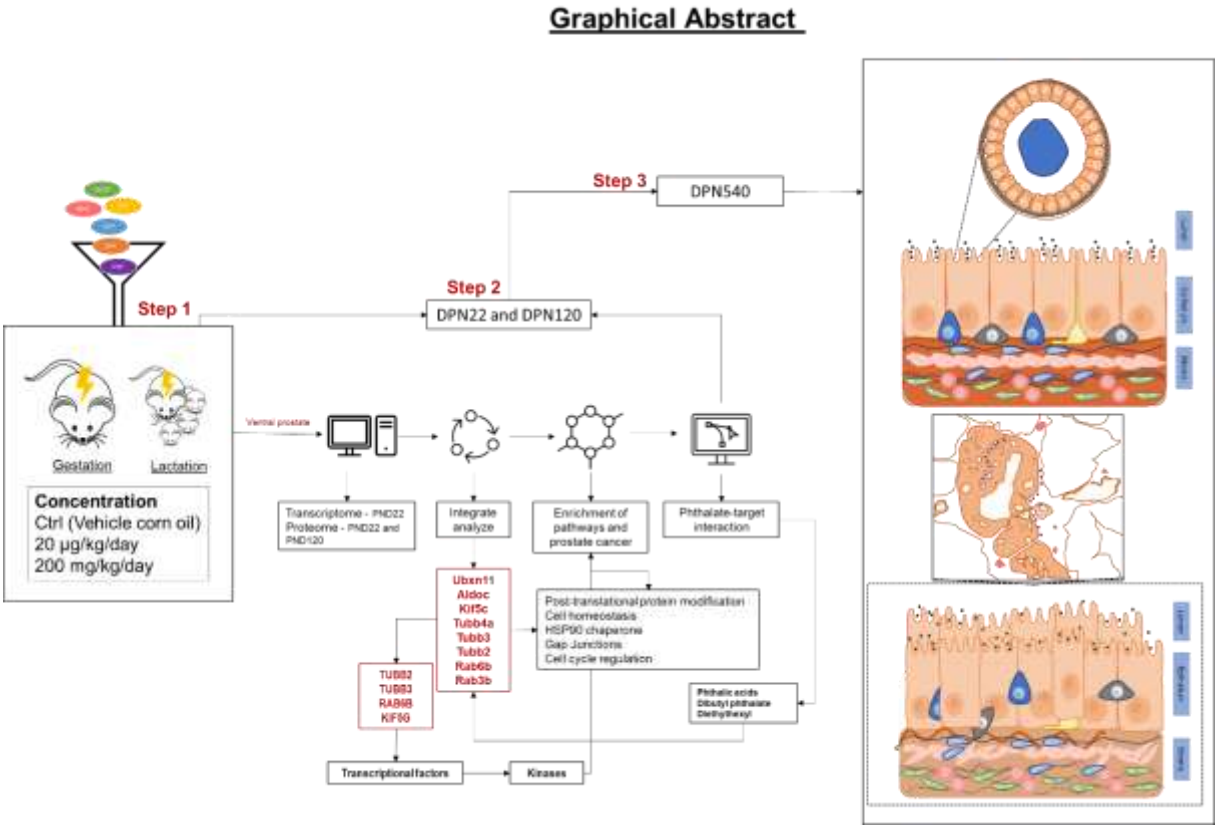

Figure 1: The graphic abstract presents the 3 steps of the investigation. The first step refers to the experimental protocol, in which pregnant rats were exposed from gestational to lactational day to phthalate mixture. The second step refers to the transcriptome and proteomic analyzes in the ventral prostate of the offspring (PND22 and PND120) and the in silico analyzes that were generated from the integrative analysis of OMICs results. Finally, the third stage refers to the histopathological analysis found in the old children.

Integrated transcriptome and proteome analysis shows potential biomarkers of prostate cancer in offspring of pregnant rats exposed to a phthalate mixture during gestation and lactation

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Abstract

As the second leading cause of death for cancer among men worldwide, prostate cancer (PCa) prevention and detection remain a critical challenge. One aspect of PCa research is the identification of common environmental agents that may increase the risk of initiation and progression of PCa. Endocrine disrupting chemicals (EDCs) are strong candidates for risk factors, partially because they alter essential pathways for prostate gland development and oncogenesis. Phthalates correspond to a set of commercially used plasticizers that humans are exposed to ubiquitously. Here, we show that maternal exposure to a phthalate mixture interferes with the expression profile of mRNA and proteins in the ventral prostate of offspring and increases the susceptibility to prostate adenocarcinomas in aged animals. The data highlight Ubxn11, Aldoc, Kif5c, Tubb4a, Tubb3, Tubb2, Rab6b and Rab3b as differentially expressed targets in young and adult offspring descendants (PND22 and PND120). These phthalate-induced targets were enriched for pathways such as: dysregulation in post-translational protein modification (PTPM), cell homeostasis, HSP90 chaperone activity, gap junctions, and kinases. In addition, the Kif5c, Tubb3, Tubb2b and Tubb4a targets were enriched for impairment in cell cycle and GTPase activity. Furthermore, these targets showed strong relationships with 12 transcriptional factors (TF), which regulate the phosphorylation of eight protein kinases. The correlation of TF-kinases is associated with alterations in immune system, RAS/ErbB/VEGF/estrogen/HIF-1 signaling pathways, cellular senescence, cell cycle, autophagy, and apoptosis. Downregulation of KIF5C, TUBB3 and RAB6B targets is associated with poor prognosis in patients diagnosed with adenocarcinoma. Collectively, this integrative investigation establishes the post-

transcriptional mechanisms in the prostate that are modulated by maternal exposure to phthalate mixture during gestation and lactation.

Keywords: Phthalate mixture, maternal exposure, DOHaD, offspring, prostate, mRNA, proteome.

Abbreviations: PCa, Prostate Cancer; EDCs, Endocrine Disrupting Chemicals; DOHaD, Developmental Origin of Health and Disease; GD, Gestational day; PND, Post-Natal day; DEHP, Bis (2-ethylhexyl) phthalate, DEP, Biethylphthalate; DBP Di-n-butylphthalate, DiBP, Diisobutylphthalate; BBzP, Bbutylbenzylphthalate; DiNP, Diisononylphthalate, VP, Ventral prostate, DER, Differentially Expressed mRNA and DAP, Differentially Expressed Protein.

Introduction

The prostate is the main secretory gland of the male genital tract. Secretions from the prostate as well as the seminal and bulbourethral grands are responsible for maintaining sperm viability (Prins and Lindgren, 2014; Verze et al., 2016). Diseases affecting the prostate gland are increasingly common, including prostatitis, which is more common in men of reproductive age than older men, and benign prostatic hyperplasia (BPH), which is considered an aging disease (Key, 1995; Marker et al., 2003; Verze et al., 2016). In the last decade, prostate cancer (PCa) has become a health concern because of its aggressiveness and lethality. PCais the cancer with the highest incidence and death rate among men across the world (Rebello et al., 2021; Siegel et al., 2021).

Individual genetic status is partially a determinant factor for cancer development; however, lifestyle and the environment to which the individual has been exposed during life are important and enhance the risk for pathological conditions in adulthood and senescence (Alcantara Santos et al., 2020; Soubry, 2018; Walker and Ho, 2012). Intrauterine and perinatal development are important periods of sensitivity of the developing urogenital tract, in which the reproductive organs are especially influenced by external factors, such as maternal metabolic diseases, dietary restriction, and exposure to environmental disruptors (Basak et al., 2020; Perobelli, 2014; Wadhwa et al., 2009). The environmental exposure during early development may be associated with repercussions in adulthood such as delayed tissue development and differentiation, as well as with increased risks for different pathologies diagnosed in adulthood (Rosenfeld, 2015; Wadhwa et al., 2009; Walker and Ho, 2012). In this context, based on the Origins of Health and Disease (DOHaD),

Willian Gardner (Gardner, 1995) proposed that PCa could originate in prenatal life and currently, this subject has been widely studied due to the growing amount of evidence about developmental diseases (Almeida et al., 2019; Gluckman et al., 2010; Skinner et al., 2010; Wadhwa et al., 2009).

Several studies indicate that constant exposure to environmental chemicals with antiandrogenic and estrogenic action known as endocrine disrupting chemicals (EDCs) increases the incidence of reproductive disorders (Basak et al., 2020; Beszterda and Franski, 2018; Chiang et al., 2017; Monneret et al., 2017; Yoon et al., 2014). Phthalates are a group of plasticizers with anti-androgenic and estrogenic effects that have non-covalent binding properties and are easily released from widely used products (Zhang et al., 2021). The versatility of products containing phthalates increases the routes of daily exposure (Basak et al., 2020; Wang and Qian, 2021). In this sense, several studies have associated exposure to single phthalates or mixtures of phthalates with reproductive disorders (Curi et al. 2019; Istvan et al. 2021; Bao et al. 2011; Hannon et al. 2015; Kay, Bloom, and Foster 2014), dysfunction in hormone-dependent organs (Ahmad et al., 2014; Bao et al., 2011; Barlas et al., 2020; Meling et al., 2020), and other diseases (Beszterda and Franski, 2018; Ma et al., 2017; Yang et al., 2017; Yong et al., 2016). Maternal exposure to EDCs, particularly phthalates, has been linked with late repercussions of reproductive disorders, multigenerational effects, and transgenerational effects (Basak et al., 2020; Dutta et al., 2020; Gill et al., 2021; Gonsioroski et al., 2022; Skinner, 2016).

Previous studies have shown that perinatal exposure to individual phthalates causes preneoplastic lesions in adult rats (Scarano et al. 2009; X. Wang et al. 2017b; Peixoto et al. 2016). A prior study also showed that exposure to an environmentally relevant phthalate mixture from gestational day 10 to 21 postnatally day 21 downregulates a set of genes and miRNAs in the prostates of rats (Scarano el at., 2019). Since previous studies, including ours, have reported that different types of exposure to individual or mixtures of phthalates cause epigenetic modulation (Grindler et al. 2018; Skinner 2016; Scarano et al. 2019; Warner et al. 2021), we expanded previous studies by performing an integrative analysis of the prostate transcriptome and proteomic profile in prostates from the offspring of mothers exposed to a mixture of phthalates during gestation and lactation relating the molecular changes observed after weaning and in adulthood and pointing out the possible consequences. In addition, for the first time, we demonstrate that perinatal exposure to a phthalate mixture increases the incidence of prostate tumors with age. Collectively, the data highlight possible biomarkers and molecular events induced by phthalates that may increase susceptibility to oncogenesis.

Material and Methods

Experimental Design

Sprague-Dawley rats (male and female), weighing on average 300g, were supplied by the Multidisciplinary Center for Biological Research (CEMIB/UNICAMP) and they were kept during the experimental period in the Bioterium of Small Mammals from Department of Structural and Functional Biology, Institute of Biosciences (UNESP). The rats were bred under controlled conditions of light (12 hours of light/12 hours of dark) and temperature (average of 23°C -25°C) and water and food were provided *ad libitum.* This study was carried out in accordance with the Brazilian Council on Animal Experimentation Control (CONCEA) and the protocol was approved by the Ethical Committee on Animal Use of the Institute of Biosciences, São Paulo State University (Protocol: 1040/CEUA).

After identifying the presence of sperm in vaginal fluid, the pregnant rats were randomly divided into three experimental groups (n= 10/group): control group: vehicle-corn oil; 20 µg/kg/day exposed group and 200 mg/kg/day exposed group. The doses used in this study were selected based on a prior study designed with three different doses of phthalate mixture: 20 µg/kg/day, 200 µg/kg/day and 200 mg/kg/day (Scarano et al. 2019), in which no important differences were observed in the effects of the two lowest doses on the prostate. The lowest dose was selected to mimic daily human exposure levels based on the amount of DEHP, and the higher dose was selected to compare our results with available single phthalate studies (Zhou and Flaws, 2017). The phthalate mixture was diluted in tocopherol-stripped corn oil (vehicle control). Pregnant and lactating female rats were treated from gestational day (GD) 10 to post-natal day (PND) 21 either with the phthalate mixture or with the vehicle (control group) as described previously by Scarano and collaborators (Scarano et al. 2019), and so, the offspring were exposed by a maternal route though the placenta and milk. After birth, the number of newborns per litter was reduced to eight (1: 1 ratio of males to females), and litters with fewer than six pups were euthanized. At PND21, the offspring were weaned and at PND22, eight offspring per group (2/litter) were euthanized (pentobarbital anesthesia followed by cardiac puncture) and ventral prostates (VP; n=4) were collected for transcriptome and proteomic analysis. The other offspring were kept under normal housing conditions and untreated until adulthood and senescence. At PND120, eight offspring (different litters) were euthanized, and the VP were selected for the proteomic analysis (n=3). At PND540, eight offspring (different litters) were euthanized, and the VP (n=8) were collected for histopathological evaluation: fixed in methacarn for 3 hours (Puchtler el at., 1970), sectioned (5

µm), and the cuts were stained by Hematoxylin & Eosin. Three different levels of each VP fragment were analyzed in a double blinded assay by WRS and an experienced veterinarian pathologist. Presence of adenocarcinoma *in situ* was verified to calculate the incidence in each group and the groups were compared using Fischer exact test and statistical difference was considered when $p<0.05$.

The mixture included the proportions of phthalates established by Zhou and Flaws (2017). Zhou and Flaws based the proportion of the phthalate mixture according to the proportions of the metabolites detected in urine samples from pregnant women. The proportion was 35% DEP (diethyl phthalate), 21% DEHP (Bis (2-ethylhexyl) phthalate), 15% DBP (di-n-butyl phthalate), 15% DiNP (di-isononyl phthalate, 8% DiBP (diisobutyl phthalate) and 5% BBzP (butylbenzyl phthalate) (Zhou et al., 2017a, 2017b).

Mass Spectometry

For the mass spectrometry, VP samples from each age (PND22 and PND120; n=3) were used. The analysis was performed using a nanoACQUITY UPLC system (Waters, Milford, MA) coupled to a Xevo Q-TOF G2 mass spectrometer (Waters, Milford, MA). The nanoACQUITY UPLC system is equPPIed with a Trap Columm (responsible for chromatography; Trap Columm: 100Å, 5 µm, 180 µm x 200 mm) previously equilibrated with 99.9% phase A (0.1% formic acid in water) at a flow of 5 µl/min and a HSS T3 M-Class type column (analytical column; Acquity UPLC HSS T3 M-Class column 75 µm x150 mm; 1.8 µm) (Waters, Milford, MA), previously equilibrated with 93% mobile phase A and mobile phase B (0.1% formic acid in ACN). Peptides were separated by a linear gradient of 7–85% mobile phase B for 70 min with 0.35 µL/min flow rate; the column temperature was maintained at 45 °C. The Xevo G2 Q-TOF mass spectrometer was operated in positive nano-electrospray ion mode and data were collected using MSE method in elevated energy (19-45V). Source optimal conditions include capillary voltage, 2.8kV; sample cone, 40V; extraction cone, 3.0V and source temperature 100°C. However, source conditions may have varied due to detector and lockspray voltage setups. Data acquisition occurred over 70 min, and the scan range was 50-1500 Da. The lockspray was run with a [Glu1] fibrinopeptide solution (1pmol/µL) at a flow rate of 0.3 µL/min, as a reference ion in positive mode at m/z 785.8427. ProteinLynx GlobalServer software (PLGS) version 3.03 (Waters, Milford, MA) was used to process and search the LC-MSE continuum data. The identification of VP proteins was performed using the ion accounting algorithm incorporated into the software and a search of the Rattus norvergicus database from the UniProtKB catalog (Universal Protein Resource)

(https://www.uniprot.org/). All samples were injected in triplicates. This process was performed in Biochemistry laboratory of São Paulo University, Bauru-SP.

After filtering using the statistic patterns used by the expert center which considers the expression value with significance of $p<0.05$, the data were analyzed considering the following parameters: proteins with statistical difference of <0,05 were considered upregulated and proteins with statistical difference of <0.95 were considered downregulated.

RNA extraction mRNA

The extractions and purification of mRNA and mRNA purification were performed as previously described by Scarano et al, 2019 (Scarano et al., 2019). Briefly, the extractions were performed using Trizol (Ambion, USA) methods and then, total RNA was quantified using a NanoDrop (Thermo Scientific, USA) and the quality of the total RNA was measured with ribosomal RNAs by the RNA integrity number (RIN) using the 2100 Bioanalyzer system (Agilent, USA). Only RNA samples with RIN > 7 were used for subsequent analysis.

mRNA purification, library construction, and sequencing RNAs

Total RNA was sequenced using the HiSeq2500 platform (Illumina). An aliquot of total unfractionated RNA was submitted for library construction and sequencing. During library preparation, Ribo-Zero rRNA Removal Kit (Illumina, USA) was used for rRNA depletion. Messenger RNA purification and library construction were carried out with total RNA using the TruSeq Standard mRNA Sample Preparation Kit (Illumina), following the manufacturer's specifications. The sequencing was performed with the HiSeq Sequencing System.

The high sequencing analysis was performed following methods previously published by Scarano *et al* (2019). Briefly, the mRNA raw reads were carried out by FastQC v0.11.5 (Andrews, 2014) to inspect for low quality reads and adapter. The single-end were trimmed with trim-galore v0.5 and Cutadapt v1.8.1(martin, 2011 using default parameter. The preprocessed reads were aligned to *Rattus norvegicus* genome (assembly 6.0) using BWA and quantified using HTSeq. The filter standard for accounting for differentially expressed genes was to consider genes with an appropriate expression level with at least 5 normalized counts.

Integrative analysis of mRNA and proteomic profile

The data from differentially expressed mRNA (DER) published by Scarano *el at*., 2019, employing the same experimental design, were used here to perform the comparative analysis with differentially abundant protein (DAP). The proteome profile was obtained using methods described before. Here, the results obtained from DAP and DER were used to investigate targets commonly altered in the offspring of mothers exposed to a mixture of phthalate during gestation and lactation and to determine the later repercussions of the phthalate mixture exposure in adult offspring (PND120). The differentially expressed mRNA profile was obtained only on PND22 (data published), whereas the DAP profile was obtained for animals on PND22 and PND120. At PND22, the mRNA and protein data were crossed considering the deregulated targets in transcriptome and proteomic. Thereafter, common deregulated targets were crossed to get the common targets in the different ages and treatment groups (PND22 and PND120; 20µg/kg/day and 200mg/kg/day).

Functional enrichment and analysis

After identifying the targets differentially expressed in transcriptome and proteomic analysis on PND22 in both treatment groups and identifying the proteins that remained altered in the offspring on PND120, an enrichment analysis was performed to understand the interference of these targets on different pathways. All identified targets were analyzed together. The KOBAS 3.0 tool gathers data from different databases such as experimental validation data, computational prediction methods, and public text collections. Here, we used data of enrichment analysis REACTOME (www.reactome.org/) and KEGG (www.genome.jp/kegg) databases to perform the pathway enrichment analyses. The selected pathways were selected by FDR value and the data were represented by $-\log_{10}$ (FDR). The mentioned database gathers information from experiments performed in the human and animal experimental investigation and the data refer to genes and proteins differentially related to different biological pathways and processes. The Enrichment analysis was performed using Rattus Norvergicos (rno or RNO) as organisms for obtaining the data.

The second filter was made to understand the functional interaction among the targets identified in both treatment groups. For obtained the interaction analysis, the data were analyzed in STRING database (string-db.org/). The database builds the network and enriches analyses from protein data but gene data is often plotted to perform the analysis since the names and acronyms of gene and proteins are the same.

Soon after, a new enrichment analysis was performed to investigate the ontological terms associated with target dysregulation. The enrichment analysis was performed using the data extracted from the KOBAS 3.0 tool (kobas.cbi.pku.edu.cn/).

The K2Kweb tool (https://maayanlab.cloud/X2K/) is a database used to measure the combination of the differentially expressed gene with transcriptional factor, protein-protein interaction, and kinases. The database uses information collected experimentally on samples from humans and animals. This database was used to perform the transcriptional factor enrichment analysis (TFEA), kinase enrichment analysis (KEA), and kinase-transcription factor interaction (KTI). The threshold for the enrichment was showed by $-\log_{10}$ (p-value). After identifying the correlation among targets and transcriptional factors/protein kinases, further enrichment analysis was performed to investigate potential pathways deregulated by this interaction. The enrichment analysis was performed using the data extracted from the KOBAS 3.0 tool (kobas.cbi.pku.edu.cn/).

Target – Phthalate interaction

The Comparative Toxicogenomics Database (CTD) gathers information regarding the different chemicals, toxic substances and carcinogens or potential carcinogens already evaluated in the literature and correlates the targets (gene/proteins) of these substances. In addiction the database makes enrichment analyses for the different diseases that may be a consequence of this interaction, which affect human health. (https://ctdbase.org/about/)

Data representation

String database (https://string-db.org/) was used to obtain the interaction protein-protein (PPI) network. Sankey was used to show the association between the transcriptome and proteome data (https://sankeymatic.com/build/). Morpheus was used to make the heatmap plot (https://software.broadinstitute.org/morpheus/). GraphPad Prism (GraphPad Software©) was used to construct the bar plots. The package “circlize’ in environment RStudio software was used to create the circos plot.

Validation TCGA and HPA

The Cancer Genome Atlas (TCGA) and The Human Protein Atlas (HPA) databases were used to investigate whether the expression of targets modulated by early exposure to the phthalate mixture were altered in prostate adenocarcinoma of human. The common targets (gene) in our study and in TCGA were represented by log_2 and FDR. The targets (protein) available in HPA tool were represented by photomicrographs showing the positive marking to selected targets. The PROGgeneV2 (http://www.progtools.net/gene/results.php) was used to show the association with the Prognostic to Prostate Adenocarcinoma (PRAD) with the selected targets. All the databases mentioned above are databases that use data from humans' patients.

Nomeclatura

The described nomenclature for gene and protein follows the standards established for genes and proteins in animal and human models. As the data refer to an integrative analysis of gene and protein, the nomenclature will follow the established standards when dealing only with genes and when dealing with proteins. When the data are described as targets (gene/protein), the nomenclature used will be referent to protein, because the authors consider that exploring the proteins, which are functionally active in the organism, will help to elucidate the effect of the identified alterations with functional consequences for the morphophysiology and pathological conditions of the prostate gland. The same pattern was adopted when the data are referred to the analyzes for animals (*Rattus Norvergicous*) and humans (*Homo Sapiens*).

Results

To verify whether perinatal exposure to phthalate mixture during gestational and lactational development was able to induce tumors in aged offspring, histopathological analysis was performed at PND540. This analysis showed a significant increase in the incidence of *in situ* carcinomas (Fig. 1) in animals treated with 200 mg/kg/day compared to the control group (c=0 vs. 200mg/kg/day = 37.5%, $p<0.05$). There was no significant difference between control and animals exposed to the mixture at 20µg/kg/day.

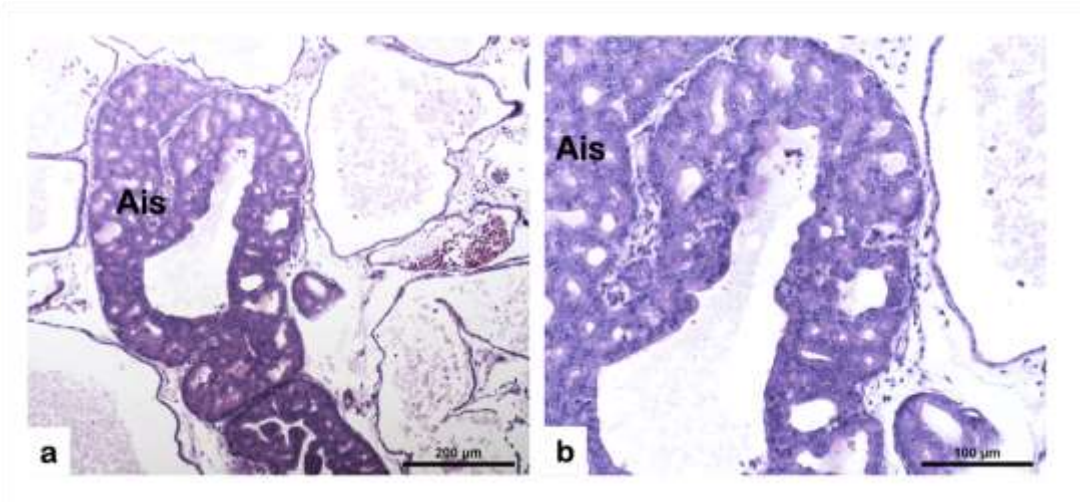

Figure 1: Histological sections of the prostate of animals at PND540 perinatally treated with 200 mg/kg. The figures are representative of the adenocarcinoma *in situ* with stratified epithelium with cell pleomorphism forming vascularized micro acini into the acini lumen.

Integrative analysis from the prostate transcriptome and proteome of offspring exposed to a phthalate mixture during gestation and lactation highlighted a set of eight common differentially expressed mRNA and proteins (*Aldoc* (ALDOC), *kif5c* (KIF5C) , *Rab3b* (RAB3B) , *Rab6b* (RAB6B), *Tubb4a* (TUBB4A), *Tubb2b* (TUBB2B), *Tubb3* (TUBB3), and *Ubxn11* (UBXN11) in the offspring exposed to 20µg/kg/day at PND22 and eight common differentially expressed mRNA and proteins (*Aldoc* (ALDOC), *L1cam* (L1CAM), *kif5c* (KIF5C), *Rab3b (*RAB3B*), Rab6b* (RAB6B), *Tubb2b* (TUBB2B), *Tubb3* (TUBB3) and *Tubb4a* (TUBB4A) in the offspring exposed to 200mg/kg/day at PND22 (Fig. 2). *Kif5c* (KIF5C), *Tubb4a* (TUBB4A), *Tubb2b* (TUBB2B), *Rab6b* (RAB6B), *Rab3b* (RAB3B) were commonly deregulated to both treatment groups. However, the *Ubxn11* (UBXN11) (gene and protein) was differentially expressed only in offspring of mothers exposed to 20µg/kg/day at PND22 and the *L1cam* (L1CAM) (gene and protein) was differentially expressed only in the 200mg/kg/day treatment group at PND22 and PND120 (fig. 2). The *Kif5c* (KIF5C) (gene and protein) was differentially expressed in both treatment groups only at PND22 (fig. 2). In parallel, the data obtained from proteome profiling in adult male offspring of exposed mothers (PND120) showed that seven targets remained deregulated in adulthood, with five targets commonly deregulated in both ages and treatment groups (RAB3B, RAB6B, TUBB2B, TUBB3, TUBB4A) and two targets commonly deregulated only in 200mg/kg/day treatment group (ALDOC AND L1CAM) (fig. 2).

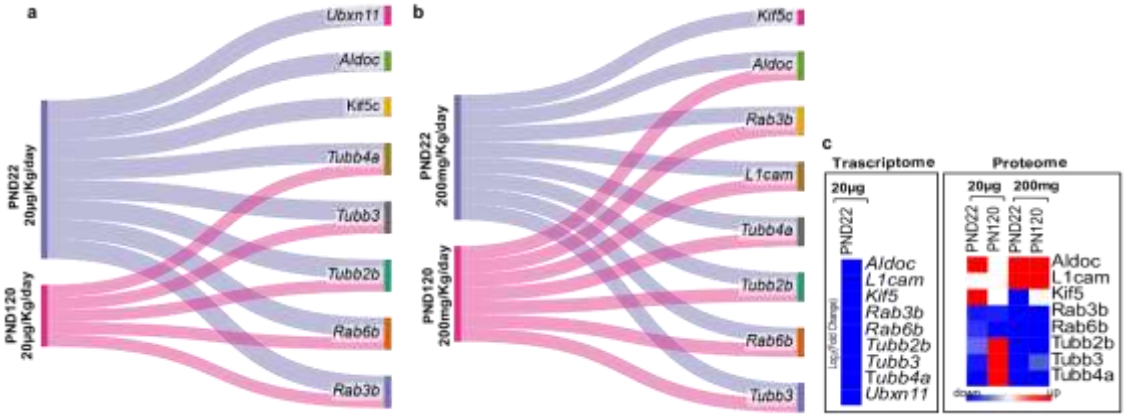

Figure 2: Sankey plot associating the targets differentially expressed in transcriptome (PND22) and differentially abundant in proteome (PND22 and 120) that remained altered in both treatments and ages. In the group exposed to 20µg/kg/day of phthalate mixture it was possible to identify eight targets (*Aldoc, kif5c, Rab3b, Rab6b, Tubb4a, Tubb2b, Tubb3,* and *Ubxn11*) commonly deregulated in both transcriptome and proteome at PND22 and five of these targets (*Rab6b* and *Rab3b, Tubb2b, Tubb3, Tubb4a*) remained deregulated in the proteome at PND120. In the group exposed to 200mg/kg/day, it was possible to identify eight targets (*Aldoc, L1cam, Kif5c, Rab3b* and *Rab6b, Tubb2b, Tubb3,* and *Tubb4a*) differentially expressed in both transcriptome and proteome at PND22 and seven targets (*Aldoc, Rab3b, Tubb4a, Tubb2b, Rab6b and Tubb3*) remained deregulated in the proteome at PN120. The commonly deregulated targets in both treatment groups were *Tubb4a, Tubb3, Tubb2b, Rab6* and *Rab3b*. **a.** Sankey plot showing the differentially expressed targets in VP of offspring exposed to 20µg/kg/day at PND22 (transcriptome and proteome) and PND120 (proteome). **b.** Sankey plot showing the differentially expressed targets on VP of offspring exposed to 200mg/kg/day at PND22 (transcriptome and proteome) and PND120 (proteome). **c.** Heat map showing the genes and proteins status. For genes data were normalized to $\log_2$ fold change and for proteins data are separated into p-value (Up, $p<0.05$ and down, $p > 0,95$). The data comes from transcriptome analysis for animals on PND22 exposed to 20ug;/kg/day (published by Scarano 2019). Also, at right, a heat map showing the protein abundance status at both ages (PND22 and PND120) of animals exposed to the treatments (red: upregulation and blue: downregulation).

The enrichment analysis was performed using all targets (*Rattus Norvergicus, protein*), including the target L1cam, which was exclusive of offspring exposed to 20µg/kg/day and Ubnx11, which was exclusive of offspring exposed to 20mg/kg/day. The results showed that five targets were common in different pathways of interest (*Tubb3, Rab3b, Tubb2b, Tubb4a* and *L1cam*) (fig. 3a-b). This analysis identified three terms involved with cell cycle, and terms associated with gap junction, the recycling pathway of L1, the HSP90 chaperone cycle for steroid hormone receptors, hemostasis, and post-translational protein modification (PTPM) (fig. 3a-b).

Enrichment analysis of potentially deregulated pathways as a function of deregulation of differentially expressed targets in this investigation showed that at least 29 pathways can be modulated by deregulation of these targets (Table 1). The PPI network showed interaction among four proteins (Tubb3, Tubb2b, Tubb4a and Kif5c - *Rattus Norvergicus, protein*) commonly deregulated in both treatment groups (fig. 4a). These targets were involved in ontological terms associated with interference in the cell cycle such as mitotic cell cycle, microtubules, GTPase activity and GTP binding (gene and protein) (fig 4b).

Table 1: Table showing enrichment analysis data for pathways modulated by the differentially expressed targets. The enrichment analysis was performed using rattus norvergicos as organism (rno or RNO).

REACTOME		
Term	$-\log_{10}$(FDR)	Target
RNO-437239 - Recycling pathway of L1	5,879426	Tubb3, Tubb4a, L1cam, Tubb2b
RNO-190840 - Microtubule-dependent trafficking of connexons from Golgi to the plasma membrane	4,809668	Tubb2b, Tubb4a
RNO-5626467 - RHO GTPases activate IQGAPs	4,400117	Tubb2b, Tubb4a
RNO-8955332 - Carboxyterminal post-translational modifications of tubulin	4,400117	Tubb2b, Tubb4a
RNO-9668328 - Sealing of the nuclear envelope (NE) by ESCRT-III	4,400117	Tubb2b, Tubb4a
RNO-9646399 - Aggrephagy	4,268411	Tubb2b, Tubb4a
RNO-6811436 - COPI-independent Golgi-to-ER retrograde traffic	4,066513	Tubb2b, Tubb4a
RNO-5620924 - Intraflagellar transport	4,044793	Tubb2b, Tubb4a
RNO-3371497 - HSP90 chaperone cycle for steroid hormone receptors (SHR)	3,958607	Tubb2b, Tubb4a
RNO-983189 - Kinesins	3,920819	Tubb2b, Tubb4a
RNO-8852276 - The role of GTSE1 in G2/M progression after G2 checkpoint	3,69897	Tubb2b, Tubb4a
RNO-380320 - Recruitment of NuMA to mitotic centrosomes	3,60206	Tubb2b, Tubb4a
RNO-6807878 - COPI-mediated anterograde transport	3,420216	Tubb2b, Tubb4a
RNO-6811434 COPI-dependent Golgi-to-ER retrograde traffic	3,420216	Tubb2b, Tubb4a
RNO-9648025 - EML4 and NUDC in mitotic spindle formation	3,366532	Tubb2b, Tubb4a
RNO-5610787 - Hedgehog off state	3,309804	Tubb2b, Tubb4a
RNO-2500257 - Resolution of Sister Chromatid Cohesion	3,29243	Tubb2b, Tubb4a
RNO-5663220 - RHO GTPases Activate Formins	3,221849	Tubb2b, Tubb4a
RNO-2132295 - MHC class II antigen presentation	3,075721	Tubb2b, Tubb4a
RNO-109582 - Hemostasis	2,886057	Tubb3, Tubb4a, L1cam, Tubb2b
RNO-2467813 - Separation of Sister Chromatids	2,886057	Tubb2b, Tubb4a
RNO-597592 - Post-translational protein modification	1,737549	Rab3b, Tubb4a, Tubb2b, Tubb3
RNO-168256 - Immune System	1,313364	Aldoc, Tubb4a, Tubb2b, Tubb3
KEEG		
no04540 - Gap junction	3,366532	Tubb2b, Tubb4a
rno05010 - Alzheimer disease	3,366532	Kif5c, Tubb4a, Tubb2b, Tubb3
rno05012 - Parkinson disease	3,366532	Kif5c, Tubb4a, Tubb2b, Tubb3
rno05014 - Amyotrophic lateral sclerosis	3,366532	Kif5c, Tubb4a, Tubb2b, Tubb3
rno05016 - Huntington disease	3,366532	Kif5c, Tubb4a, Tubb2b, Tubb3
rno05020 - Prion disease	3,366532	Kif5c, Tubb4a, Tubb2b, Tubb3
rno04145- Phagosome	2,853872	Tubb2b, Tubb4a

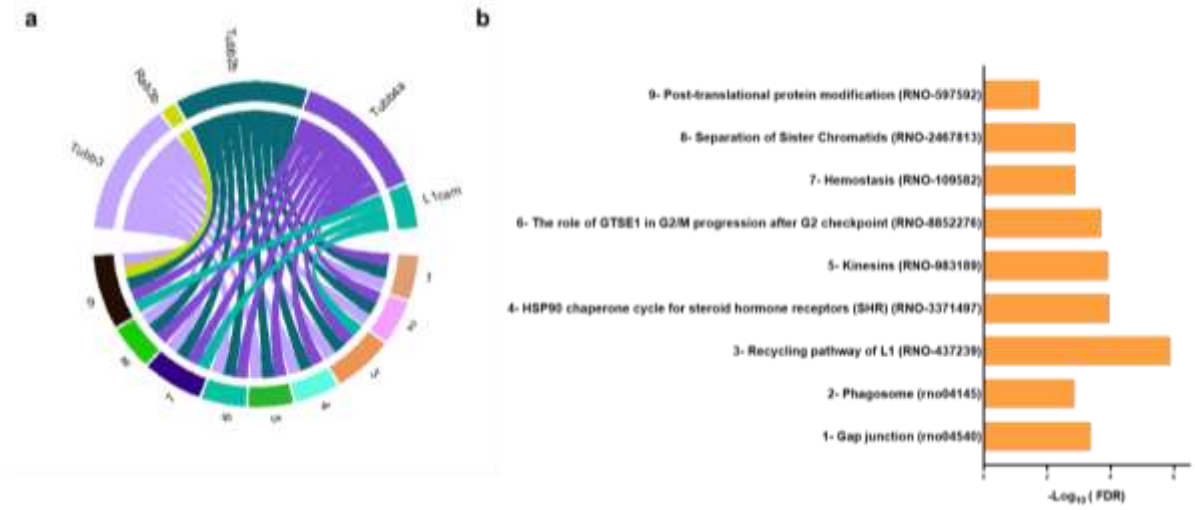

Figure 3: a. Circos Plot highlighting the relationship between targets and cell pathways. The first enrichment analysis was done considering all differentially expressed targets, including *Lcam1* and *Ubxn11* targets to understand how the interaction between them was possible. **b.** Bar graph representing the main pathways influenced by the deregulated targets differentially expressed in offspring exposed to a phthalate mixture (20µg/kg/day and 200mg/kg/day) at PND22 (transcriptome and proteome) and PND120 (proteome). The graph was made considering the -$\log_{10}$ of FDR. The enrichment analysis was performed using rattus norvergicos as organisms for obtaining the data.

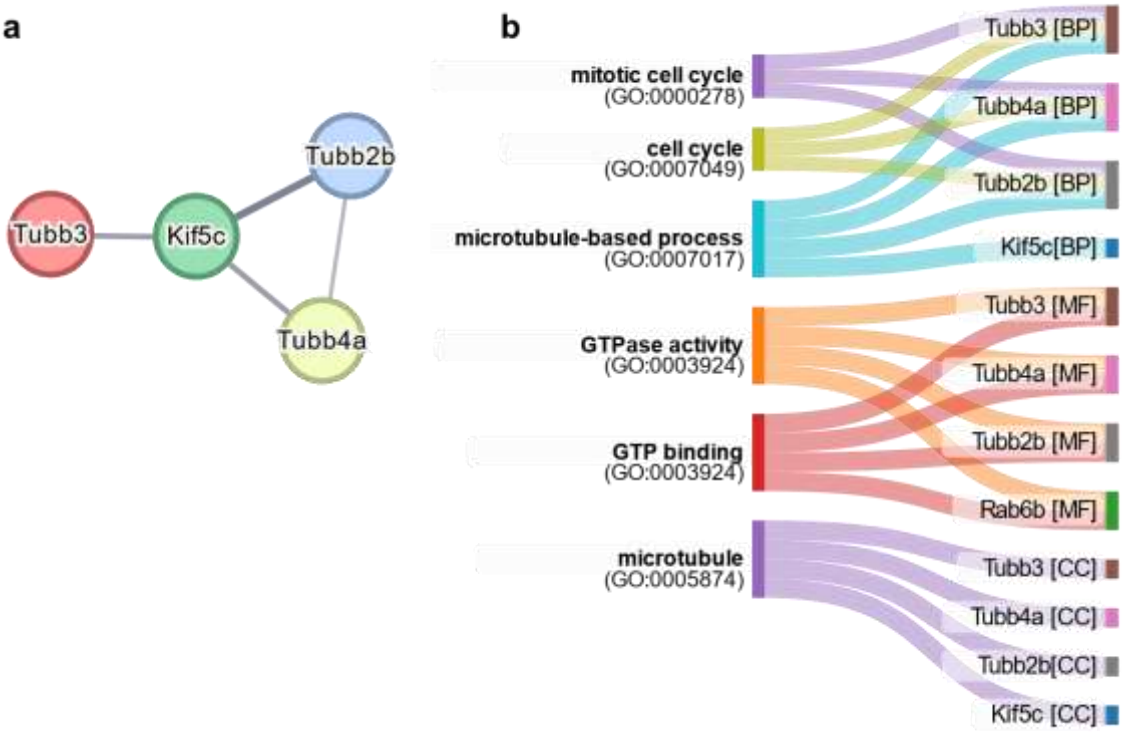

Figure 4: a. The PPI network shows the interaction of the targets differentially expressed on VP of offspring exposed to a phthalate mixture. The PPI was made in string database. **b.** Sankey plot showed the association of targets differentially expressed in VP with the ontological terms (BP: Biological process; MF: Molecular function and CC: Cellular component). The enrichment analysis was performed using rattus norvergicos as organisms for obtaining the data.

The differentially expressed targets identified interact with transcriptional factors (TF). This association pointed to influences in the activity of protein kinases, essential for cell signaling activities, such as maintaining control of the cell cycle (Fig. 5 a-b and Supplementary Figure 2). In contrast, the enrichment performed considering the pathways potentially deregulated as a consequence of the Target -Factor transcriptional - kinases interaction pointed to signaling pathways commonly deregulated in prostatic pathologies such as PI3K-AKT, cGMP-dependent protein kinase G (cGMP-PKG), estrogen, tumor necrosis factor alpha (TNF-α), ras-proximate 1 (Rap1), rat sarcoma virus (Ras), vascular endothelial growth factor (VEGF), cellular senescence; autophagy, and immune system signaling pathway (fig. 6).

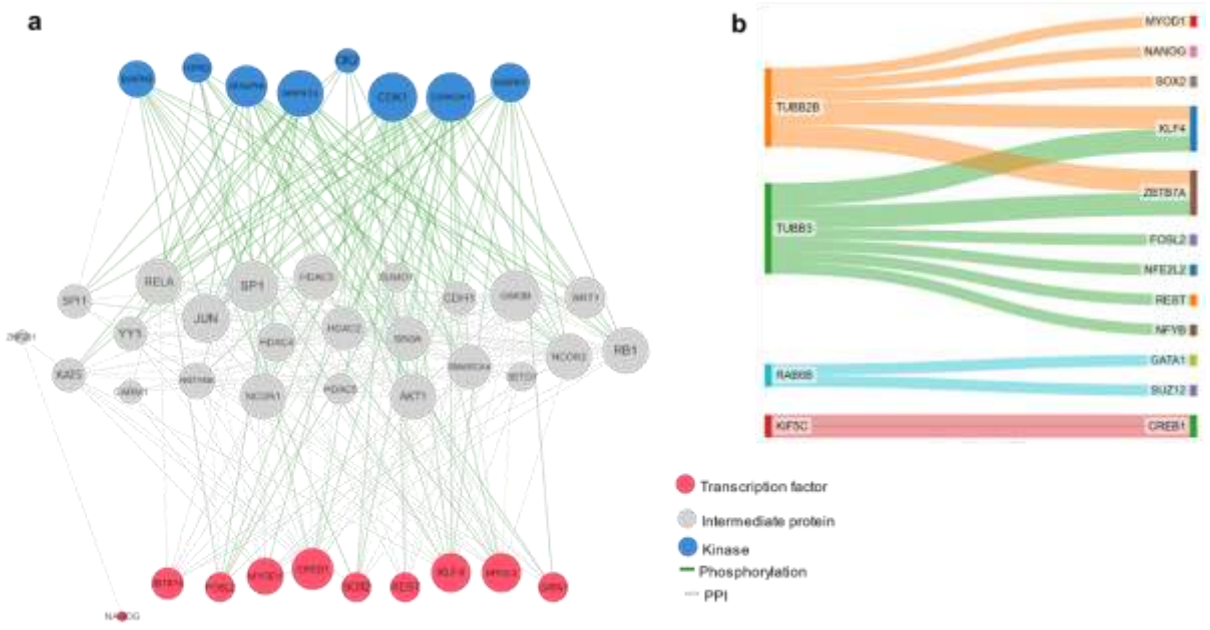

Figure 5: a. Kinase-Transcription Factor Interaction (KTI) network. Red color is referent to transcription factor; Blue color is referent to kinase proteins; grey color is referent to intermediate proteins and green color is referent to phosphorylation. The data were obtained using the *k2kweb* tool. **b.** Sankey plot pointing the relation among the differentially expressed targets with the *TFEA* data. The database uses information collected experimentally on samples from humans and animals.

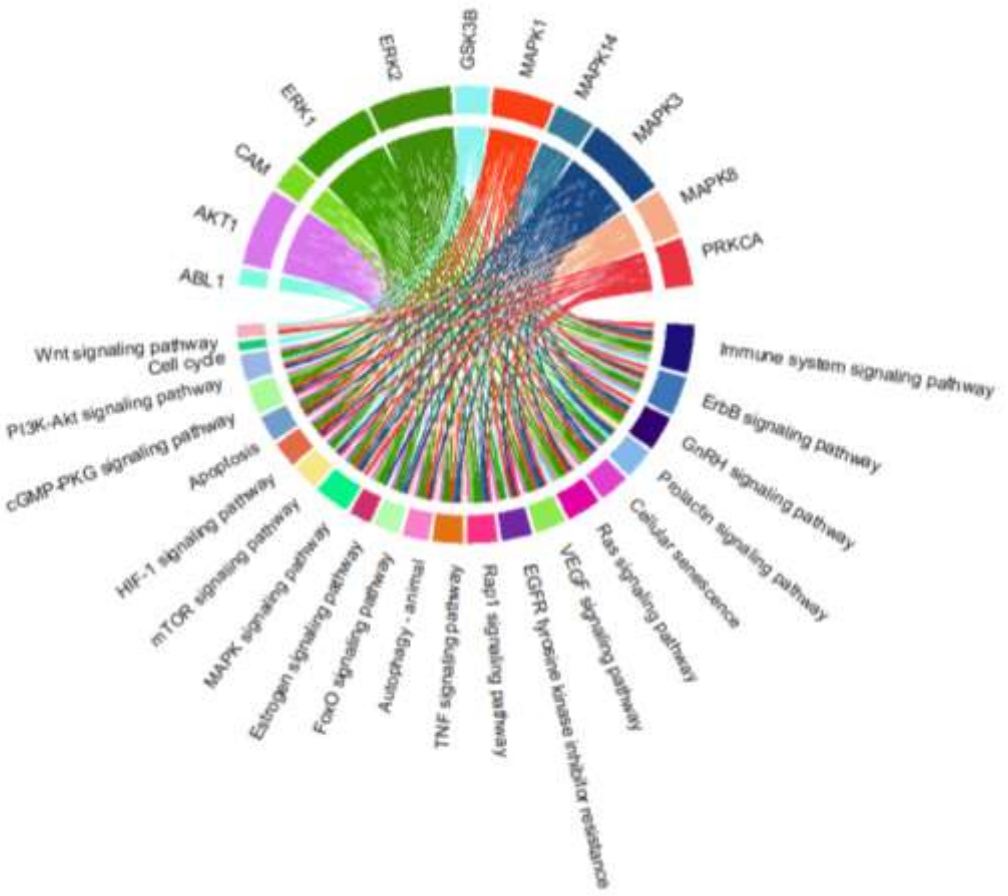

Figure 6: Circos plot showing the top twenty-two pathways regulated by the seventeen-kinesis highlighted in the KEA Enrichment that can be modulated by the targets pointed out in this investigation (TUBB3, TUBB2A, RAB6B and KIF5C). The nomeclature refers to protein descriptions for humans because there is much more enrichment from humans' investigation than animal studies for this analysis.

Combining differentially expressed targets identified in this investigation of potential targets with the *TCGA* database data, the results showed six common genes identified in prostatic adenocarcinoma (PRAD) and downregulated in this investigation (*UBXN11, KIF5C, RAB3B, RAB6B, TUBB3 and L1CAM* – Homo sapiens, gene). In contrast, comparing the results with the data available in *HPA* database, it was possible to identify positive reactivity for seven common proteins in prostate adenocarcinoma (fig.7). Furthermore, the analysis in *PROGgeneV2* pointed to the up-regulation of three genes (*ALDOC, TUBB2B AND UBX11* – Homo sapiens, protein) and decrease in expression of four genes (*KIF5C, RAB3B, RAB6B, TUBB3*), likely with worse cancer prognosis (Supplementary Figure 3).

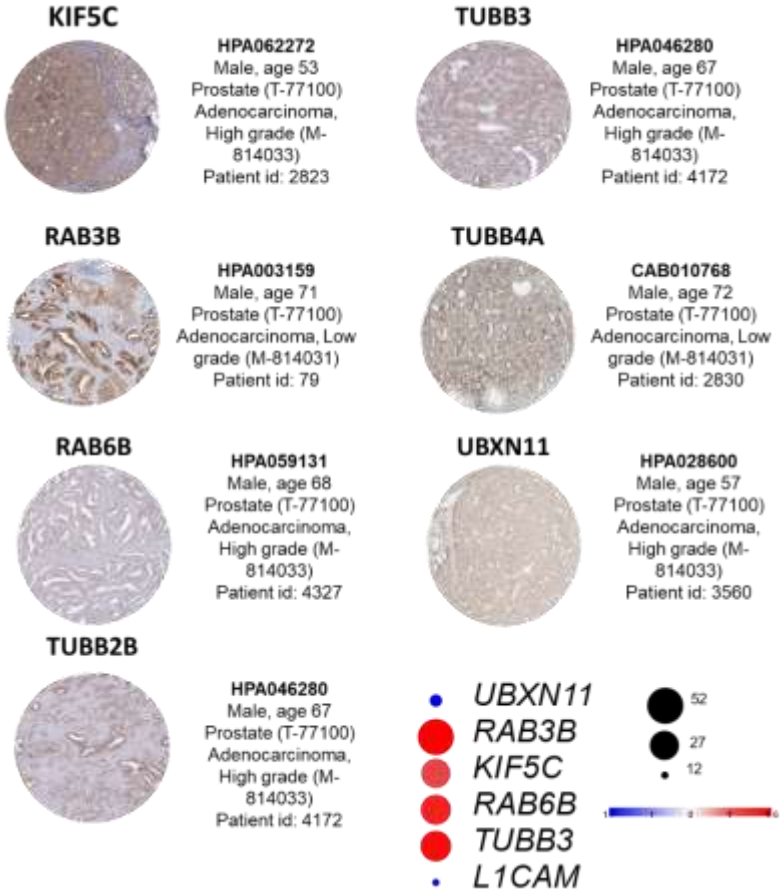

Figure 7: Histological photomicrograph showing the positive reaction (by immunohistochemistry) of seven proteins in prostate adenocarcinoma (KIF5C, RAB3B, RAB6B, TUBB2, TUBB3, TUBB4A, UBXN11–*Homo sapiens*, protein). On the right side of the figure, a heatmap representing the deregulated expression of the six genes in PRAD (*UBXN11, KIF5C, RAB3B, RAB6B, TUBB3 AND L1CAM –Homo sapiens*, gene) was observed. The data used in this representation considers log2(fold change) and FDR. The data about the proteins expression was obtained in HPA database and the data obtained to deregulated gene was obtained in TCGA database.

Discussion

The integrative analysis of mRNA and proteins that were deregulated in the offspring after phthalate mixture exposure during gestation and lactation can improve our knowledge about maternal influence in the organogenesis of the prostate during a susceptible window of development. Previously, we described that maternal exposure to a phthalate mixture increased the incidence of reproductive disorders in both male and female offspring (Scarano et al. 2019; Gonsioroski et al. 2022), including multigenerational effects in

female offspring (Gonsioroski et al. 2022). Here, we showed that the maternal exposure to a phthalate mixture modulated targets *Aldoc, kif5c, Lcam1, Rab3b, Rab6b, Tubb4a, Tubb2b, Tubb3,* and *Ubxn11* in different proportions at both low (20 μg/kg/day) and high (200 mg/kg/day) doses in prepubertal and adult offspring. In addition, the results pointed to the regulation of potentially oncogenic targets in the prostate in both treated groups and ages, reinforcing the results previously obtained in previous publication, which showed inflammatory and premalignant histopathological changes in prostates of offspring after maternal exposure to di-n-butyl-phthalate (DBP) during the gestation and lactation (Scarano et al. 2009; Peixoto et al. 2016). Here, using the phthalate mixture in a maternal exposure model, was observed an increase in the incidence of adenocarnoma *in situ* in aged offspring.

The Comparative Toxicogenomic database showed that the targets modulated by the mixture in this study have been described as interacting with different phthalates. Toxigenomic assays have associated different phthalates with endocrine system disorders, urologic and male genital diseases, adenocarcinoma, and prostatic diseases (Singh and Li 2011). In most cases, epigenetic modifications can be associated with disorders in later life, but environmental exposures are also known to increase susceptibility to these pathological conditions (Dutta et al. 2020; Ho et al. 2017). According to Sigh and Li (2011), the effects of early phthalate exposure are associated with both the embryonic period and time of exposure. In our experimental model, offspring were exposed during the most critical windows for urogenital tract development, and therefore, it was expected that the treatment would modulate important pathways related to prostate development. The histopathological results showed that the exposure to a phthalate mixture in this critical period was able to increase the susceptibility to prostate adenocarcinoma *in situ*, suggesting that important pathways involved in urogenital development and oncogenesis may be disturbed by an environmental stressor.

Phthalates are known to bind endogenous ligands and consequently to interfere in sexual development (Watkins et al., 2014), metabolism (Wittassek and Angerer, 2008; Zhang et al., 2020), development, and stress response (Schug et al., 2011). The placenta is an organ with high metabolic activity and it is responsible for endocrine control (Tang et al., 2020). Endocrine disruptors such as phthalates cross the placental membrane (Schug et al., 2011; Tang et al., 2020) and the relation of phthalate exposure in the placenta is associated with losses in signaling pathways such as PPAR, EGF, EGFR, PI3K/AKT and ERK (Tang et al., 2020). The consequences of this interference can impair placental development, efficiency, and disturbances in tissues differentiation. In addition, diesters of phthalate such

as DBP, DEHP, and DiNP and their metabolites were detected in human breast milk in different concentrations (Fan et al., 2019; Frederiksen et al., 2007). *In vivo* investigation showed that maternal gestational and lactational phthalate exposure impacted the offspring (Ahmad et al., 2014; Curi et al., 2019; Gonsioroski et al., 2022; Sekaran and Jagadeesan, 2015; Wang et al., 2017b). Data presented in this manuscript support the DOHaD hypothesis, which associates pathological disorders with insults during in the main developmental windows of the individual (Gardner 1995; Barker 1997; Soubry 2018; Wadhwa et al. 2009).

Integration of the results from the transcriptome and proteomic analyzes showed that regardless of the interaction among the nine targets differentially expressed at PND22, *Kif5c, Tubb4a, Tubb2b, Tubb3* and *L1cam* targets were related to post-translational protein modification (PTPM) and *Tubb4a, Tubb2b, Tubb3* and *L1cam* targets were important for regulation of homeostasis processes. In contrast, *Tubb4a, Tubb2b, Tubb3* were related to pathways that modulate cell cycle and gap junction structure/function. In tumor conditions, the homeostasis process is impairment as a consequence of the reduction on signaling cascade that controls cytokine/growth factor-independent growth, cell-cell adhesion, and alteration in extracellular matrix (Orend, 2005).

Here, the results pointed to *Tubb3, Rab3b, Tubb2b, Tubb4a* and *L1cam* as potentially deregulated by maternal phthalate exposure and these targets were associated with post-translational protein modification (PTPM), or post-translational modification (PTM). Disorders in different isoforms of tubulins (Tubbs) have been associated with PTMs and human cancer (Lopes and Maiato, 2020). PTM consists of phosphorylation, acetylation, glycosylation, lipidation, and other modifications (Khoury et al., 2011) and was observed in different models of EDCs exposure (Kim et al., 2022). According to Li et al. (2020), DBP was able to cause obesity when mice were treated during gestation by a mechanism associated with endoplasmic reticulum stress (Li et al., 2020). Moreover, phthalates can modulate post-translational modification of histones, which could affect epigenetic marks during development (Xin et al., 2015). Therefore, one of our hypotheses is that phthalate exposure could modulate PTMs and increase dysregulation in cell signals related to prostate development and differentiation.

Different studies using isolated or mixed phthalates have demonstrated alterations in genes related to the cell cycle *in vivo* (Craig et al., 2013; Gonsioroski et al., 2022; Meling et al., 2020) and in *in vitro* (Cavalca et al., 2022; Di Lorenzo et al., 2018; Zhou and Flaws, 2017). Flaws and collaborators using isolated phthalates (Craig el at., 2013) or a mixture (Zhow, and Flaws, 2017) that was the same as used in

this work, demonstrated changes in cell cycle regulatory proteins, stopping the cell cycle in antral follicles. Di Lorenzo et al. (2018) demonstrated that DBP was able to alter the cell cycle by stopping some phases by acting on regulatory proteins, in an AR-independent and ER-dependent mechanism in LNCaP prostatic cells. Recently, Cavalca and collaborators (2022) showed that prostate cells exposed to a mixture of monoesters of phthalate interfere in the expression of genes involved directly with cell cycle pathway. In this sense, it is possible that the phthalate mixture can modify the cell cycle and potentiate the errors during cell division, which could contribute to the oncogenic process.

Gap junctions are extremely important in epithelial tissue, including prostate epithelium because they maintain cell-cell interaction. In prostate differentiation, gap junctions are responsible for controlling epithelial cell proliferation and prostate budding (de Freitas et al., 2016; Scarano et al., 2018; Thriene et al., 2018). However, structural disturbances in adjacent cells through the connexins and cytoskeletal components of gap junctions may be related to prostate cancer development, as well as tumor progression and invasion (Czyz et al., 2012). Gap junction stability is directly related to alterations in structural proteins such as tubulin, N-cadherin, α-β Catenin (Czyz et al., 2012). DEHP has been shown to deregulate cell-cell communication by decreasing or inhibiting gap junction pathways in different types of cells (Di Lorenzo et al., 2018; Du et al., 2021). In addition, the DEHP exposure was able to impair intercellular junctions, such as gap and tight junctions in Sertoli and Leydig cells in the testes of fetal and young rats (PND30) (Di Lorenzo et al., 2020; Sobarzo et al., 2009), suggesting that exposure during prostate development may make the epithelium more instable and susceptible to dysplasia in adulthood.

The KTI (Kinase-Transcription Factor Interaction) network showed eight transcription factors (TFs) that can be phosphorylated by one or more kinases, and that *Kif5c, Tubb2b, Rab6b,* and *Tubb3* were the differentially expressed targets with the highest interaction with the TFs. In this sense, this correlation among targets highlighted in this investigation with potential TFs and kinases allowed the establishment of an association with pathways commonly altered in pathological conditions, especially in prostate cancer. According to Samaržija (2021), pathways such as PI3K-AKT signaling are the main targets of epigenetic and post-translational modifications (PTMs) in prostate cancer. Furthermore, pathological conditions such as different types of cancer are associated with dysregulation in steps of post-translational modification by alteration in tumor suppressor and oncogenic proteins for TFs or signaling molecules (Samaržija, 2021).

Among the TFs, KLF4 (Transcription Factor 4) and ZBTB7A (Zinc Finger and BTB Domain containing 7A) were evidenced by potential interaction with *Tubb2b* and *Tubb3*. The ZBTB7A is a TF that

act as proto-oncogene by regulation of tumor suppressors that inhibit tumor invasion (Wang et al., 2013). The KLF4 also is a TF associated with cell proliferation, differentiation, apoptosis, and pluripotent stem cells, especially in epithelial cells. Further, KLF4 has an important role during differentiation and organogenesis of different tissues (Ghaleb et al., 2005; Ghaleb and Yang, 2017). According to enrichment analysis, KLF4 interacts with 10 important kinases and is involved in maintaining the integrity of the cell cycle and may have a tumor suppressor effect (Ghaleb et al. 2005). In prostate cancer, the expression of KLF4 was associated with proliferation and tumor progression (Shin et al., 2014) and down-regulation was associated with an increase aggressiveness in tumor cells (Zhang et al., 2019). Further, 2,4-DTT, one type of EDC, was able to downregulate KLF4 expression in breast cancer cells (Kalinina et al., 2021). In this sense, it would be possible that early exposure to phthalate mixture is associated with cell cycle damage, increasing the susceptibility to develop prostate disorders. According to the survival data presented in this investigation, the downregulation of KIF5C, RAB6B and TUBB3 may be associated with a worse prognosis in prostate adenocarcinoma (PRAD), which draws attention to the late repercussions of exposure, mainly in individuals more susceptible to prostatic lesions.

Our study highlights new and important findings by presenting a new approach considering posttranscriptional events after phthalate exposure by OMICs analyses. However, our study has some limitations. The mechanisms and the TF–Kinases interactions explored in the *in silico* analysis have not been deeply tested in vitro or in vivo models. Functional tests were not performed to mimic or block the pathways identified in this study. Despite this, the more frequent tumor phenotype in old animals exposed to the mixture, the differentially expressed sets of genes and proteins, make our study an important advance in developmental environmental toxicology. Future studies should delve deeper into the mechanistic and epigenetic aspects associated with the model addressed in this investigation.

Conclusion

Collectively, our data indicate that maternal exposure to a mixture of phthalates during pregnancy and lactation may influence cellular pathways associated with development, differentiation, maintenance of epithelial phenotype and cell survival in the offspring prostate. Additionally, many of the targets remained altered until adulthood, which together with other genetic and epigenetic factors, may increase the oncogenic potential in the prostate, explaining the increased incidence of tumors in aged offspring exposed

to the mixture. Finally, this study demonstrates the important actions of phthalates as stressors during prostate development as well in increasing the risk for oncogenesis.

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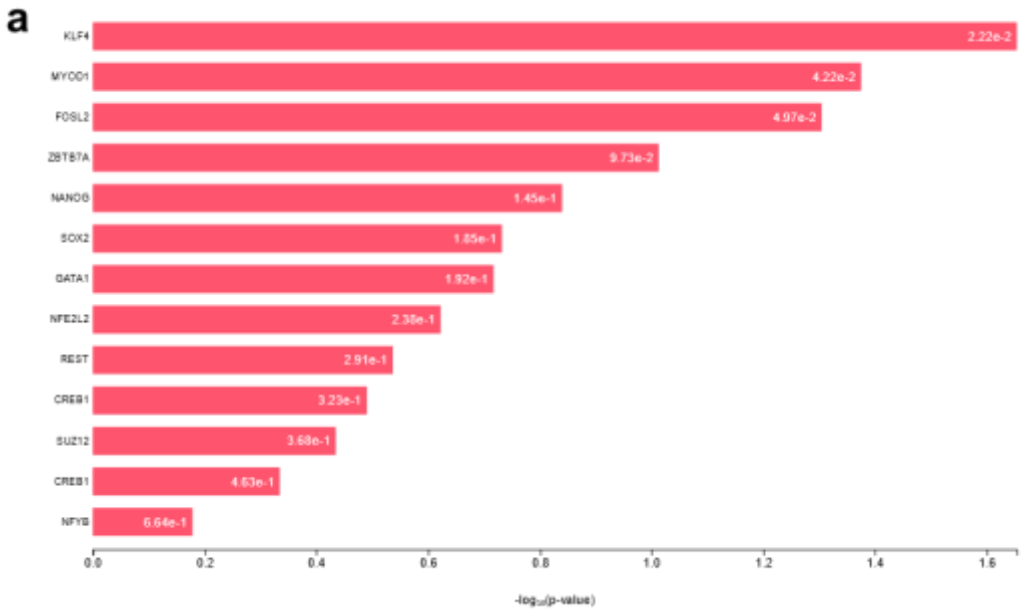
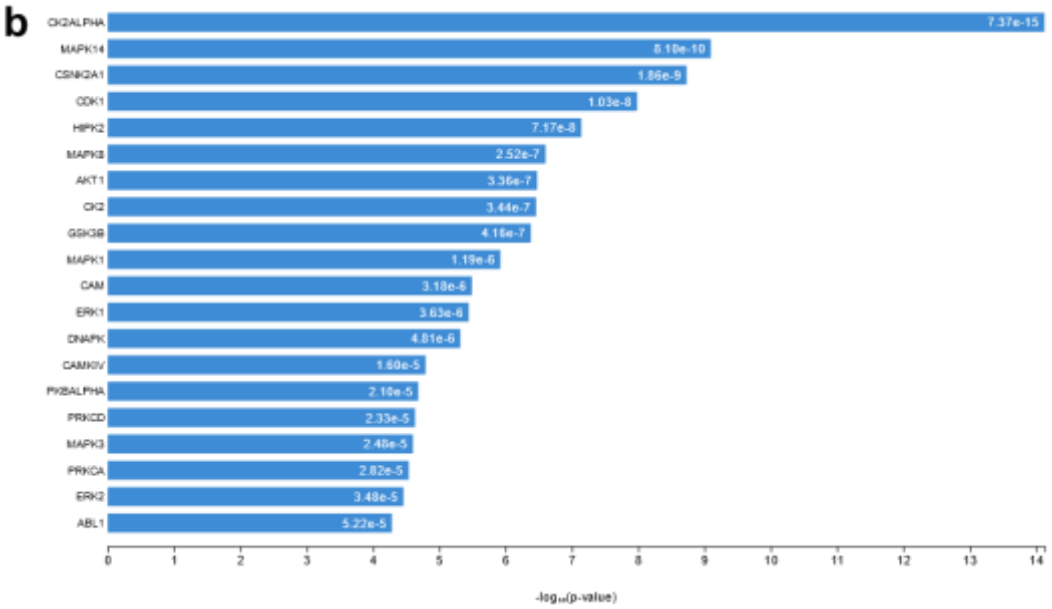
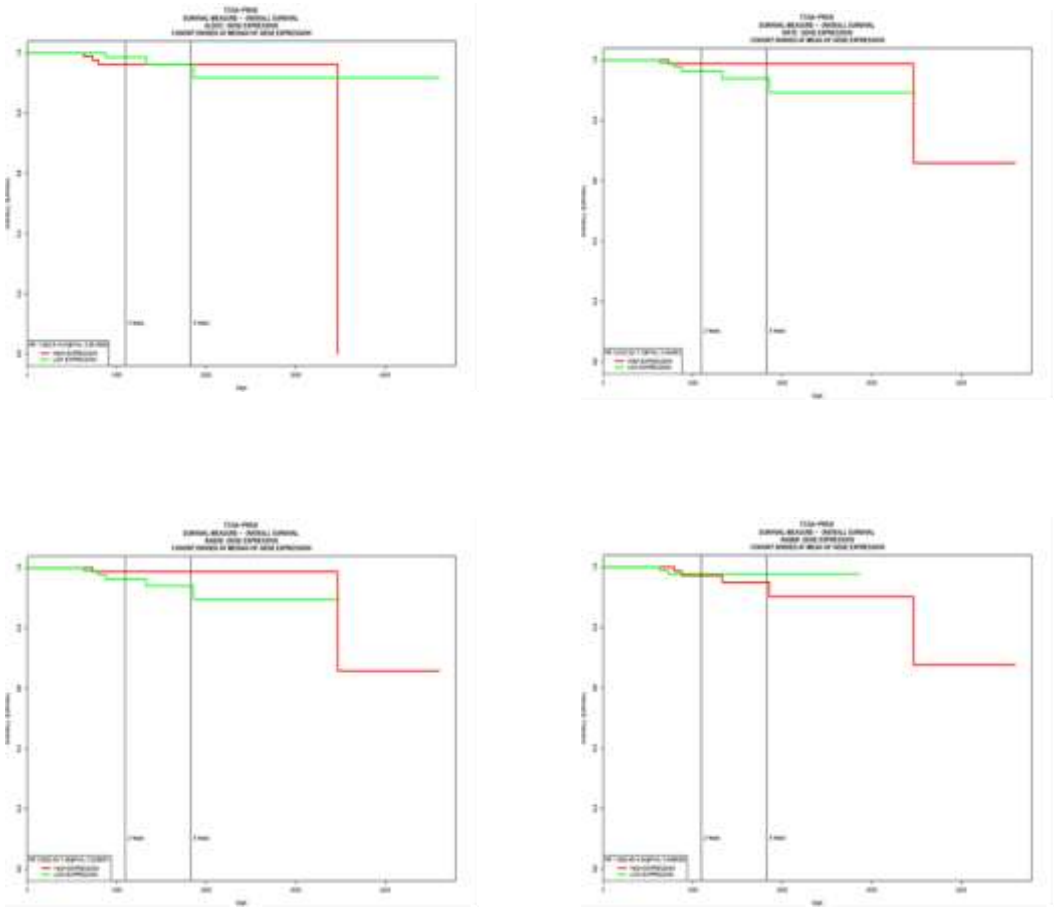
Supplementary Figures

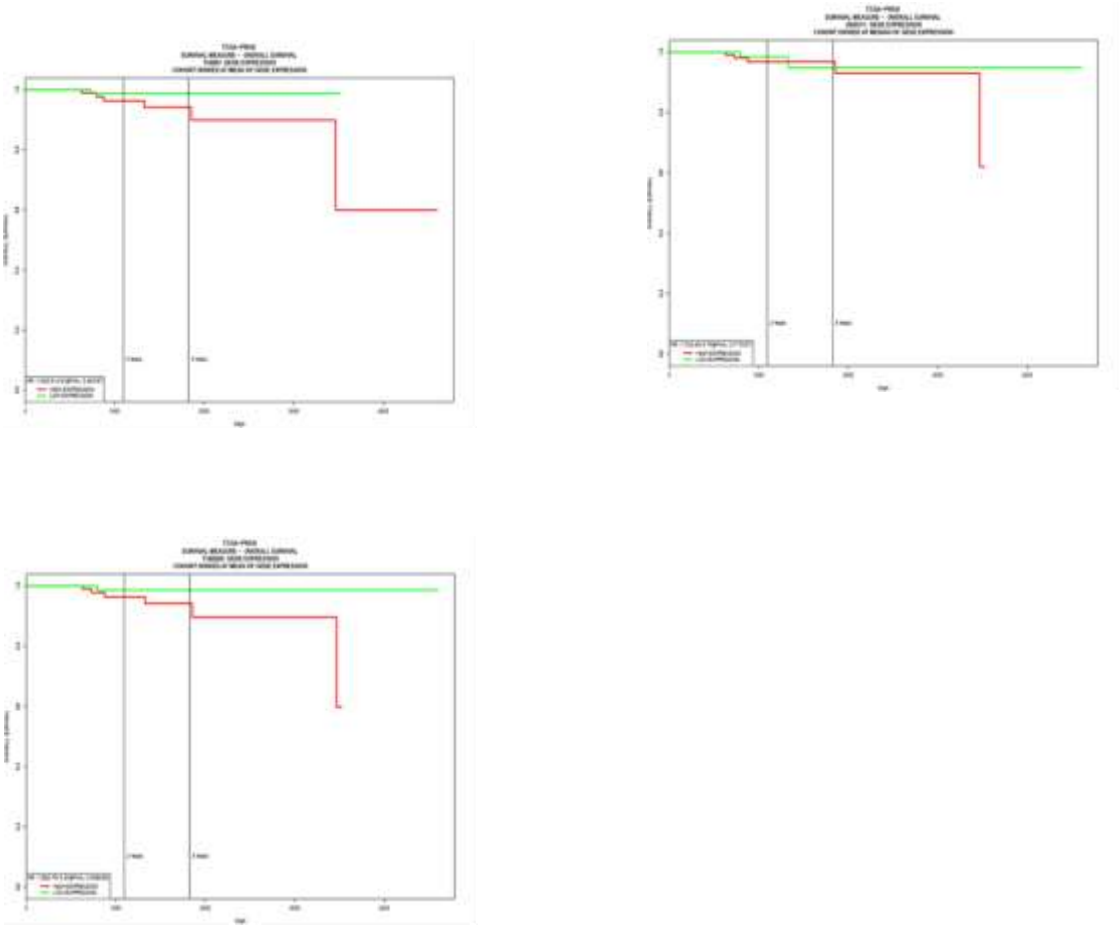
Figure 1: a. Veny plot built on the comparative toxicogenomic database platform showing the relationship of the phthalates DBP, DEHP and phthalic acid with the differentially expressed targets in the transcriptome and proteome of this investigation**. b.** Sankey plot highlighting the targets that was describe by interaction with DBP, DEHP and Phthalic acid.





Supp. Fig. 2: a. Bar graph showing the *Transcriptional Factor Enrichment Analysis (TFEA*). In this analysis it was possible identify that relation among the target differentially expressed identified in this study with thirteen transcriptional Factors (TFs). **b.** Bar graph showing the *Kinase Enrichment Analysis (KEA*): twenty kinases can be regulated by one or more TFs.





Supp. Fig. 3: Survival graphs showing the relation of up-expression of three gene (*ALDOC, TUBB2B* and *UBX11*) and down-expression of four gene *(KIF5C, RAB3B, RAB6B* and *TUBB3*) with the worst prognostic. The data of the gene expression in PRAD was obtained in TCGA database.

Capítulo IV

Artigo científico

Lista de figuras – Capítulo IV

Prenatal exposure to a phthalate mixture alters prostatic proteome profile in f1 and f2 generations of rats

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Abstract

The programming of the offspring through maternal insult in a critical window of development can generate late and transgenerational modifications. Endocrine disrupters (EDs) act as environmental factors and trigger a series of epigenetic changes in development, causing reproductive, neurological, and behavioral imbalances, among others. Phthalates are chemical substances widely used in the plastics and cosmetics industry. Extensive studies show the presence of different phthalates in maternal-fetal tissue. Here, the prostate proteomic profile from descendants (F1 and F2) was mapped to set up the perturbations resulting from maternal exposure to a phthalate mixture in a similar proportion to that found in the urine of pregnant women. Pregnant rats were randomly distributed at three experimental groups (C: control (vehicle); T1: 20µg/kg/day and T2: 200mg/kg/day (by gavage). The pregnant rats were treated from gestational day 10 (GD10) to postnatal day 21 (PND21). The F1 offspring was euthanized in two different moments: F1-PND22 and F1-PND120. On PND90 treated males and females were mated with no exposed animals to generate F2. F2 descendants from treated F1 mothers (F2-PND22-M) and fathers (F2-PND22-F) were euthanized on PND22. The proteomic profile showed that the maternal exposure to a mixture phthalate could change and keep proteins deregulated among ages and generations. These proteins enriched molecular pathways extremely essential to morphogenesis, differentiation, and maintenance of prostate gland in F1 and F2 descendants. Additionally, our results showed the gender influence in the transmitting acquired modifications from F1 to F2, and interestingly, F2-descendants from exposed F1-male presented more deregulated

proteins than offspring from F1-females. Finally, our data showed that many altered proteins among ages and generations are involved in prostate oncogenesis and probably some of them are responsible for prostate disorders observed in previous studies; however, they need to be deeply investigated.

key words: Phthalate mixture, offspring, DOHaD, proteome and prostate cancer.

Abbreviation: PCa, Prostate Cancer; EDCs, Endocrine Disrupting Chemicals; DOHaD, Developmental Origin of Health and Disease; GD, Gestational day; no-coding RNA molecules (sncRNAs), PND, Post-Natal day; DEHP, , DEP, Biethyl phthalate; DBP Di-n-butyl phthalate, DiBP, Diisobutyl phthalate; BBzP, Butyl benzyl phthalate; DiNP, Diisononyl phthalate, BPA, Bisphenol A, MEHP mono (2-ethylhexyl) phthalate, VP, Ventral prostate, IPP Protein-Protein Interaction network (IPP), HPA, The Human Protein Atlas.

Introduction

The prostate gland has an important contribution in the survival of the male germ cell and in the reproductive successful (Sánchez et al. 2013). Furthermore, the gland is hormone dependent and is susceptible to perturbation in different phases of the development. In rodents and humans, prostate morphogenesis starts *in utero* and finishes completely only in pre-pubertal phase (Marker et al. 2003; Gail S. Prins and Putz 2008; Gail S Prins et al. 2007). this sense, the environmental conditions, which individuals are exposed during the intrauterine and postnatal development, can be responsible to alter and increase the susceptibility of different disorders prostatic in adulthood and in the senescence (X. Wang et al. 2017a; Alcantara Santos et al. 2020).

There are a growing number of the research showing that gestational period can generate developmental perturbations with late repercussions, based in the Barker hypothesis (D. J. Barker et al. 1989). Currently, there is an understanding that not only the adverse maternal conditions can permanently program the offspring, but there are

other developmental critical periods including perinatal, neonatal, lactational, prepubertal and pubertal periods, since the late organogenesis of different tissues occur in those moments. (Soubry 2018; Almeida et al. 2019; S. M. Ho et al. 2017).

All the hormone dependent organs are susceptive to be altered by direct interference of the Endocrine Disrupting Chemicals (EDCs) in any moment of life, but the fetus and the newborn represent vulnerable populations to exposure to EDCs, both are critical window of the development and chronic exposure is potentially damage (Annamalai and Namasivayam 2015; Repouskou et al. 2019; Chiang, Mahalingam, and Flaws 2017; Wellerson R. Scarano et al. 2018).

Phthalates are commonly used as plasticizers to increase the flexibility of plastic products. Nowadays, it is possible identify use of phthalate in different products as cosmetics, pharmaceutical, children toys, food packaging, clothing, cleaning materials, insecticides and others (Qureshi et al. 2016; Schettler et al. 2006a). The structural characteristic of these molecules does not allow covalently bound to the products in which they are used, this makes contaminate route diverse (Bosnir et al. 2003; Fromme, n.d.). As EDCs, Phthalate has been especially studies for their antiandrogenic activity (Fisher 2004).

Phthalate has a damage action in reproductive system when isolated or in combination with another toxic substance (Pant et al. 2008; Y. H. Zhang et al. 2008; Meling et al. 2020; Warner et al. 2019). Previous study showed increase in the prevalence of prostatic disorders in offspring of rats exposed to phthalate in intra-uterine development (Wellerson R Scarano et al. 2019; S.-M. Ho et al. 2017a; X. Wang et al. 2017a). Experimental study has associated fetal and neonatal exposure to phthalates exposure with an increase to susceptibilities at prostatic carcinogenesis and more incidence and severity of prostatic lesions (Wellerson Rodrigo Scarano et al. 2009; Peixoto et al. 2016; X. Wang et al. 2017a). Recently, it was showed the maternal exposure also can increase the susceptibility to mammary carcinogenesis in offspring (T. de Freitas et al. 2021a). Furthermore, the phthalate mixture was able to modulate the expression of microRNA and mRNA of prostate of the offspring maternal exposure

(Wellerson R Scarano et al. 2019). These results display an important posttranscriptional damage.

Recently, there are a growing number of the research showing that the gestational period can generate maternal insults with late repercussions, based in the finding of Barker (D. J. Barker et al. 1989). Nowadays, there is an understanding about maternal condition can permanently program the offspring and the most of this research are investigating another critical period of the development, including perinatal, neonatal, postnatal and puberal development, because of the late organogenesis of the different tissues and the late repercussions in the quality of life and association with the prevalence of disease of present time, which is know how “Developmental origins of health and Disease” (DOHaD) hypothesis (Soubry 2018; Almeida et al. 2019; S. M. Ho et al. 2017).

The maternal insults can determine the quality of offspring life, modulating important p molecular parameter as epigenetic and posttranscriptional aspects, which can modify genes and proteins expression altering the development, differentiation and maturation of organs and tissues. Previous results showed phenotypic alterations in prostate after isolated phthalate and mixture exposure, however, a few studies have elicited molecular pathways involved in those effects. Additionally, multigenerational, and transgenerational effects have been shown to be an extremely important approach in toxicological. Therefore, we hypothesized that prenatal exposure to an environmentally relevant phthalate mixture would modify multigenerational and transgenerational effects on prostate proteomic profile. To test our hypothesis, we dosed pregnant and lactating female rat with different concentration of phthalate mixture and evaluated the male offspring born to these exposed dams for two generations. The F2 analyzed males were obtained from both F1 exposed and males and females to compare possible variable gender dependent.

2. Material and Methods

2.1. Experimental Design

Sprague-Dawley rats (male and female), weighing average 300g, were supplied by Multidisciplinary Center for Biological Research (CEMIB/UNICAMP) and they were kept during the experimental period in the Biotherium of Small Mammals from Department of Structural and Functional Biology, Institute of Biosciences (UNESP). The rats were bred under controlled conditions of light (12 hours of light/12 hours of dark) and temperature (average of 23°C -25°C) and received water and food *ad libitum.* This study was carried out in accordance with the Brazilian Council on Animal Experimentation Control (CONCEA) and the protocol was approved by the Ethical Committee on Animal Use of the Institute of Biosciences, São Paulo State University (Protocol: 1040/CEUA).

After identifying the presence of sperm in vaginal fluid, the pregnant rats were randomly divided into three experimental groups (n=10/group): Group C: control group (vehicle: corn oil); T1: 20µg/kg/day and T2: 200mg/kg/day of the mixture of phthalates diluted in corn oil by gavage). The phthalate mixture was diluted in tocopherol-stripped corn oil (vehicle control). The lowest dose was selected to mimic daily human exposure levels based on the amount of DEHP, and our higher dose was selected to compare our results with available single phthalate studies (Zhou et al., 2017). The animals from T1 and T2 received the respective doses of the phthalate's mixture in the following proportion: 21% DEHP (Bis (2-ethylhexyl) phthalate), 35% DEP (Diethyl phthalate), 15% DBP (Di-n-butyl phthalate), 8% DiBP (Diisobutyl phthalate), 5% BBzP (Butylbenzyl phthalate), and 15% DiNP (Diisononyl phthalate).The treatment period was from gestational day 10 (GD10) to postnatal day 21 (PND21) as described previously by Scarano (2019) (Wellerson R Scarano et al. 2019).

Pregnant and lactating females treated with the mixture were designated as F0 generation. The offspring from F0 generation were named F1 generation. Eight pregnant female rats were used in each group to produce F1 generation. After birth, the number of F1 offspring per litter was reduced to eight (1: 1 ratio between males and females whenever possible), and litters with fewer than six pups were euthanized. To guarantee the independence of treatments and litters, the experimental unit was the litter and not the individual (Brandt et al. 2014). Eight adult males and females from F1 were used in

each group to produce F2. Therefore, in this study to obtain F2 generation, F1 males and females were mated with non-treated animals.

The experimental design was conducted into three steps: gestational and lactational exposure (first step), F1 adulthood evaluation (second step), and F2 evaluation produced by F1 males and females (third step). In the end of first step, on PND21, F1 animals were weaned and on PND22 four animals by litter were euthanized (pentobarbital anesthesia followed by heart puncture) and the ventral prostate (VP) was collected to proteomic analysis.

The other F1 animals were kept under normal housing conditions and without treatment until adulthood. On PND90, F1 males and females were mated with unexposed animals to produce F2 generation (named as PND22-M, from F1 treated mothers; and PND22-F, from F1 treated fathers). On PND120, four F1 animals (different litters) were euthanized (pentobarbital anesthesia followed by heart puncture) and the VP was collected to proteomic analysis (second step). To the third step, F2 male descendants from F1 generation were analyzed. On PND22, F2 males were euthanized as described earlier and VP was collected to proteomic analysis (Fig. 1).

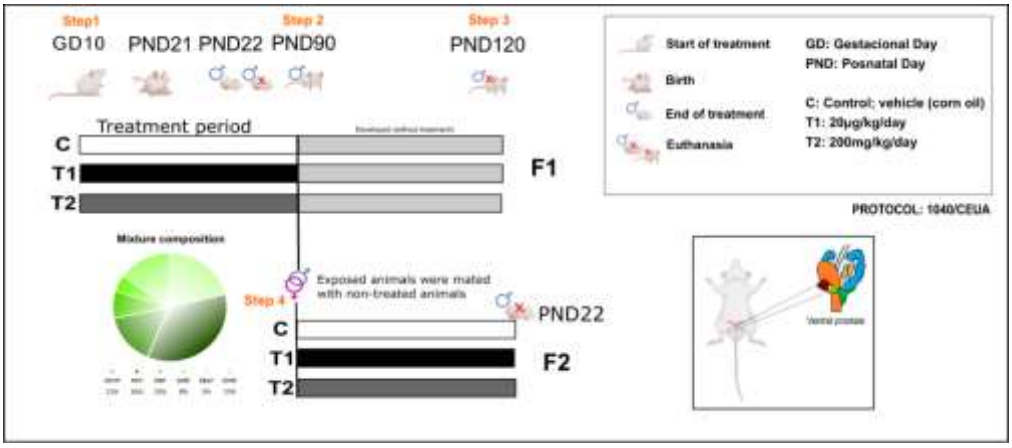


Figure 1: Detailed experimental design (n= 4/litters). The VP sample was collected in three moment F1 - PND22: F1 - PND120, and F2 - PND22-M and PND22-F. All the animal picture used in this figure was obtained BioRender platform.

2.2 Mass Spectrometry

2.2.1 Sample extraction

The protocol was performed according to parameters used in Biochemistry laboratory of São Paulo University, Bauru-SP. Briefly, for protein extraction, the VP sample of all the timepoint of this investigation was carried out by homogenizing in extraction buffer containing 0.01 M Tris-HCl, 0.005 M phenylmethylsulphonyl fluoride, 1% protease inhibitor, 0.065 M dithiothreitol, 8 M urea (in a proportion of 30mg tissue/100 µg buffer). The homogenate was vortexed for 2-3 min and centrifuged for 15 min at 9,690 g and 4°C. The supernatant was recovered, and the total protein was quantified by Bradford assay using the BSA standard (Bradford 1976). Samples were grouped to constitute three pools of 50µg proteins each in a total of 50µL (1 µg/µL). In the next, the samples were incubated for 60 min at 37°C with 10µL of 50 mM ammonium bicarbonate and 25 µL of 0.2% surfactant solution, followed by incubation with 2.5 µL of 0.1 M dithiothreitol for 40 min at 37°C. Carbamidomethylating was performed with 2.5µL of 0.3 M iodoacetamide, incubated for 30 min at room temperature, and protected from light. After, samples were subjected to proteolytic digestion overnight at 37°C using 0.05 µg/µL trypsin diluted in 0.05 M Ammonium bicarbonate, followed by incubation with 10µL trifluoroacetic acid 5% for 90 min at 37°C. The samples were centrifuged at 14,000 RPM 4ºC for 30 minutes. After this step, the samples were desalted using Sep-Pak Vac C18 (Waters Manchester, UK) columns, reduced in a concentrator, and maintained at -20°C until the time of analysis by mass spectrometry.

For the mass spectrometry four VP samples from each time (by group were used. The analysis was performed in a nanoACQUITY UPLC system (Waters, Milford, MA) coupled to a Xevo Q-TOF G2 mass spectrometer (Waters, Milford, MA). The nanoACQUITY UPLC system is equipped with a Trap Column (responsible for chromatography; Trap Column: 100Å, 5 µm, 180 µm x 200 mm) previously equilibrated with 99.9% phase A (0.1% formic acid in water) at a flow of 5 µl/min and a HSS T3 M-Class type column (analytical column; Acquity UPLC HSS T3 M-Class column 75 µm x150 mm; 1.8 µm) (Waters, Milford, MA), previously equilibrated with 93% mobile phase A and mobile phase B (0.1% formic acid in ACN). Peptides were separated by a linear gradient of 7–85% mobile phase B for 70 min with 0.35 µL/min flow rate; the column

temperature is maintained at 45 °C. The Xevo G2 Q-TOF mass spectrometer was operated in positive nano-electrospray ion mode and data were collected using MSE method in elevated energy (19-45V). Source optimal conditions include capillary voltage, 2.8kV; sample cone, 40V; extraction cone, 3.0V and source temperature 100°C. However, source conditions may vary due to detector and lockspray voltage setups. Data acquisition occurred over 70 min, and the scan range was 50-1500 Da. The lockspray was run with a [Glu1] fibrinopeptide solution (1pmol/µL) at a flow rate of 0.3 µL/min, as a reference ion in positive mode at m/z 785.8427. ProteinLynx GlobalServer software (PLGS) version 3.03 (Waters, Milford, MA) was used to process and search the LC-MSE continuum data. The identification of islet proteins was performed using the ion accounting algorithm incorporated into the software and a search of the Rattus norvegicus database from the UniProtKB catalog (Universal Protein Resource) (http://www.uniprot.org). This process was performed in the Biochemistry laboratory of São Paulo University, Bauru-SP. All samples were injected in triplicates. It was used the p-value ($p<0,05$ – downregulated and $p>0.95$ – upregulated) and “exclusivity” of the proteins into each group to determine differentially expressed proteins among the experimental groups (C-T2).

2.3 Functional enrichment

After filtering the up and down regulate proteins, the data was crossed using the venny tool (http://bioinformatics.psb.ugent.be/webtools/Venn/) to compare the proteins that remained unregulated in the two ages of F1 and in the offspring (F2) in all the treatment groups compared to the control group. The data was analyzed in the STRING tool (string-db.org/) to obtain the protein-protein interaction network (PPI). The IPP network was constructed using all active interaction sources and highest confidence (0.9) and high confidence (0.7) to confirm the strength of the data.

The KOBAs 3.0 (kobas.cbi.pku.edu.cn/) was used to determine the enrichment of pathways and ontological terms related to the differentially expressed proteins in our experimental groups. This tool gathers information from three different data banks, KEEG (www.genome.jp/kegg), PANTHER (pantherdb.org) and REACTOME

(www.reactome.org/cgi-bin/eventbrowser_st_id?ST_ID=R-RNO-1430728), and all of them was used to obtained the results. The enrichment pathways were performed using the statistical method: hypergeometric test/Fisher's exact test (p-Value) and FDR correction method: Benjamini and Hochberg (adjusted p-value). It was selected the pathways with corrected p-value (<0,05) and the data was represented in $-\log_{10}$ (p-Value corrected). The REVIGO toll (http://revigo.irb.hr) was used to make a cluster of the ontological terms (p-Value) and the represented graph was made in RStudio.

2.4 Data representation

We used the GraphPad Prism (GraphPad Software) to construct the Bar plots; the webserver Venny 2.1.0 (bioinfogp.cnb.csic.es/tools/venny/) to obtain the Venn diagrams; The package "circlize' in environment RStudio software to create the circus plot and the SnakeyMatic (sankeymatic.com/) to create Alluvial diagram.

2.5 Validation

"The Human Protein Atlas" platform (www.proteinatlas.org/) was used to investigate and validate proteins identify in this research in the normal and in the tumor sample of human patient with different age.

3. Results

The proteomic profile showed that the phthalate mixture exposure (both doses) was able to deregulate 118 proteins at F1-PND22 (33 up and 85 downregulated); 190 proteins at F1-PND120 (13 up and 177 downregulated); 172 proteins at F2-PND22-M (10 up and 162 downregulated) and 221 proteins at F2-PND22-F (190 up and 31 downregulated) (Figures 2a and b). For this exploratory study, the results will be presented comparing ages and generations, and only differentially expressed proteins between treatment (T1 and T2) and control will be considered.

When we compare upregulated proteins among ages and generations two by two, 1 protein was commonly upregulated in both ages in F1-PND22 and PND120, 2

proteins were upregulated in F1-(PND120 and F2-PND22-F), 5 proteins were upregulated in F1-PND22, and F2-PND22-F and 1 protein was commonly upregulated in F2-PND22-F and F2-PND22-M (Figures 2a and Supplementary Table 1). There was no functional interaction among the five upregulated proteins in F1-PDN22 and F2-PND22-F (Supplementary Fig 11a and Table 2), but when the individual proteins are investigated, is possible establish the functional importance by the cluster of interaction with another proteins (Supplementary Fig 1b-c).

The Pfn1, an actin-binding protein, was the only protein upregulated after the mixture exposure in both F1-PND22 and F1-PND120, while the immunoglobulin complex (Igg-2a) was upregulated in F1-PND120 and F2-PND22-F (Supplementary Table 1). Five proteins (Gdi2, Hnrnpa2b1, Myl4, Prdx1, Rps7) were upregulated in both F1-PND22 and F2-PND22-F, and Csrp1 was positively regulated in F2-PND22-F and F2-PND22-M after the phthalate mixture exposure (Fig. 2a and Supplementary Table 1).

There were observed 54 commonly downregulated proteins among ages and generations after the exposure (Figure 2b). When we compare downregulated proteins among ages two by two, 7 proteins were commonly downregulated in F1-PND22 and F1-PND12). At first generation, the functional interactions are between 2 Nucleoside diphosphatase kinase (Nme), proteins associated with synthesis of nucleoside triphosphates and interaction between 2 tropomyosin (Tpm), proteins that act stabilizing cytoskeleton actin filaments (Fig. 1b and Supplementary Table 1 and Fig2).

Comparing F1 and F2 generations, early phthalate mixture exposure was able to downregulate 38 proteins in F1-PND22 and F2-PND22-F, and 16 proteins in F1-PND22 and F2-PND22-M and deregulated 31 proteins in F2-PND22-F and F2-PND22 (Fig 2b and Supplementary Table 1). These results were briefly explored to investigate the main common alteration among the generation and the alteration transmitted by the exposed parent (Fig2b). Additionally, it was identified 6 downregulated proteins in the comparing F1-PND120, and F2-PND22-F and the treatment negatively regulates 24 proteins F1-PND120 and F2-PND22-M (Figure 2b; Supplementary Table 1). Additionally, it was observed downregulation in 35 proteins in F1-PND22, F2-PND22-2 and F2-PND22M; 18 proteins in F1-PND120, F2-PND22-F and F2-PND22M; 6 proteins in F1-PND22, F1-

PND120 and F2-PND22-M; 22 proteins in F1-PND120, F2-PND22-F and PND22-M (Supplementary Table 1)

The proteomic profile showed that the phthalate mixture exposure (both doses) was able to deregulate 118 proteins at F1-PND22 (33 up and 85 downregulated); 190 proteins at F1-PND120 (13 up and 177 downregulated); 172 proteins at F2-PND22-M (10 up and 162 downregulated) and 221 proteins at F2-PND22-F (190 up and 31 downregulated) (Figures 2a and b). For this exploratory study, the results will be presented comparing ages and generations, and only differentially expressed proteins between treatment (T1 and T2) and control will be considered.

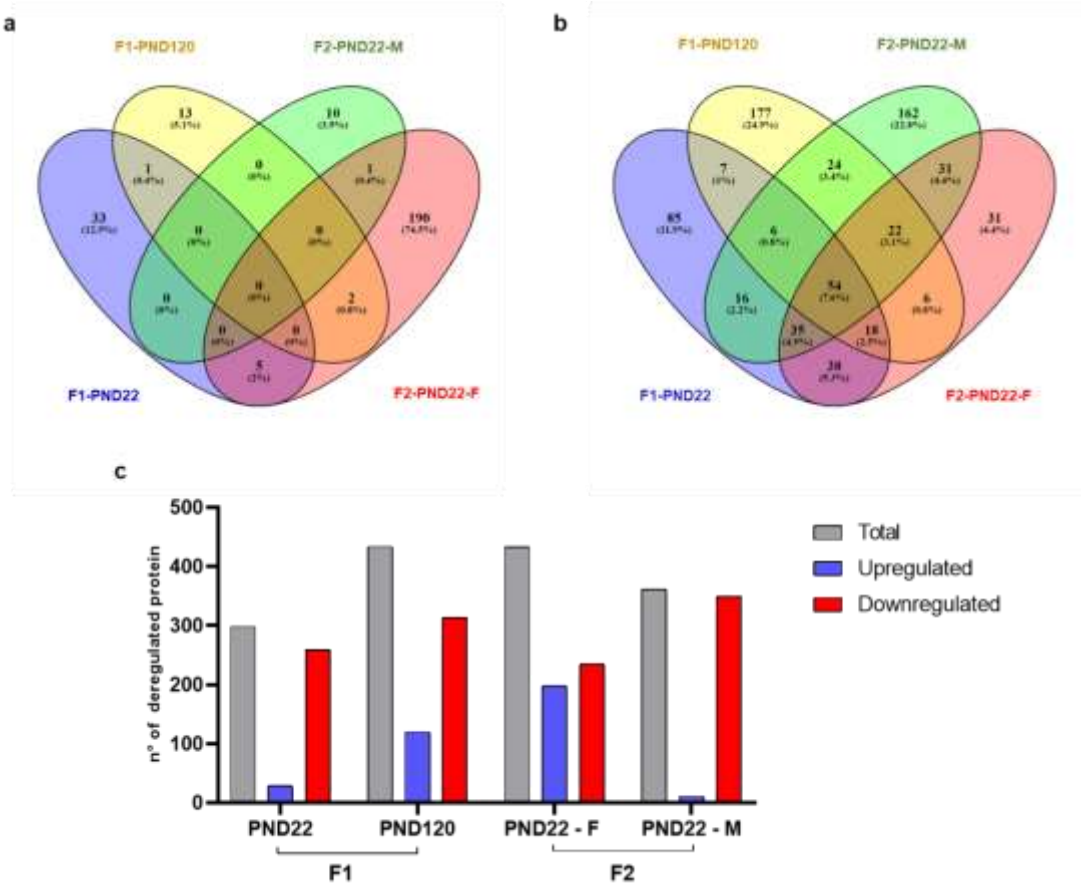
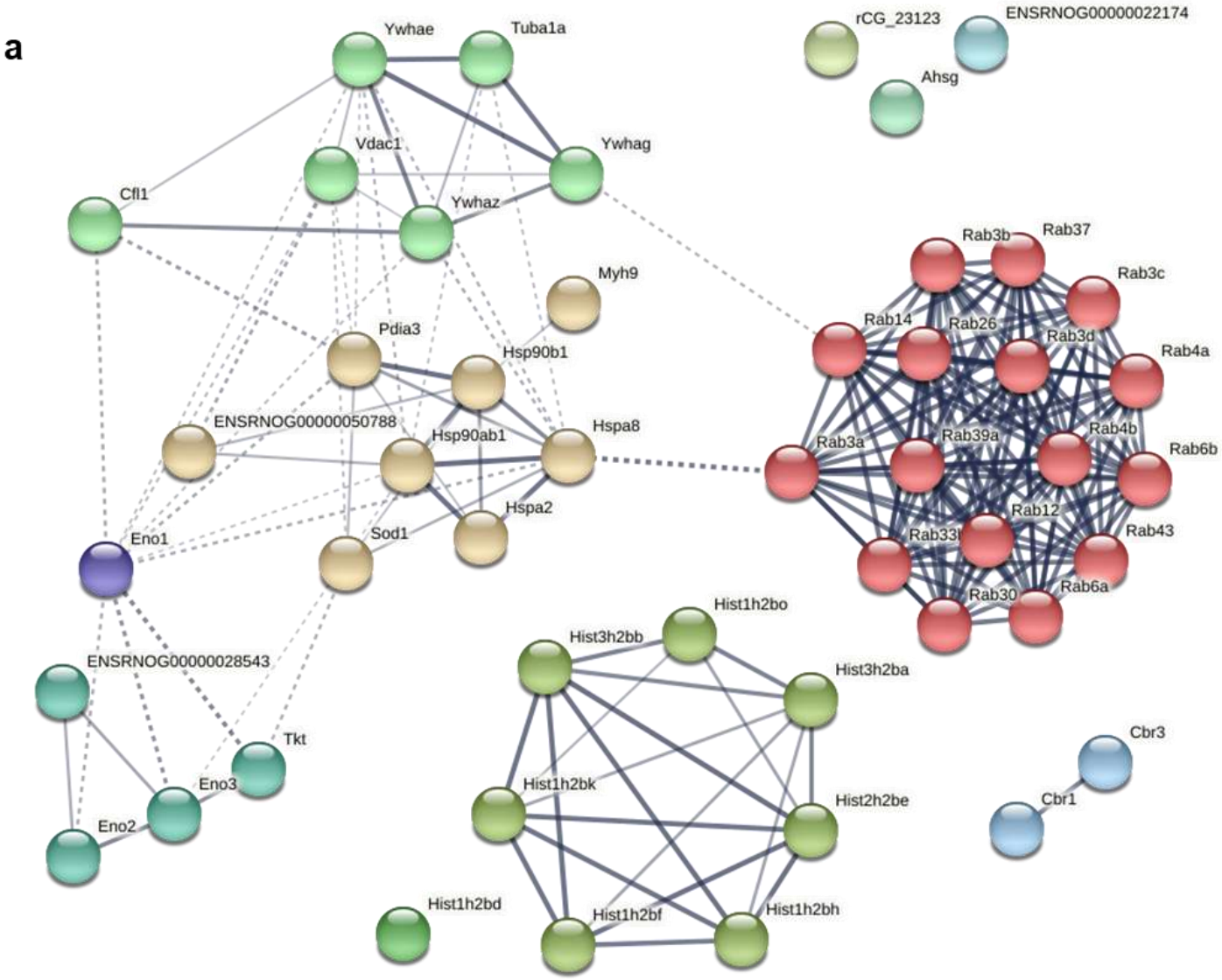


Figure 2: Venn diagram - The graph showed all the upregulated **(a)** and down-regulated **(b)** protein in all ages and generation in both groups of treatment. These results point 54 common proteins differentially expressed among the Ctrl, T1 and T2 group in all the animals on F1-PND22 and PND120 and F2-PND22-F and PND22-M. **(c)** Bar chart showed the distribution of the deregulated protein by age in both generations.

The protein-protein interaction (PPI) network showed all differentially expressed proteins after the treatment among ages and generations (Fig 3a and Supplementary Table 1). In the PPI is possible identify five important cluster of proteins as Rab, Histone (Hist), Chaperone (Hsp), Tyrosine 3-Monooxygenase/Tryptophan 5-Monooxygenase Activation Protein Gama (Ywhag), Enolase (Eno), carbonyl reductase (Cbr) and other proteins (Fig. 3a).

The enrichment analyzed was made by cluster of protein. From the more important clusters, four of them enriched for important cell pathways as EGF, FGF, PI3k-AKT, Estrogen and MAPK signaling, metabolism, cellular response, Cell cycle, signaling by WNT and others (Fig. 3b-c).

The ontological terms used to analyze the environmental intracellular condition showed that independently of the protein cluster, there were terms likely to functional mechanism of the cell survival and differentiation (Fig. 3d). In addition, it was selected some proteins (12 protein, 2 proteins by cluster) to evidence positive reaction in normal (fig.4), and in tumor cell (Fig.5) of men in different age. The association among these proteins with patient life expectancy with diagnostic adenocarcinoma (Supplementary fig.3).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Ahsg
Vdac1
Ywhag
Cfl1
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Rab3b
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Rab26
Rab3d
Rab4a
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Hspa8
Rab3a
Rab39a
Rab4b
Rab6b
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Rab43
Rab30
Rab6a
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Hist3h2ba
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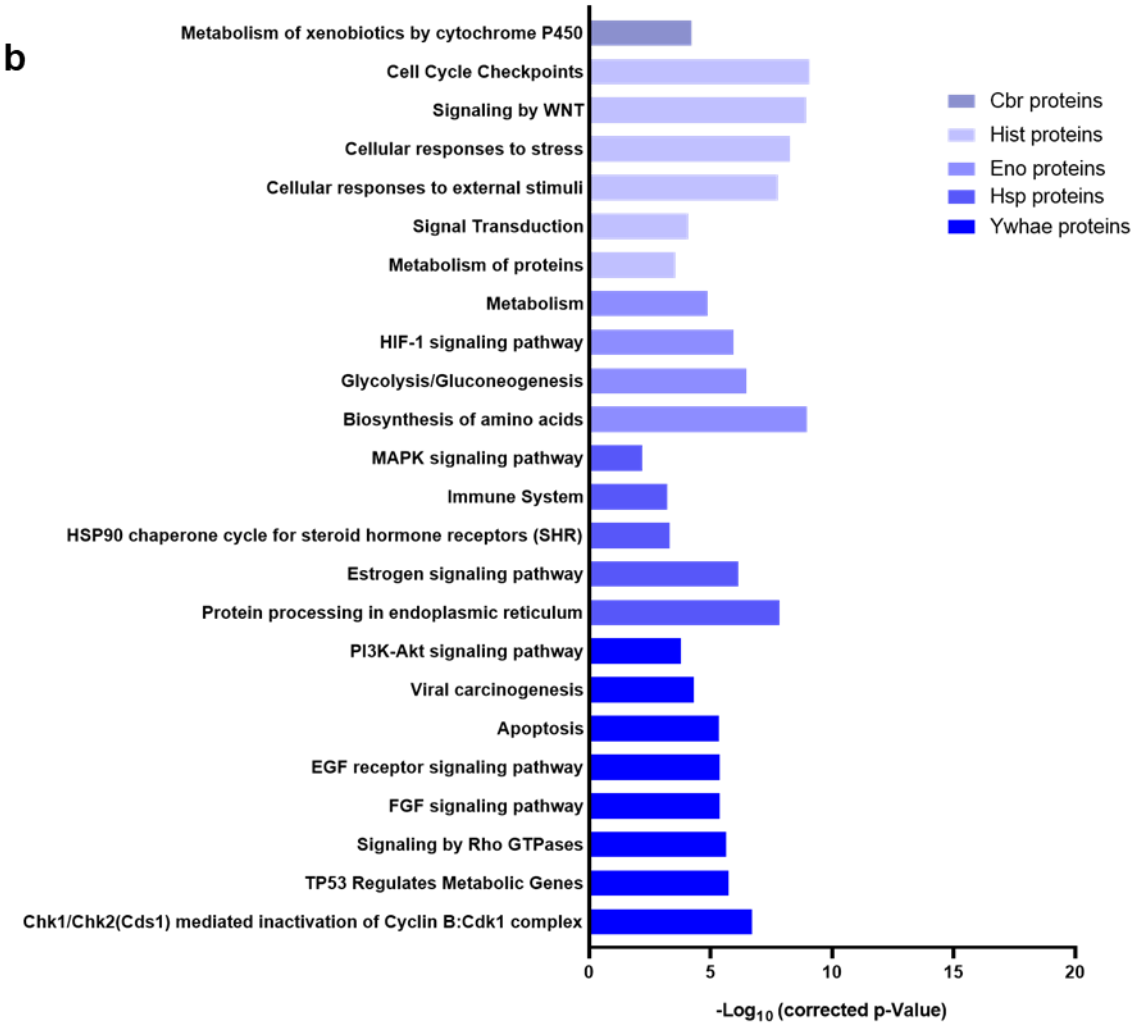
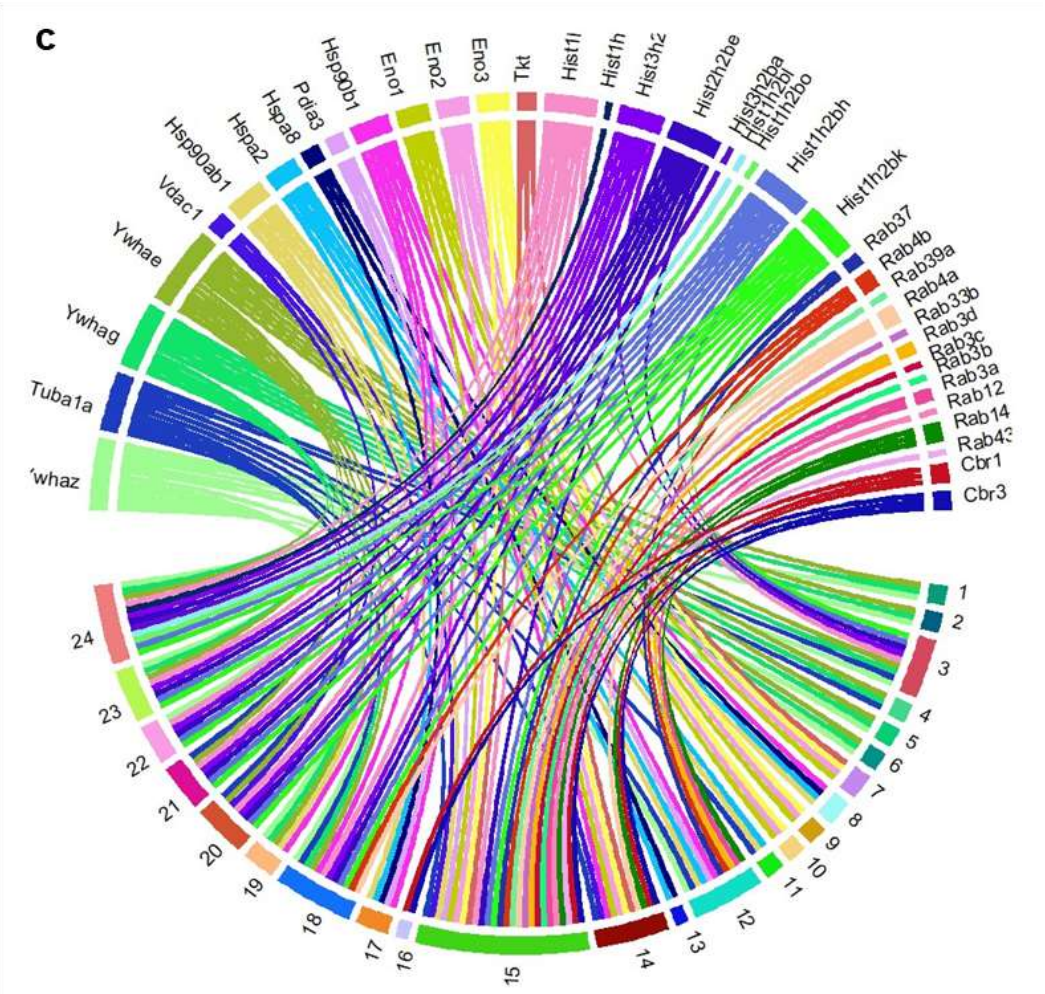

b
Metabolism of xenobiotics by cytochrome P450
Cell Cycle Checkpoints
Signaling by WNT
Cellular responses to stress
Cellular responses to external stimuli
Signal Transduction
Metabolism of proteins
Metabolism
HIF-1 signaling pathway
Glycolysis/Gluconeogenesis
Biosynthesis of amino acids
MAPK signaling pathway
Immune System
HSP90 chaperone cycle for steroid hormone receptors (SHR)
Estrogen signaling pathway
Protein processing in endoplasmic reticulum
PI3K-Akt signaling pathway
Viral carcinogenesis
Apoptosis
EGF receptor signaling pathway
FGF signaling pathway
Signaling by Rho GTPases
TP53 Regulates Metabolic Genes
Chk1/Chk2(Cds1) mediated inactivation of Cyclin B:Cdk1 complex
Cbr proteins
Hist proteins
Eno proteins
Hsp proteins
Ywhae proteins
0
5
10
15
20
-Log10 (corrected p-Value)



1 - Chk1/Chk2(Cds1) mediated inactivation of Cyclin B:Cdk1 complex
2 - TP53 Regulates Metabolic Genes
3 - Signaling by Rho GTPases
4 - FGF signaling pathway
5 - EGF receptor signaling pathway
6 - Apoptosis
7 - Biosynthesis of amino acids
8 - Estrogen signaling pathway
9 - Glycolysis/Gluconeogenesis
10 - HIF-1 signaling pathway
11 - HSP90 chaperone cycle for steroid hormone receptors (SHR)
12 - Immune System
13 - MAPK signaling pathway
14 - Metabolism
15 - Metabolism of proteins
16 - Metabolism of xenobiotics by cytochrome P450
17 - Protein processing in endoplasmic reticulum
18 - Signal Transduction
19 - PI3K-Akt signaling pathway
20 - Cellular responses to external stimuli
21 - Cellular responses to stress
22 - Signaling by WNT
23 - Cell Cycle Checkpoints
24 - Viral carcinogenesis

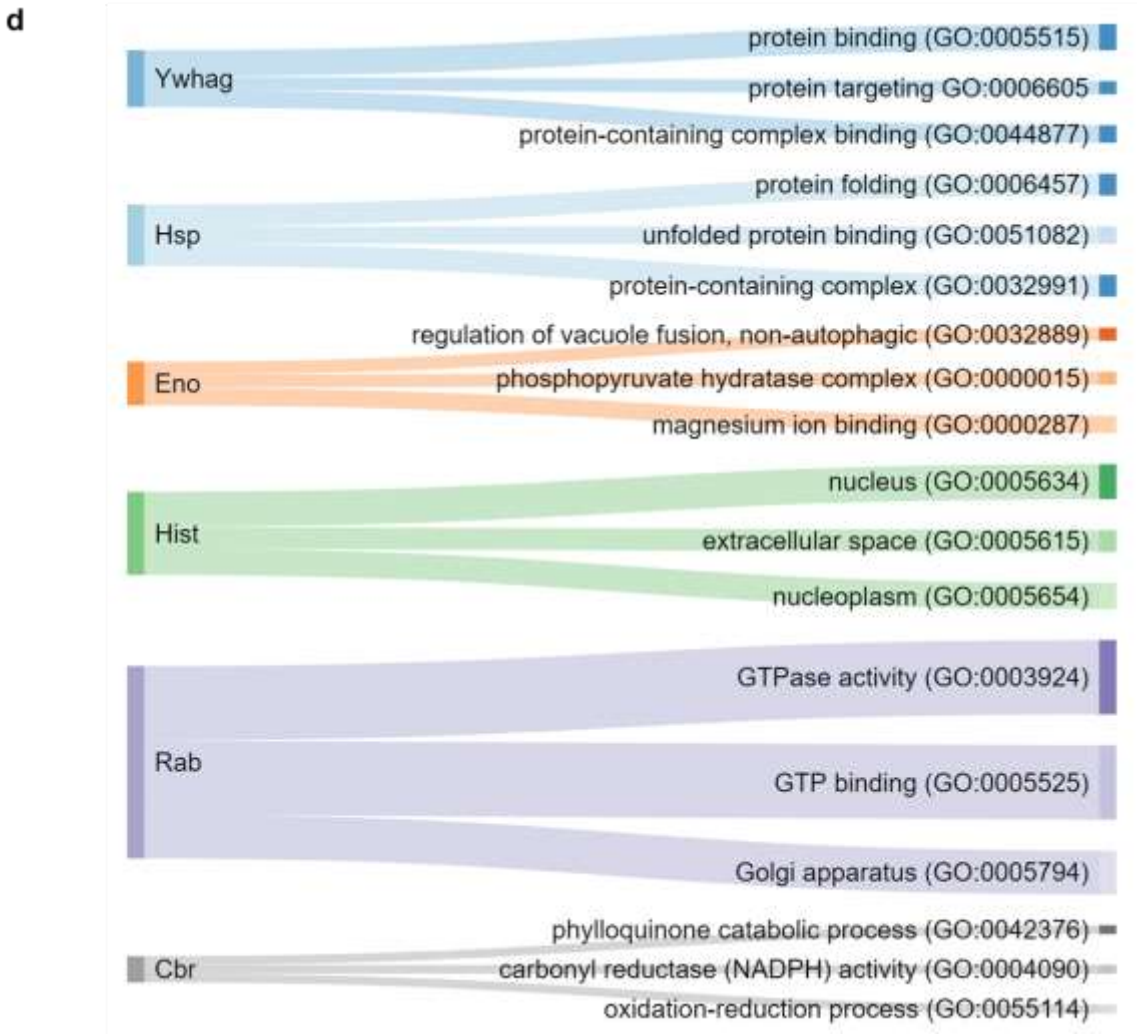


Figure 3: a-The protein-protein interaction (PPI) network of all the proteins differentially expressed in the VP of the animal's control and treated group in the different ages and generations. The PPI network was constructed into the STRING databases, and the thickness among proteins (nodes) showed the degree of confidence of the interaction. In this PPI is possible to see important clusters of Rab, Histone, and Hsp proteins and interactions with other proteins. **b-** Enrichment of pathways that the proteins differentially expressed in all the VP sample of the animals on F1-PND22 and PND120 and F2-PND22-F and PND22-M are involved. The enrichment was obtained in the KOBAs 3.0 platform, and the data are expressed in $-\log_{10}$(corrected p-Value). **c-** The circus plot graph showed the protein interaction with the select pathways. **d-** The graph highlights the three main ontological terms enriched in each of the protein clusters.

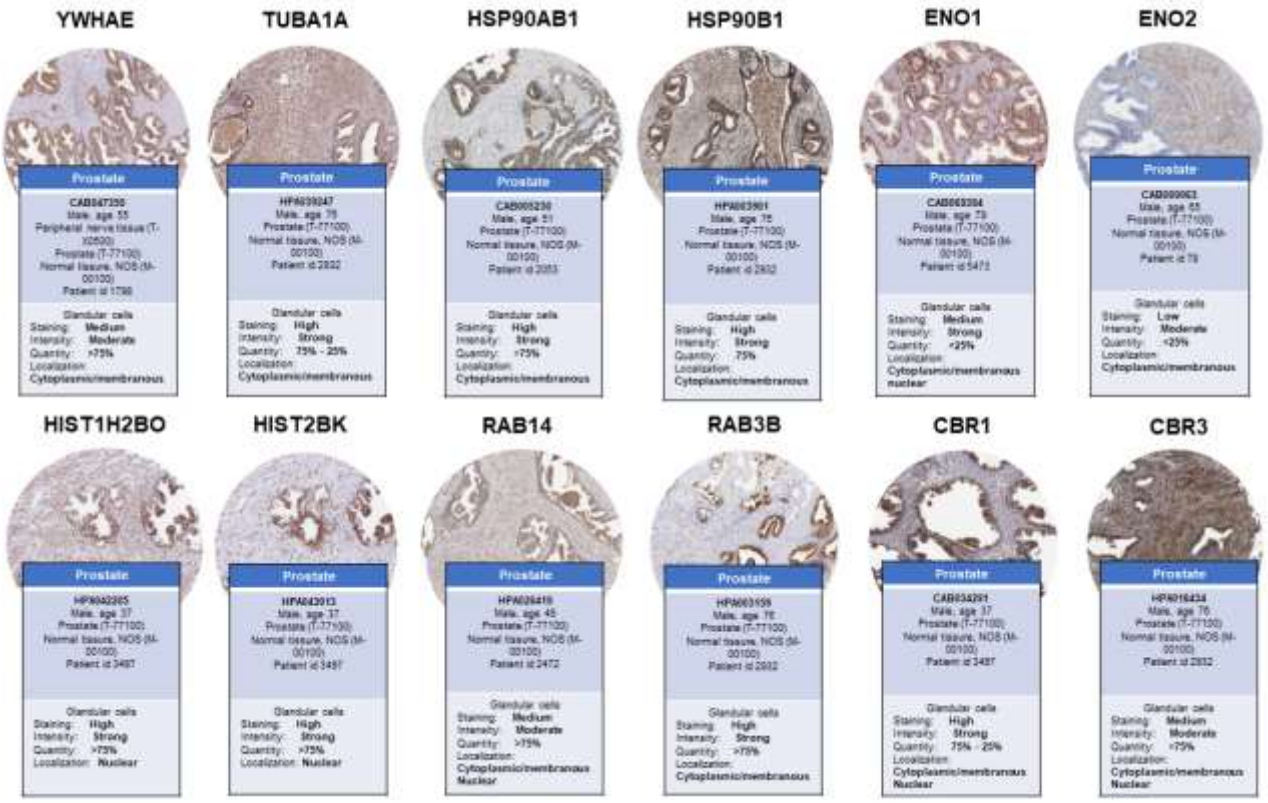


Figure 4: Photomicrograph of positive reaction (by Immunohistochemistry) for twelve-protein on normal prostate of different human patients with different age. These proteins were identified in this study. All photomicrographs were obtained in "The Human Protein Atlas" platform. These proteins are downregulated in F1-PND22 and PND120 and F2-PND22-F and PND22-M comparison.

Figure 5: Photomicrograph of positive reaction (by Immunohistochemistry) for twelve-protein on prostate cancer of different human patients with different ages. These proteins were identified in this study. All photomicrographs were obtained in "The Human Protein Atlas" platform. These proteins are downregulated in F1-PND22 and PND120 and F2-PND22-F and PND22-M comparison.

The investigation of the deregulated proteins between generation (F1 and F2) showed that there was an interesting result comparing the offspring from exposed F1 male (38 proteins), from exposed F1 female (16 proteins) and from the offspring (F2) independently of the F1 descendant's gender that was exposed (31 protein).

The deregulated proteins in comparison between the exposed offspring from F1-mother or father pointed that downregulation of these proteins had influence on functional pathways as metabolism of proteins post-translational protein modification and tight junction (Fig. 7b). The differentially expressed proteins after the treatment in the offspring from exposed F1 mother (16 proteins) showed influence on pathways related with nutritional support and in the scavenging aberrant molecules, as in O_2/CO_2

exchange in erythrocyte and Binding and uptake of ligands by Scavenger Receptors pathways (Fig. 7b). The offspring from exposed F1-father evidenced many proteins associated with signaling and cell communication like cGMP-PKG and extra-nuclear estrogen signaling, and RAF/MAP kinase cascade as well as signaling by nuclear receptor and WNT pathways (Fig. 7b).

The ontological terms were represented in clusters to show the general view of the data obtained to each of the comparison (Fig. 8a-b). In F2-PND22-M vs. F2-PND22-F (both F1 descendants) comparison, there were evidenced terms associated with binding of different molecules with cellular compartment, as cytosol and basement membrane (Suppl. Fig. 3c). The F1-PND22 vs. F2-PND22-M comparison probable has alteration in transcription, mRNA splicing and peroxidase activity (Suppl. Fig. 3b). Finally, in F1-PND22 vs. F2-PND22-F comparison, there were related with binding molecular functions as antioxidant activity, histone, heat shock, ubiquitin protein ligase binding, and cellular response to DNA damage (Suppl. Fig. 3a). The F1-PND22 and F2-PND22-M probably have alteration in transcription, mRNA splicing and peroxidase activity (Suppl. Fig. 3b). And in F2-PND22-F and F2-PND22-M there are terms related to the binding of different molecules with cellular compartment, as cytosol and basement membrane (Suppl. Fig. 8c).

The validation of the results obtained in proteomic profile in F1 x F2 investigation showed positive expression of 12 proteins (4 of each comparison) identified in normal and tumor sample on prostate of men in different age (Fig.9), in the incidence on tumor cell (fig. 10), and the relation between these proteins with survival patient with diagnostic to prostate cancer (Supplementary fig. 4) showed the relation between low protein expression with tumor cell and poor prognostic.

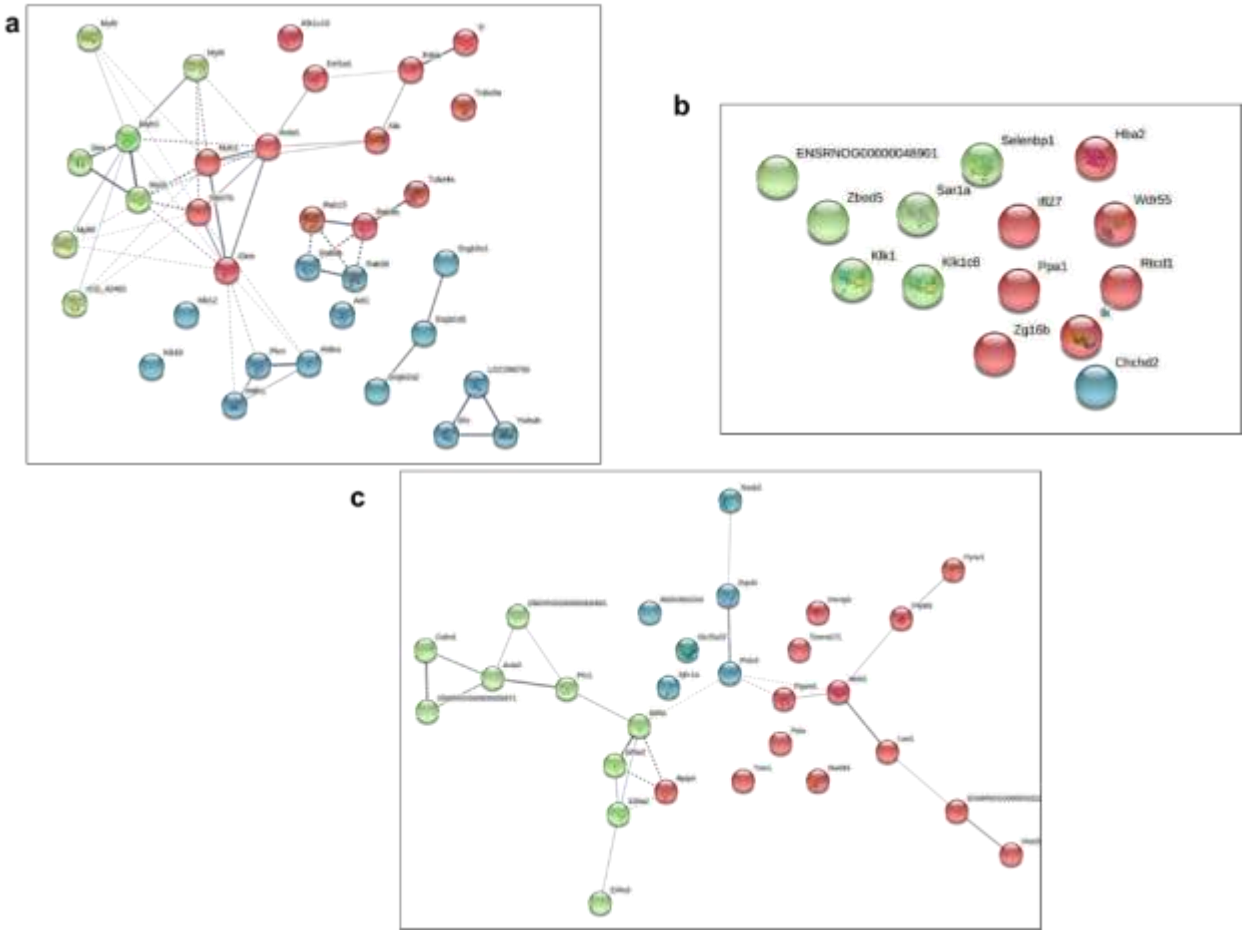
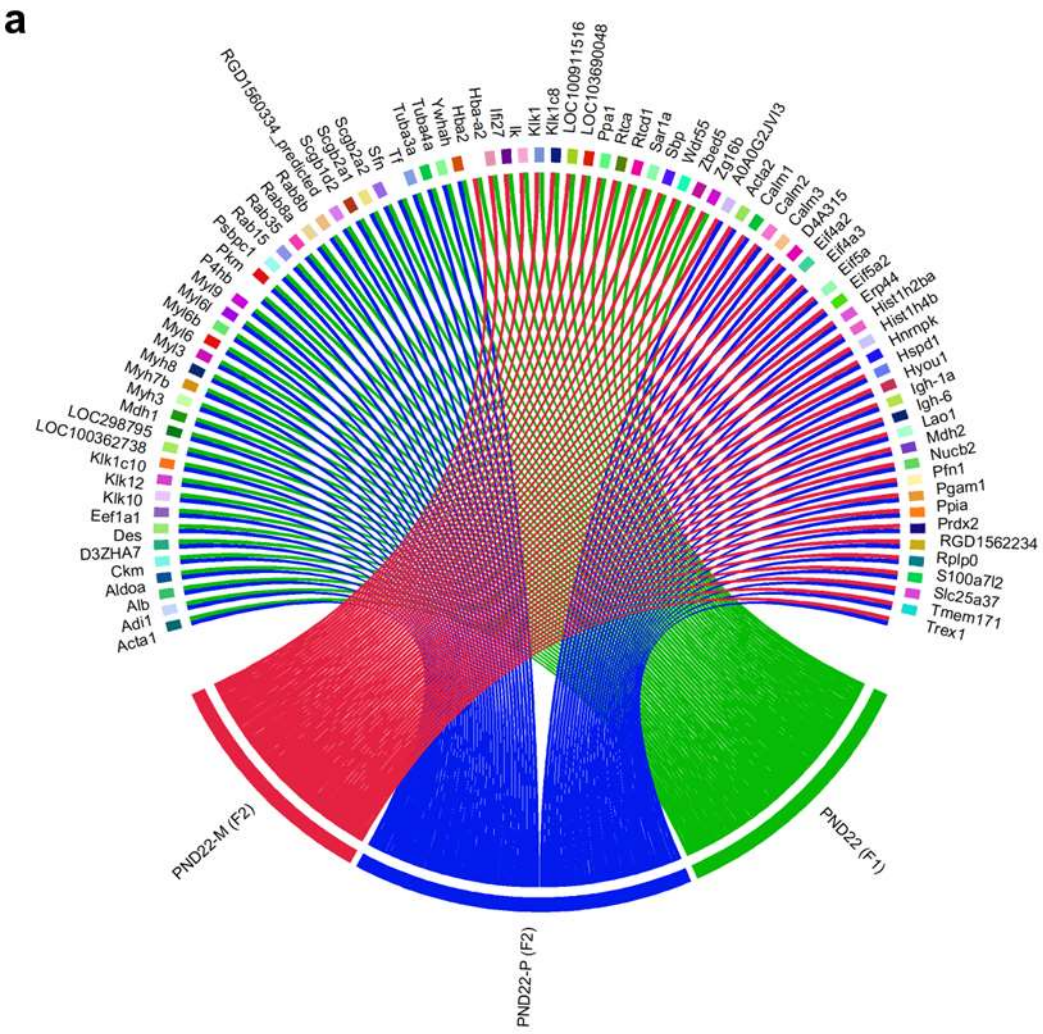


Figure 6: a- The PPI network showed the proteins differentially expressed in the VP of the animal's control and treated group in both generations. **a.** PPI network of functional interaction in F1-PND22 and F2-PND22-F. **b.** PPI network of functional interaction in F1-PND22 and F2-PND22-F. **c.** PPI network of functional interaction in F2-PND22-F and PND22-F.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RGD1560334_predicted
Rab8b
Rab8a
Rab35
Rab15
Psbpc1
Pkm
P4hb
Myl9
Myl6l
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Myl6
Myl3
Myh8
Myh7b
Myh3
Mdh1
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Zbed5
Zg16b
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Acta2
Calm1
Calm2
Calm3
D4A315
Eif4a2
Eif4a3
Eif5a
Eif5a2
Erp44
Hist1h2ba
Hist1h4b
Hnrnpk
Hspd1
Hyou1
Igh-1a
Igh-6
Lao1
Mdh2
Nucb2
Pfn1
Pgam1
Ppia
Prdx2
RGD1562234
Rplp0
S100a7l2
Slc25a37
Tmem171
Trex1
PND22-M (F2)
PND22-P (F2)
PND22 (F1)

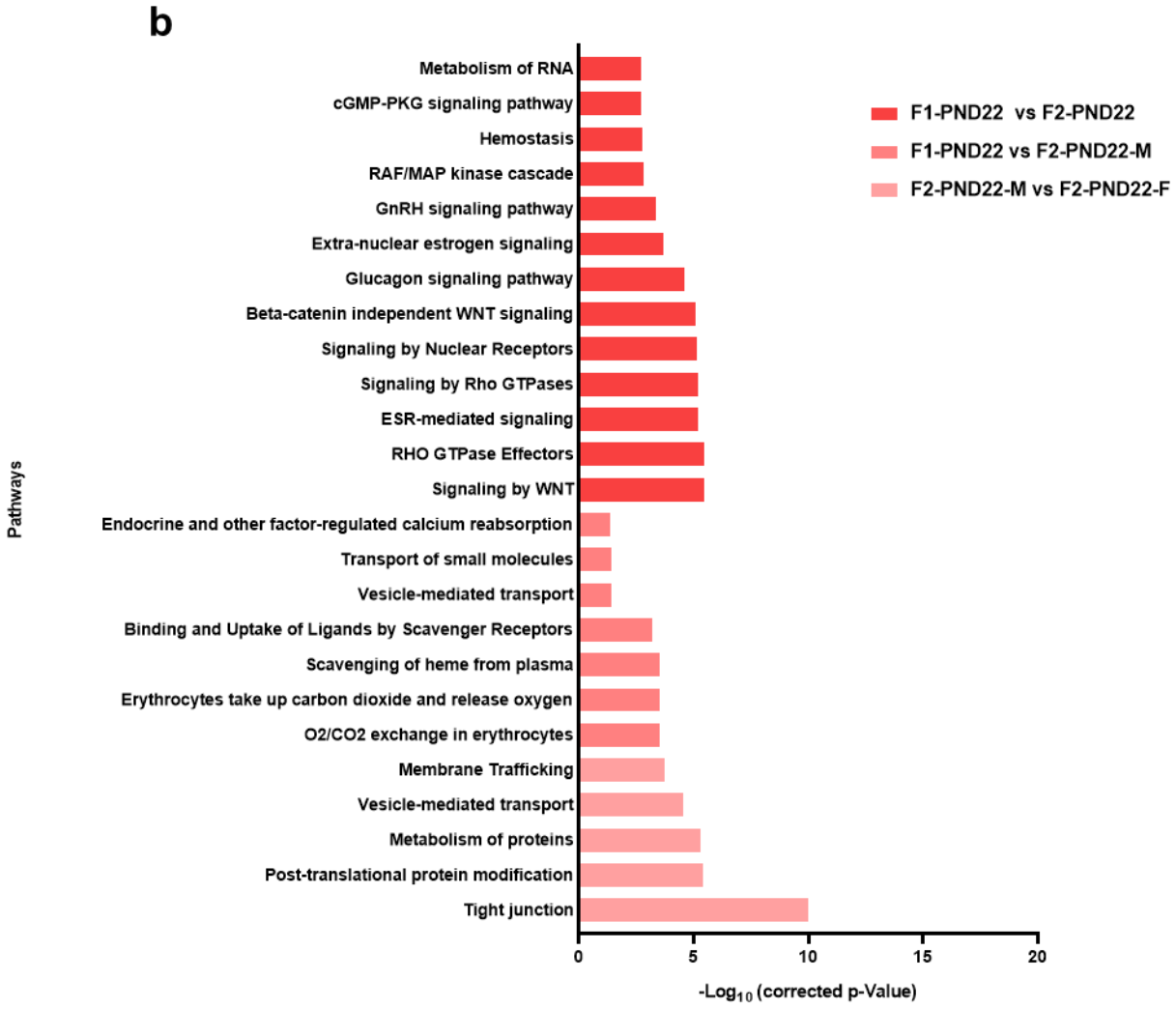


Figure 8: a. The circus plot of all the proteins that remained differentially expressed in VP of the animals in both generations. **b-** Enrichment of pathways that the proteins differentially expressed in all the VP sample of the animals on F1-PND22 and F2-PND22-F and PND22-M are involved. The enrichment was obtained in the KOBAs 3.0 platform, and the data are expressed in -$\log_{10}$(corrected p-Value). **d-** The graph highlights the three main ontological terms enriched in each of the protein clusters.

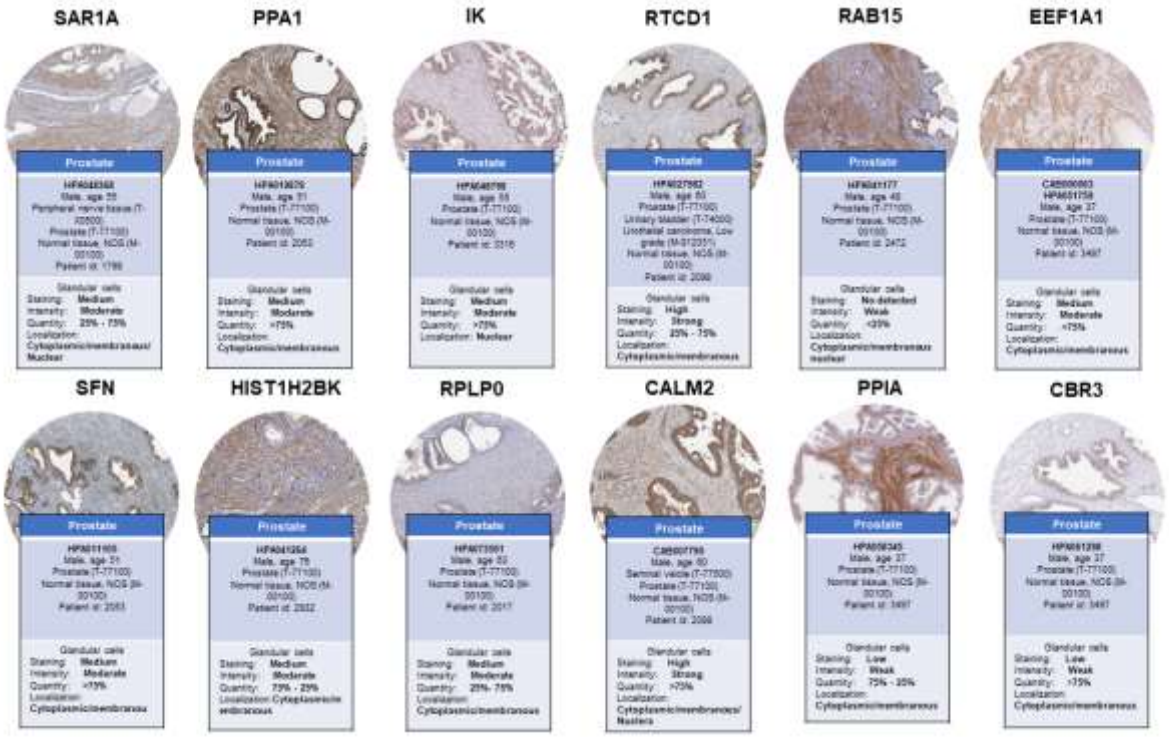


Figure 9: Photomicrograph of positive reaction (by Immunohistochemistry) for twelve-protein on normal prostate of different human patient with different age. These proteins were identified in this study in the comparison F1-PND22 vs F2-(ND22-P)- (SAR1A, PPA1, IK, RTCD1,); F1-PND22 vs F2-PND22-M - (RAB15, EEF1A1, SFN, HIST1H2BK) and F2-(PND22) vs F2-PND22-M - (RPL0, CALM2, PPIA, CBR3). All photomicrographs were obtained in “The Human Protein Atlas” platform.

Figure 10: Photomicrograph of positive reaction (by Immunohistochemistry) for twelve-protein on prostate cancer of different human patient with different age. These proteins were identified in this study in the comparison F1-PND22 vs F2-PND22-P - (SAR1A, PPA1, IK, RTCD1,); F1-PND22 vs F2-PND22-M - (RAB15, EEF1A1, SFN, HIST1H2BK) F2-PND22 vs F2-PND22-M - (RPL0, CALM2, PPIA, CBR3). All photomicrographs were obtained in “The Human Protein Atlas” platform.

4. Discussion

Though few, different studies showed that early exposure to phthalate mixture have an important harm and late repercussion in reproductive system (Repouskou et al. 2019; Wellerson R Scarano et al. 2019; Zhou, Gao, and Flaws 2017a). Prostate have been little studied comparing to the other male reproductive organs, however previous studies with isolated phthalates or in mixture have shown that prostate responds in long terms increasing adaptive and proliferative lesions (Wellerson R Scarano et al. 2019; S.-M. Ho et al. 2017a; Wellerson Rodrigo Scarano et al. 2009). This data evidence a mechanism of fetal programing in human and other animals exposed during intrauterine and postnatal development. Here, the proteomic profile showed important molecular changes after the perinatal exposure, modifying proteins related with morphogenesis,

differentiation, and maintenance of prostate in F1 and F2 descendants. Additionally, our results were able to show important differences between molecular profile in F2 descendants from F1 mothers and fathers exposed, evidencing the gender multigenerational and transgenerational influence.

Recently we showed that the maternal exposure to the same phthalate mixture used in this study not altered expressively the weight of pregnant rats and the offspring (Wellerson R Scarano et al. 2019). The Pfn1, an action-binding protein, can be inhibit androgen receptor aggregation and the interacts with estrogen receptor interfering in estrogen response (Kanaujiya et al. 2013; Shao et al. 2008) and affect angiogenesis (Fededa, Gerlich, and Dambournet 2012). Pfn1was the only protein that remained unregulated between the animals on F1-PND22 and F1-PND120.

There was no molecular interaction among the five upregulated proteins (Gdi2, Hnrnpa2b1, Myl4, Prdx1, Rps7) in F1-PDN22 and F2-PND22-F, which does not mean that they don´t drive to molecular changes. Rab GDP dissociation inhibitor beta (Gdi2) regulates Rho GTPase and is commonly investigate to therapeutic target in prostate cancer (PCa) (C. Liu et al. 2021) and in other types of cancer (B. Zhang 2006; Sun et al. 2007). Additionally, the heterogeneous nuclear ribonucleoprotein A2/B1 (Hnrnpa2b1) is an important biomarker to cancer. This protein regulates the β-catenin protein in prostate cancer (Stockley et al. 2014), regulate E-cadherin expression and modulate epithelial-mesenchymal transition (EMT) in lung cancer (Tauler et al. 2010) and control the invasive cell migration and proliferation (Moran-Jones et al. 2009; David et al. 2010; M. K. Kim et al. 2021; Dasari et al. 2019).

Peroxiredoxin 1 (Prdx1) interact with AR and increase AR transcription by hypoxia and by ligand stimulus (Feng et al. 2020). Also, according to the authors, in PCa the hypoxia or reoxygenation favors Prdx1 activity AR independent antioxidant machinery (Feng et al. 2020). Lastly, the ribosomal protein S7 (Rsp7), an important molecule to post-transcriptional ribosome synthesis, also is a good biomarker to prostate carcinogenesis. Male rats exposed to 2-N-hydroxylamino-1-methyl-6-phenylimidazo [4,5-b] pyridine isolate or in combination with another substances, as testosterone or 5α-dihydrotestosterone (carcinogenic promoter), showed altered expression in RSP7

protein(TELIH BOYIRI, RICHARD I. SOMIARI, STEPHEN RUSSELL 2009). As well as hnrnpa2b1, the RSP7 protein promoting EMT by regulated E-cadherin and N- cadherin expression (Wen et al. 2019). This protein can be regulated in tumorigenesis and progression of PCa cell line (C. Zhang et al. 2019b; 2019a). Maternal exposure to DPB decreased N-cadherin an increased E-cadherin and β-Catenin in urethral epithelial of offspring (Zhu et al. 2020). and maternal exposure to DEHP increased the susceptibility to PCa in offspring (X. Wang et al. 2017a). In this sense, it is possible that our findings contribute to the understanding on the possible mechanisms that contribute to prostate carcinogenesis after phthalate exposure.

Our results showed that there were more downregulated proteins than positively regulated proteins in all the age and generations after the treatment, and probably, the abundance of negatively regulated proteins would have relation with the epigenetic modulation as miRNA upregulation and increase in DNA methylation (Wellerson R Scarano et al. 2019). All the 54 proteins deregulated after the phthalate mixture exposure and during postnatal development (F1) and that remained altered in F2 generation showed the importance of the maternal exposure in influencing multigenerational and transgenerational transmission of molecular changes and will be discussed as a group below.

Enzymes of cytochrome P450 are involved in phthalate biotransformation and in the nephrotoxicity and hepatotoxicity phthalate-induced (Y. Z. Zhang et al. 2018; H. Wang et al. 2020). This study found proteins involved in xenobiotics metabolism by cytochrome P450 (Cbre1 and Cbr2). These results probably show that chronic exposure affects peripheral organs, which can be associated with imbalance of enzyme P450 hemostasis and failure of the body to eliminate phthalates. This alteration can influence the cell's ability to fight against stressor mechanisms, since pathways as cellular response to stress and cellular response to external stimulus are enriched by different proteins.

Eight different deregulated proteins (Ywhaz, Ywhag, Ywhae, Hist1h2bk, Hist3h2bb, Hist2h2be, Hist1h2bh and Hist1h2bk) after phthalate mixture exposure were enriched to the cell cycle checkpoints and Chk1/Chk2(Cds1) mediated inactivation of

Cyclin B: Cdk1 complex. In addition to being involved in epigenetic regulation, cyclin-dependent kinase 1 (Cdk1) is responsible to stimulating or clocking histone mark (Michowski et al. 2020),, alteration in this complex can point to DNA damage and apoptotic process. On the other hand, the Cdk1 is responsible to active androgen receptor (AR) phosphorylation at Ser-81 *in vitro* (S. Chen et al. 2006).

Our results showed that deregulated Ywhae and Hsp cluster of proteins were enriched for pathways like MAPK, Estrogen, PI3k-Akt, FGF and EGF signaling are hormone–dependent and essential to normal development of the prostate as well as in pathological conditions (Lin and Wang 2010). FGF, for example, is an essential pathway to prostatic morphogenesis and homeostasis prostatic (Lin et al. 2007), in stromal-epithelial interaction and communication (Giacomini et al. 2021), to stimulating EFG-MAPK, PI3k-Akt, Ras-Raf in normal and tumor tissue (Eswarakumar, Lax, and Schlessinger 2005; Lin and Wang 2010), and alteration in FGF and MAPK pathways are frequently related with progression of prostate cancer (PCa) in both *in vivo* and *in vitro* models (Bluemn et al. 2017; Acevedo et al. 2007). Maternal exposure to DEHP is able to activate PI3K/Akt/mTOR signaling to stimulate proliferation and differentiation of testicular cells in offspring and in their descendants (J. Zhang et al. 2020). Additionally, Akt is one of the way to stimulate the survival and protecting germ cells after toxic exposure (Rasoulpour et al. 2006; J. Zhang et al. 2020)(Rasoulpour et al. 2006; J. Zhang et al. 2020)(Rasoulpour et al. 2006; J. Zhang et al. 2020). DEHP was describe by promote the overactivation of PI3k signaling in folliculogenesis (Hannon, Brannick, Wang, and Flaws 2015) and stimulate autophagy process by PI3K/Akt/mTOR signaling in testicular cells after a low dose of exposure, and apoptosis, by STAT3/P53 signaling in a high dose of exposure. Maternal exposure to DBP downregulated expression of MEK1/2 and Bcl-2, which altered cell proliferation and apoptosis in testis of offspring (T. Ma et al. 2017).

Here, the phthalate mixture was responsible for downregulating proteins associated with proliferation, differentiation, and survival cellular in all ages and generations. Looking to morphogenic process of the gland, is possible that these results delay the morphogenesis or trigger a disbalance in development, maturation, and

physiology of the gland, which can reflect in decrease of reproductive quality. On other hand, these pathways are responsible for controlling most of the protective mechanisms during adverse conditions caused by the different external factors. The downstream of this signaling may be a favorable environment for different prostatic disorders, such as prostate cancer.

Most of the deregulated proteins after the treatment were enriched for cell processes like metabolism of proteins, metabolism, signal transduction and by GTPases pathways, and this aspect may to point for a posttranscriptional failure, since this alteration may be related to harm in the vesicle transport signaling. Rab proteins, widely downregulated in this study, are member of the Ras superfamily of GTPase and are involved in all intracellular trafficking (Ding et al. 2017), besides the alteration in this protein can reflect in the most process dependent of GTPases activity (Goody, Rak, and Alexandrov 2005). The low expression of genes related to vesicular trafficking and secretion is a potential target of investigation to PCa. The under expression of RAB27A and RAB27B was associated with advanced PCa (Worst et al. 2017). Rats treated with different dose of DBP showed inhibition of RhoA/Rock/LIMK2 by altering Rho-GTPase signaling and harm neuroplasticity (Ding et al. 2017). Sertoli cell exposure to MBP abundant damage in phagocytic activity by modulation of RAC1 expression, which is activated by Ras (Gong et al. 2018). Together, the data obtained from the pathways and terms enrichment have pointed to an important damage in the signal transduction, translocation of protein, cell activity and metabolism, and in the capacity of the intracellular and extracellular interaction (Y. Zhang et al. 2020).

The validation in the human protein atlas showed that seven proteins downregulated by the treatment (Ywhae, Eno1, Eno2, Rab3b, HSP90ab, Hsp90B1 and CBR3) are associated with poor prognostic (Supplementary Fig.3) in tumor cell of men diagnosticated with adenocarcinoma prostatic. In normal tissues, most of them has medium or high expression. These results need to be more explored, but it is possible that this association point important way to establish the hypothesis of the maternal exposure to phthalate can increased the susceptibility of offspring to PCa development, as described by Wang and collaborators (2017). Similar results were obtained in other

study evaluating the effect of maternal diet on offspring and reinforce the hypothesis that the intrauterine microenvironment may contribute to the development of PCa at senescence (Alcantara Santos et al. 2020).

Interestingly, the results showed that the commonly deregulated proteins after the phthalate mixture exposure were higher in F2 descendants when the father (F1) was exposed than when the mother (F1) was exposed. Among the proteins commonly deregulated in F1-PND22 and F2-PND22-F, proteins that act in structural function and intracellular secretion were more abundant. These proteins enriched for pathways as metabolism of RNA, cGMP-PKG, RAF/Mar Kinase cascade and signaling as extra-nuclear estrogen, nuclear receptor and WNT. Importantly here, there was a downregulation in secretoglobulins (Scgbs), that according with UniProt platform, correspond to a part of prostatein, the main secreted glycoprotein in seminal fluid of the ventral prostate (Wu et al. 2021; Maccioni, Cabezas, and Rivero 2003). Additionally, Wnt signaling regulates prostatic morphogenesis, epithelial cell differentiation and proliferation of prostate epithelial progenitor cells, being extensively studied in prostate tumors (Kharaishvili et al. 2011). The low expression of prostatein is an important damage to the prostatic function in reproduction. The enrichment to extra-nuclear estrogen and nuclear receptor points to an important factors involved in estrogenized models during the development, which seems to be related to the estrogenic imprinting during the prostate aging (Marker et al. 2003; Gail S. Prins and Putz 2008; Gail S Prins et al. 2007; Gail S. Prins and Birch 1997).

Among the proteins commonly deregulated in F1-PND22 and F2-PND22-M, there were no functional strongly association, but most of them are involved with intracellular function and cell architecture like the kallikerin (Klk), a polypeptide involved in activation of matrix metalloproteinase; the protein rad (Ik), a component of spliceosome involved with check point machinery; secretion associated Ras Related GTPase 1B (Sar1a) and inorganic diphosphatase, that are associated with endoplasmic reticulum to Golgi (ER-Golgi) transport; RNA 3’-terminal phosphatase cyclase (Rtca) involved in RNA processing, Wd repeat -containing protein 55 (Wdr55), act in organogenesis process by modulated the rRNA synthesis and others proteins. In female, Klk expression was

responsible to regulate steroids hormones and was associated with reproductive disorders (Raimondo et al. 2021). In prostate gland, there are different type of Klk identified in normal and tumor prostate (Ramsay et al. 2008) and it was considered as a potential biomarker to prostate and breast cancer (Ramsay et al. 2008; Diamandis, Yousef, and Olsson 2004). Exposure to mono-2-ethyhexyl phthalate decreased the expression of KLK3, a gene regulated by androgen, in LNCaP cell, which can favored the progression of PCa (Yong et al. 2016). In neuronal cells, Sar1a is important to morphological development and has essential function in the anterograde transport ER-Golgi and in controlling vesicle budding. The downregulation of these proteins showed impair in intracellular communication (Z. Wang et al. 2018; Urai et al. 2018). Deregulated Sar1 protein was associated with autophagy process (Gui et al. 2019), Golgi disorganization and fragmentation (Petrosyan et al. 2015) and it was related with progression of renal cell carcinoma, liver (Zhao et al. 2020) and prostate (Boyiri et al., 2009) carcinogenesis. PPa1 was recognized as a possible biomarker to ovarian cancer (Hui Li et al. 2017) and laryngeal carcinoma (Bodnar et al. 2016). These data show that there is a tight association among the commonly deregulated proteins and oncogenesis process after the treatment in F1 and F2, leading to a possible association between the treatment and tumorigenesis. In addition, the validation showed that downregulation of unless five proteins (Sar1a, RplP0, Eef1a1, Calm2, Ik, and Rtcd1) are associated with poor prognostic in men diagnosticated with adenocarcinoma in prostate.

The commonly deregulated proteins after the treatment in F1 and F2 prostate descendants (independently of F1 gender) showed downregulation in essential proteins as calmodulin (Calm), eukaryotic initiation Factor (Eif), histones (Hist), peptidylprolyl isomerase A (Ppia), peroxiredoxin 2, among others. According to the cell pathways enrichment, in general these proteins are associated with membrane traffic and vesicle transport, as well as with post-transcriptional protein modification and tight junction. Interestingly, it was possible to observe that vesicle transport and post-transcriptional modification pathways were repeatedly altered in all comparation between generations (F1xF2). Evidences have shown that intrauterine exposure to isolated phthalates were generally associated with development of prostate disorders and breast cancer (T. de

Freitas et al. 2021b; Peixoto et al. 2016; Wellerson R Scarano et al. 2019; X. Wang et al. 2017a). Now, we could show different altered proteins that can establish molecular signals that are triggered in early life and that have long-term effects.

Conclusion

Here, the data showed evidence that maternal exposure to a phthalate mixture impairs the expression of different proteins related to prostate development, function, and oncogenesis. Some of these proteins are crucial for metabolism, cell communication, secretion and in modulation of gene expression. More studies will be necessary to associate these alterations with the development and morphophysiology of the gland, as well as with the development of prostatic disorders, as prostate cancer. However, the data provided in this investigation showed that maternal exposure to phthalates can interfere with the prostate proteome in the offspring and descendants.

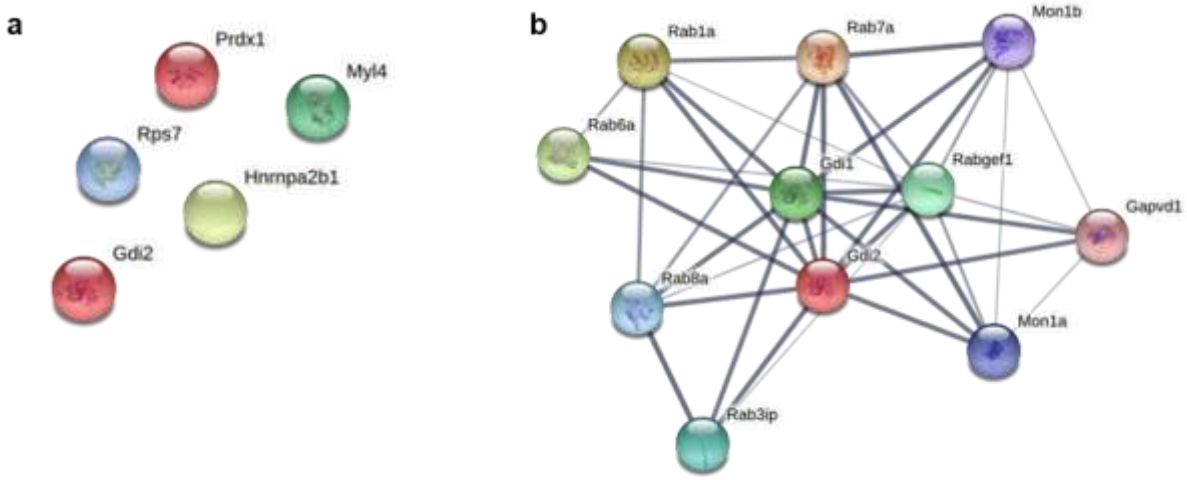
Supplementary Material

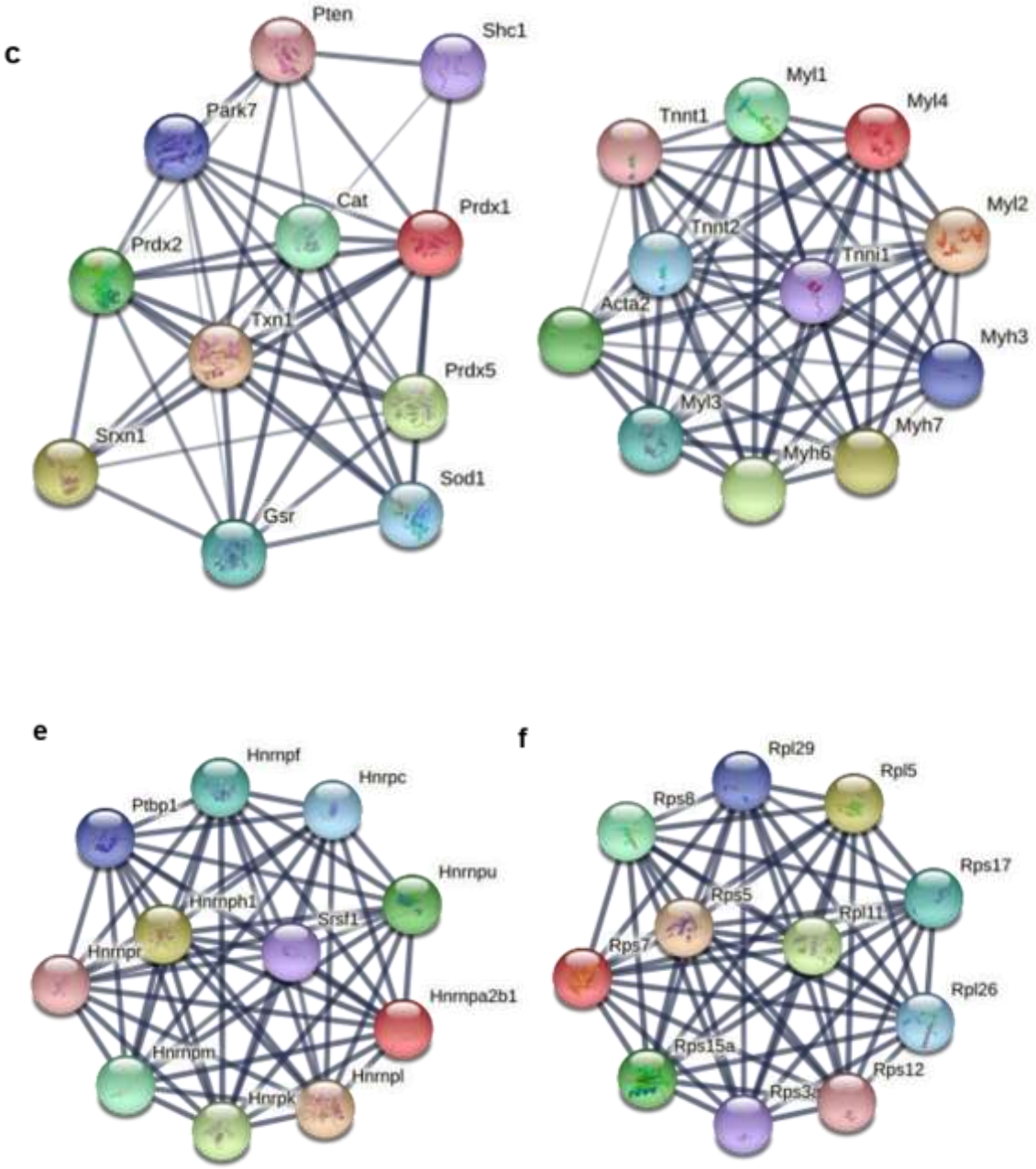
Supplementary - Table 1: Relation among the common protein up-regulated in differences age and generation

Comparison	Amount	Upregulated protein
F1-(PND22) and F1-(PND120)	1	Pfn1
F1–(PND120) and F2-(PND22-F)	2	Igg-2a, LOC679045
F1–(PND22) and F2-(PND22-F)	5	Gdi2, Hnrnpa2b1, Myl4, Prdx1, Rps7
F2–(PND22-F) and F2-(PND22-M)	1	Csrp1

Supplementary: Relation among the common protein downregulated in differences age and generation

Comparison	Amount	Downregulated protein
F1-(PND22), F1-(PND120), F2-(PND22-F) and F2(PND22-M)	54	A0A0G2JYY8, A0A0G2K099, Ahsg, Atp5f1a, Cbr1, Cbr3, Cfl1, D3ZME3, Eno1, Eno2, Eno3, Hist1h2bd, Hist1h2bf, Hist1h2bh, Hist1h2bk, Hist1h2bl, Hist1h2bo, Hist1h2bq, Hist2h2be, Hist3h2ba, Hist3h2bb, Hsp90ab1, Hsp90b1, Hspa2, Hspa8, LOC100359692, LOC102556347, M0R5J4, Myh9, Pdia3, Rab12, Rab14, Rab26, Rab30, Rab33b, Rab37, Rab39a, Rab3a, Rab3b, Rab3c, Rab3d, Rab42, Rab43, Rab4a, Rab4b, Rab6a, Rab6b, Sod1, Tkt, Tuba1a, Vdac1, Ywhae, Ywhag, Ywhaz
F1-(PND22) and F1-(PND120)	7	A0A0G2K7M1, F1M269, Nme1, Nme2, Rpl7l1, Tpm3, Tpm4
F1-PND22 and F2-PND22-F	38	Acta1, Adi1, Alb, Aldoa, Ckm, D3ZHA7, Des, Eef1a1, Klk10, Klk12, Klk1c10, LOC100362738, LOC298795, Mdh1, Myh3, Myh7b, Myh8, Myl3, Myl6, Myl6b, Myl6l, Myl9, P4hb, Pkm, Psbpc1, Rab15, Rab35, Rab8a, Rab8b, RGD1560334_predicted, Scgb1d2, Scgb2a1, Scgb2a2, Sfn, Tf, Tuba3a, Tuba4a, Ywhah
F1-(PND22) and F2(PND22-M)	16	Hba2, Hba-a2, Ifi27, Ik, Klk1, Klk1c8, LOC100911516,-LOC103690048, Ppa1, Rtca, Rtcd1, Sar1a, Sbp, Wdr55, Zbed5, Zg16b
F1-(PND22), F2-(PND22-F) and F2(PND22-M)	35	A0A0G2JX19, Actb, Actg2, Arhgdia, Calr, Crp2, Eef2, Klk1c12, Klk1c6, Klk1c9, Krt18, Krt19, LOC100360413, LOC100363782, LOC100909441, LOC103691939, LOC680121, P22k15, Prdx5, Psbpc2, Rab10, Rab1a, Rab1b, Rpsa, Scgb1d4, Tuba1b, Tuba1c, Tubb2a, Tubb2b, Tubb3, Tubb4a, Tubb4b, Tubb5, Vcp, Ywhab
F1-(PND22), F1-(PND120) and F2-(PND22-F)	18	Eef1a2, Gapdh, Gapdh-ps2, Lmna, LOC100361180, LOC100362366, LOC102549061, LOC108351137, M0R660, Myh9l1, Pbsn, RGD1564958, Rps17, Rps3, Tagln, Tpm1, Tubb6, Vim
F1-(PND22), F1-(PND120) and F2-(PND22-M)	6	Aldh2, Atp5a1, Hbe1, LOC689064, Pdia6, Tuba8
F1-(PND120) and F2-(PND22-F)	6	Ccl17, Got2, Myh11, RGD1307100, Tpm2, Vat1
F1-(PND120) and F2(PND22-M)	24	A0A0G2K689, Atp1a1, Bik, Cltc, Dbi, Dnajc3, Gpx6, H2afv, H2afz, Hist1h2ac, Hist1h2af, Hist1h2ah, Hist1h2ak, Hist2h2ab, Hnrnpa2b1, Krt10, Krt32, Lman1, LOC100910554, Mat2a, Nans, Rps7, Surf4, Vom2r31
F1-(PND120), F2(PND22-F) and (PND22-M)	22	Acly, Aldh6a1, Atp5f1b, Cap1, Copa, Hsp90aa1, D4A269, Dstn, Gstm1, Gstm4, Gstm7, Hint1, Hspa1a, Hspa1b, Hspa1l, LOC100911252, LOC296235, Park7, RGD1560831, Rplp1, Rpn1, Snd1
PND22F-(F2) and PND22-M (F2)	31	A0A0G2JVI3, Acta2, Calm1, Calm2, Calm3, D4A315, Eif4a2, Eif4a3, Eif5a, Eif5a2, Erp44, Hist1h2ba, Hist1h4b, Hnrnpk, Hspd1, Hyou1, Igh-1a, Igh-6, Lao1, Mdh2, Nucb2, Pfn1, Pgam1, Ppia, Prdx2, RGD1562234, Rplp0, S100a7l2, Slc25a37, Tmem171, Trex1

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Park7
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Myl4
Myl2
Tnnt2
Tnni1
Acta2
Myh3
Myl3
Myh7
Myh6
e
Hnrnpf
Hnrpc
Ptbp1
Hnrnpu
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Rpl29
Rpl5
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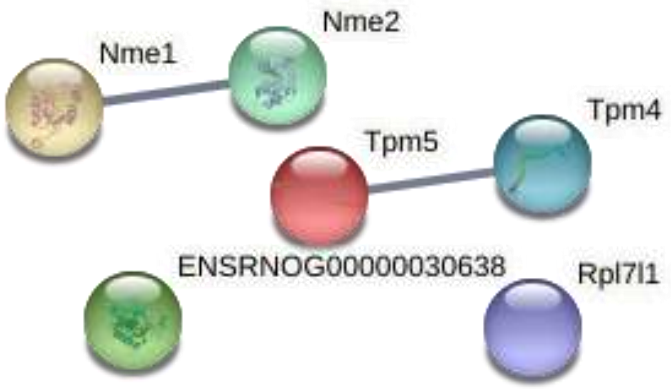
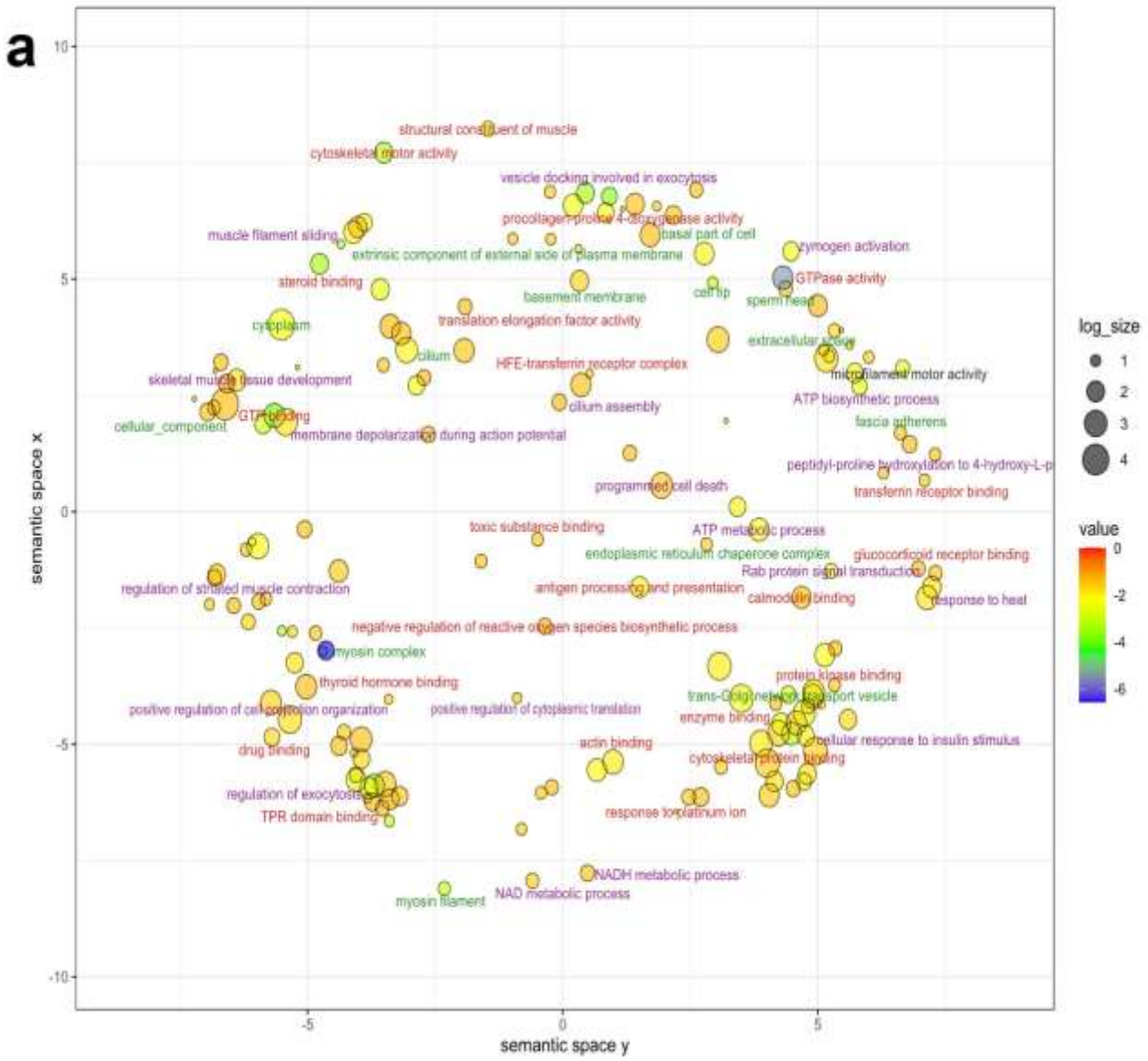
Rps17
Rps5
Rpl11
Rps7
Rpl26
Rps15a
Rps12
Rps3a

Supplementary Figure 1: a- The PPI network showed the interaction among upregulated protein in the VP of the animal's control and treated group in F1-(PDN22) and F2-(PND22-F). The PPI network was constructed into the STRING databases, and the thickness among proteins (nodes) showed the degree of confidence of the interaction. **a.** In this PPI is possible to see no interaction among the proteins with proteins not identified in this comparison. **b.** The cluster showed the strong association of Gdi2 protein with another 10 different proteins. This interaction is associated with unbalance of GDP/GTP exchange reaction. **c.** The cluster showed the strong association of Prdx1 protein with another 10 different proteins that have relation with cell protection against oxidative stress. **d.** The cluster showed the strong association of Myh4 protein with another 6 Myh and 3 Tnnt family proteins, and Acta2 protein. All these proteins act in intracellular architecture, since are associated with signaling and cellular processes **e.** The cluster showed the strong association of hnrmph1 with other proteins of the same family, Hnrnpl family protein, and Srsf, this interaction has association with alteration in nascent pre-mRNAs, packaging then into hNRNP particles. **f.** The cluster showed the strong association of Rps7 with other proteins of the same family, and this can alter the translation process.



Supplementary Figure 2: a-The PPI network showed the interaction among the downregulated in the VP of the animal's control and treated group F1-(PND22) and F1-(PND120). The PPI network was constructed into the STRING databases, and the thickness among proteins (nodes) showed the degree of confidence of the interaction. In this PPI is possible to see interaction between Nme1 and Nme2 protein, and Tpm5


a
structural constituent of muscle
cytoskeletal motor activity
vesicle docking involved in exocytosis
procollagen-proline 4-dioxygenase activity
muscle filament sliding
basal part of cell
zymogen activation
extrinsic component of external side of plasma membrane
steroid binding
GTPase activity
basement membrane
cell tip
sperm head
translation elongation factor activity
cytoplasm
extracellular space
cilium
HFE-transferrin receptor complex
microfilament motor activity
skeletal muscle tissue development
ATP biosynthetic process
cilium assembly
GTP binding
fascia adherens
cellular_component
membrane depolarization during action potential
peptidyl-proline hydroxylation to 4-hydroxy-L-p
programmed cell death
transferrin receptor binding
toxic substance binding
ATP metabolic process
endoplasmic reticulum chaperone complex
glucocorticoid receptor binding
Rab protein signal transduction
regulation of striated muscle contraction
antigen processing and presentation
calmodulin binding
response to heat
negative regulation of reactive oxygen species biosynthetic process
myosin complex
protein kinase binding
thyroid hormone binding
trans-Golgi network transport vesicle
positive regulation of cell migration
positive regulation of cytoplasmic translation
enzyme binding
cellular response to insulin stimulus
drug binding
actin binding
cytoskeletal protein binding
regulation of exocytosis
response to platinum ion
TPR domain binding
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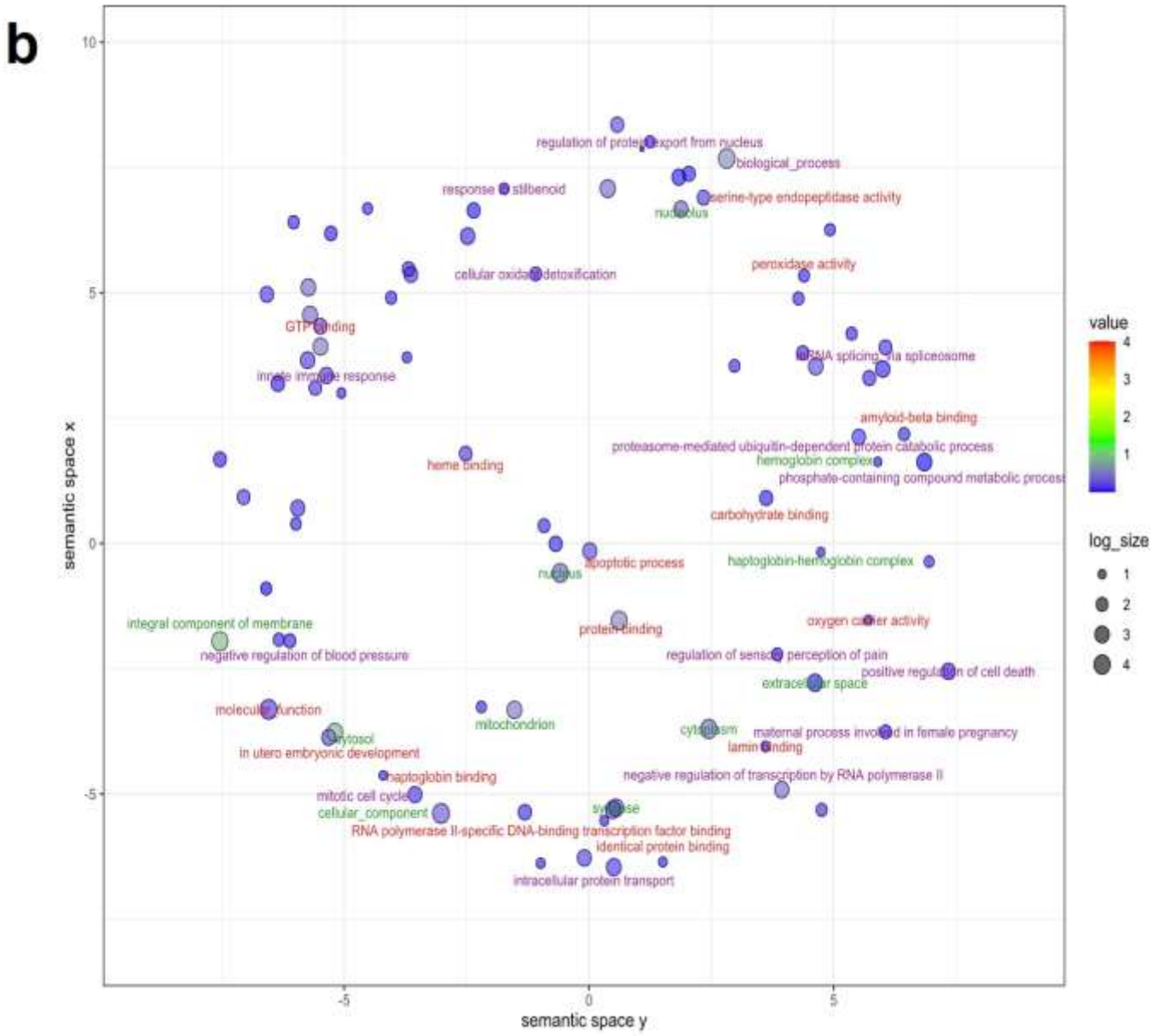
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peroxidase activity
cellular oxidant detoxification
GTP binding
innate immune response
mRNA splicing, via spliceosome
amyloid-beta binding
proteasome-mediated ubiquitin-dependent protein catabolic process
hemoglobin complex
heme binding
phosphate-containing compound metabolic process
carbohydrate binding
haptoglobin-hemoglobin complex
apoptotic process
nucleus
integral component of membrane
protein binding
oxygen carrier activity
negative regulation of blood pressure
regulation of sensory perception of pain
positive regulation of cell death
extracellular space
molecular_function
mitochondrion
cytoplasm
maternal process involved in female pregnancy
cytosol
lamin binding
in utero embryonic development
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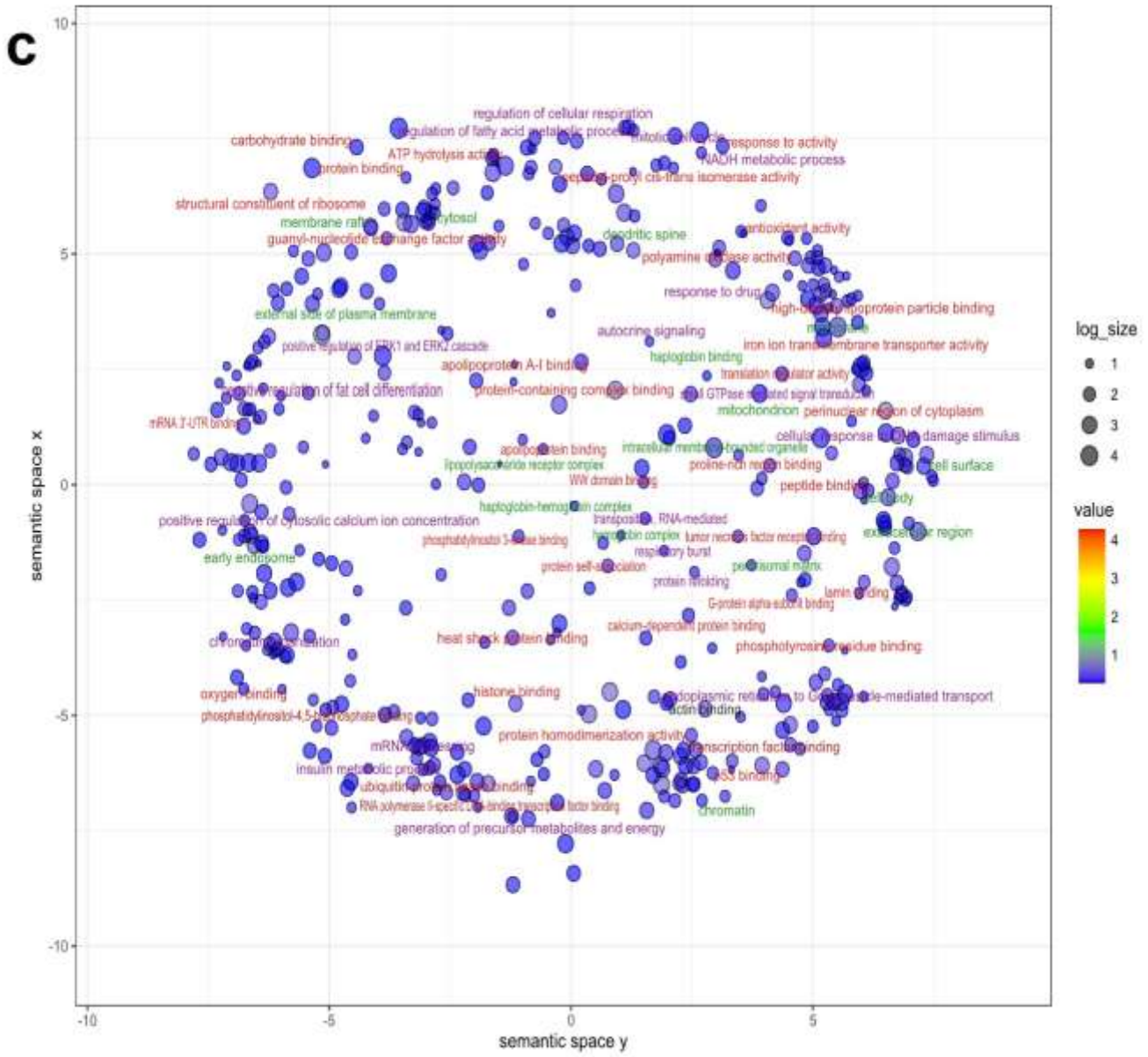
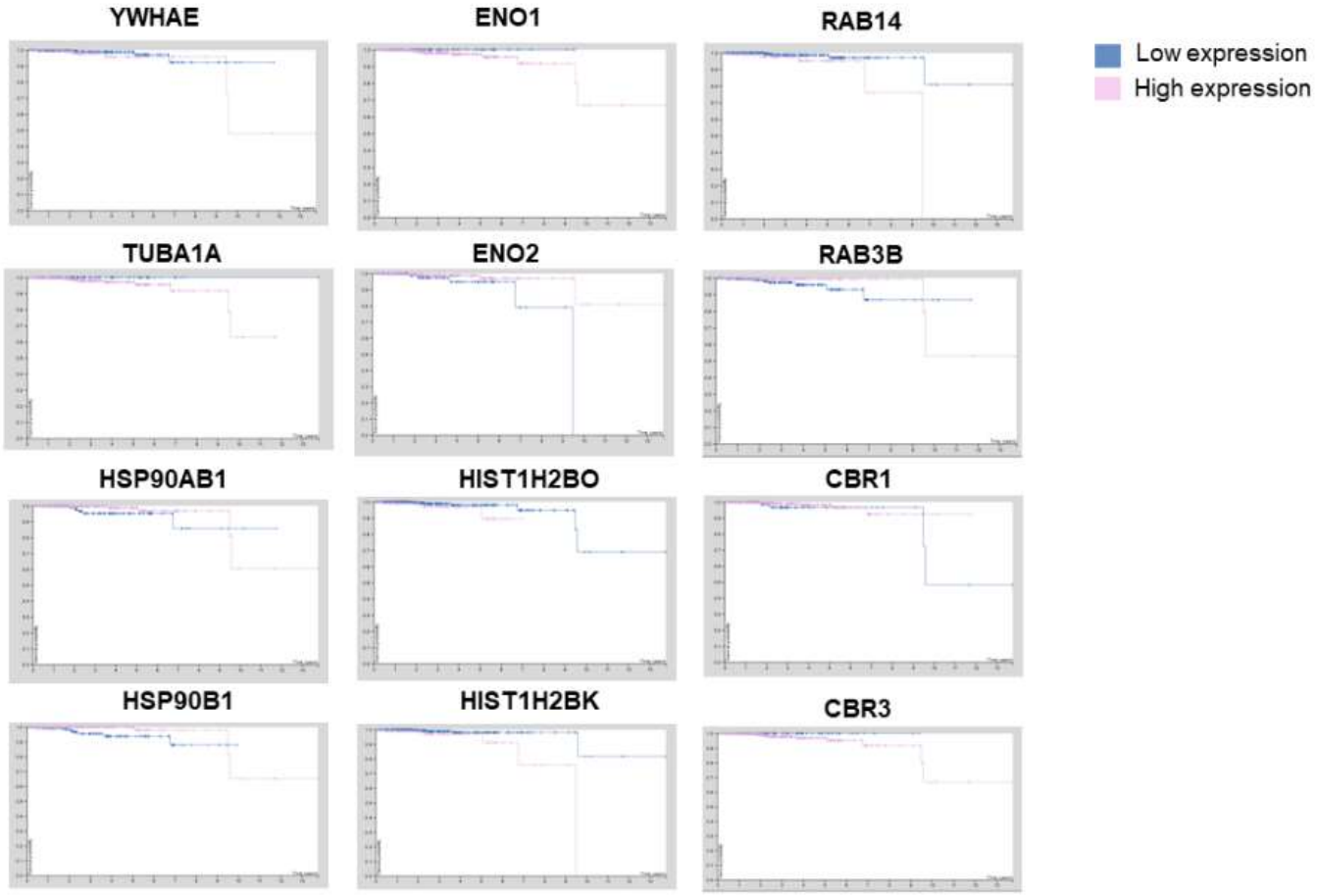
identical protein binding
intracellular protein transport



Supplementary Figure 3: a. The graph plot showed the cluster of all ontological terms: BP, CC, and MF, enrichment in the comparison among F1-PND22 vs F2-PND22-F. **b.** The graph plot showed the cluster of all ontological terms: BP, CC, and MF, enrichment in the comparison among F1-PND22 vs F2-PND22-M. **c.** The graph plot showed the cluster of all ontological terms: BP, CC, and MF, enrichment in the comparison among F2-PND22-F vs F2-PND22-M. The enrichment was made in KOBAS, and the cluster was made in REVIGO tool. The color represents BP - purple, CC - green and MF red. The abbreviation means BP: Biological process, CC: Cellular component, and MF: Molecular Function



and Tpm4 protein.

Supplementary Figure 4: graph of survival analysis of human patient with diagnostic to prostate cancer. These proteins were identified in this study in the comparison of all downregulated proteins in all the samples in F1 and F2. All photomicrographs were obtained in “The Human Protein Atlas” platform.

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Capítulo V

Resultados preliminares do projeto BEPE

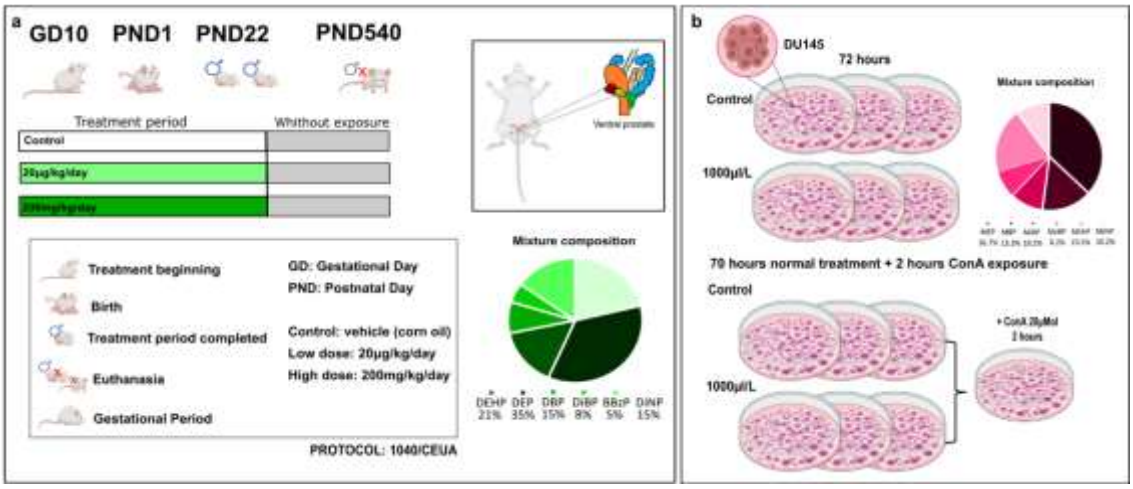

Figure 1: Experimental design. **a.** The representative diagram highlights the experimental design in the *in vivo* model. The pregnant rats were treated from DG10 to PND21, a period that corresponds to the gestation and lactation of the offspring. At the end of the treatment period, the offspring were kept in the bioterium under normal controls and without exposure to the mixture until DPN540. Abbreviations: GD: Gestational Day; PND: Postnatal day. **b.** The representative diagram showing the experimental design used to perform the culture cell test. The DU145 cells were grown in 10 cm^2 plates (triplicate) and maintained at 37 °C for 24 hours. The cells were subjected to a treatment with vehicle (0.5% DMSO) or 1000µM/L of the phthalate mixture for 72 hours and subjected to a treatment with vehicle (0.5% DMSO) or 1000µM/L. In the second moment, the cells were submitted to the same experimental design, however, with 70 hours of normal treatment and in the last to hours of the treatment the Concanamycin (ConA) was added to the culture medium to block the autophagy process, by inhibiting the degradation of lysosome.

Results

Results of the animal tissue experiment

During the experimental design the animals showed common characteristics of the aging process, which resulted in a 40% death rate of the animals in the different experimental groups. Among the treatment groups, the death rate was higher in animals exposed to the lower dose, but it is not possible to say that it was due to the treatment. With respect to the animal's body weight, the data reveal that there was no difference between the experimental groups (fig.2a). However, when the weight of the prostate

gland was analyzed, there was a significant increase in the prostate weight of the offspring of mothers exposed to higher dose (200mg/kg/day) (fig. 2b).

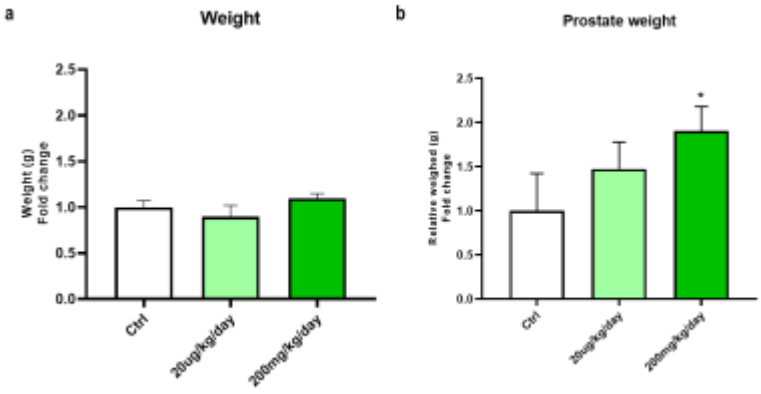

Figure 2: Representative bar graph showing the weight (b) and prostate weight (b) of senile offspring exposed to different treatment group (Ctrl: vehicle; low dose: 20µg/kg/day and high dose: 200mg/kg/day) during gestational and lactational development and evaluated only in PND540. The experimental groups were compared two by two, and the statistical analysis used was T test, followed by the Mann-Whitney test. *p<0.05.

The data obtained reveals the pattern of protein abundance profile is different among the groups (fig.3ab) and it was observed heterogeneously among samples of the same group (fig.3a). Despite this variation, it was possible to identify 82 upregulated and 78 downregulated differentially abundant proteins (DAP) in the group exposed to the highest dose of the treatment when compared to control group (S1). The protein-protein network (IPP) of the upregulated proteins (Atp2a2, Atp2a3, Alg9, Fkbp11, Ddost, Ldb3, Rpl30, Rpl8, Rpl9, Rpn1, Rps11, Rps26, Rps27l, Magt1, Ostc, Pdia4, Ptgfrn, Rnft1, Sar1a, Sec61a1, Sh3g12, Srp9 and Tnnc2) was separated into three proteins clusters (fig.4a). These proteins have influence in translation, membrane proteins complex, endoplasmic reticulum, and endomembrane system (fig.4b). What may be show a possible interference of the treatment in the accumulation of stress in the Endoplasmic Reticulum (ER), which may trigger out of control in the synthesis of membrane proteins, for example, or even in the response mechanism of the cell against the stress provoked by the treatment.

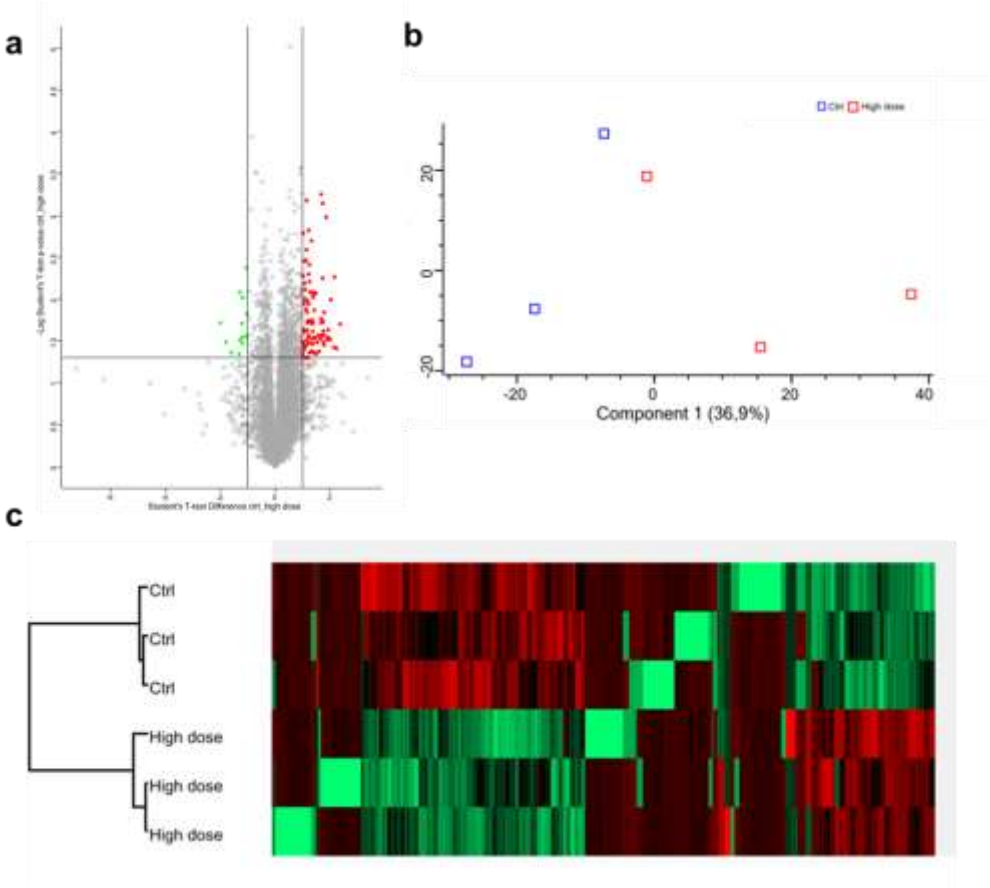

Figure 3: Representative graphs highlighting the pattern of protein abundance on VP of senile offspring (PND540) in the comparison control vs. high dose group (200mg/kg/day). **a.** Vulcan plot showing the pattern of the DAP according to the log of p-Value. **b.** PCA plot showing the cluster of samples by treatment group. **c.** Heatmap showing the differentiation of protein abundance profile in each sample and treatment group (p < value).

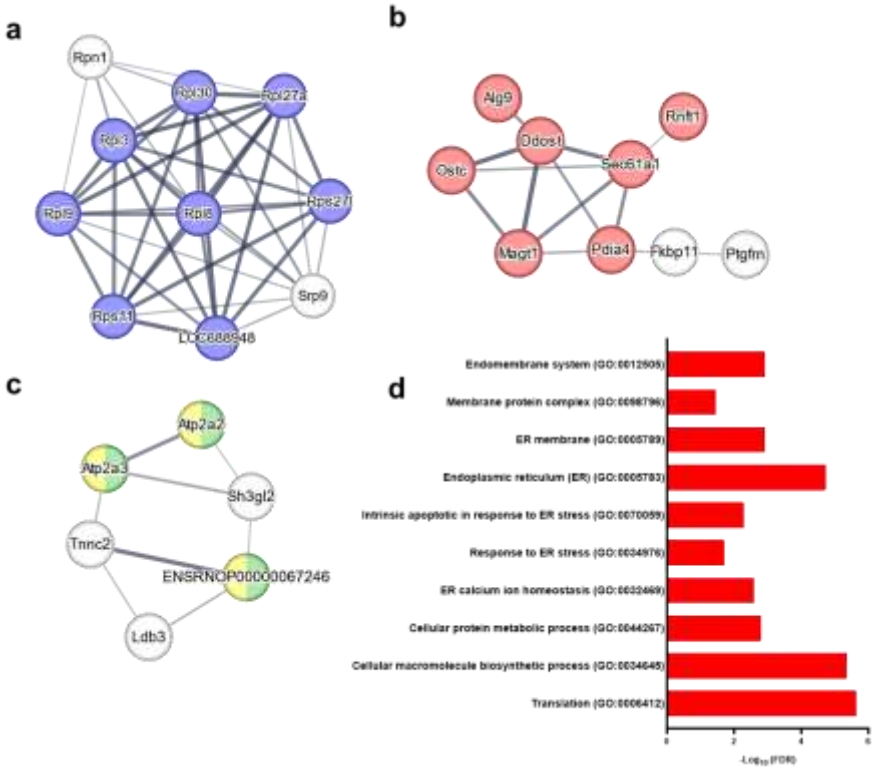

Figure 4: The IPP network shows three clusters of upregulated proteins in senile offspring of mother exposed to highest dose of the phthalate mixture (200mg/kg/day). **a.** The blue protein cluster is composed by the interaction of ribosomal protein (Rpl30, Rpl8, Rpl9, Rpn1, Rps11, Rps26 and Rps27l), which are mainly related to the translation and endoplasmic reticulum (ER). **b.** The red protein cluster, composed by the interaction of different proteins (Sar1a, Rnft1, Pdia4, Sec61a1, Ptgfrn, Alg9, Ostc, Ddost and Magt1), which are of the endomembrane system complex **c.** The green protein cluster showed proteins associated with the response of endoplasmic reticulum to stress (Atp2a2, Atp2a3 and ENSRNOP00000067246). **d.** Bar chart showing the top 10 biological processes enriched by downregulated proteins. The graph is plotted using -log10 of FDR.

Enrichment analysis for the biological pathways showed strong influence of these proteins on ribosomes, metabolic pathway, protein processing in endoplasmic reticulum, calcium signaling pathways and others, pathways that may be targets of treatment (fig 5a). In addition, the data for enrichment analysis for diseases perturbation shows at least 16 proteins that are associated with prostate cancer as well as with another types of cancer hormone dependent as Endometrial and Breast cancer and testis seminoma

(fig 5b). The strength interaction between proteins shows that when the analysis was made considering the Homo Sapiens as organism, the interaction among ribosomal proteins with other proteins that were also altered in the proteome of the animals, present a slightly different interaction from those obtained from animals, which could point to lack in animal database.

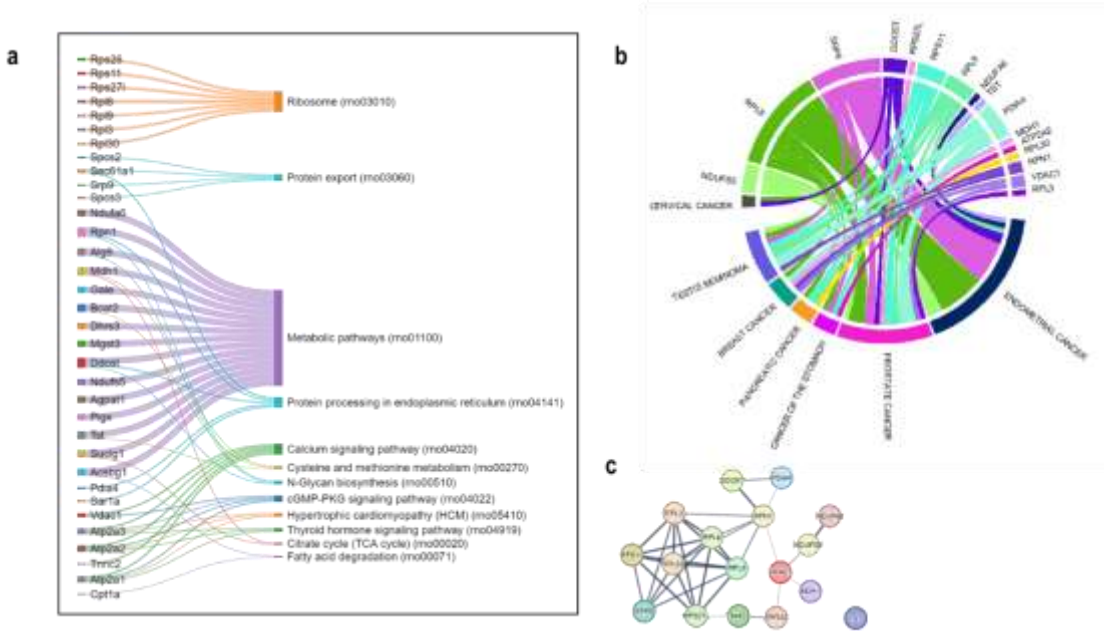

Figure 5: a. Alluvial graph showing the main pathways influenced by the upregulation of the proteins in the proteomic profile of senile offspring of mother exposed to the highest dose of the treatment (200mg/kg/day). In the graph it is possible to identify the altered pathways are ribosomes, metabolic pathway, protein processing in endoplasmic reticulum, calcium signaling pathways and others (KEGG database). The pathways were selected according to FDR. **b.** Circus plot showing the relationship of proteins in cancer conditions (Enrichr database, Diseases perturbation GEO). **c.** IPP of the ribosomal protein with other altered proteins identified in this investigation, however, the enrichment was made for *Homo Sapiens* as organism on string database.

The RPL8, DDOST, NDUFS5, RPL9, PDIA4, RPL3 are potential targets because these proteins were identified in the sample of patients with prostate adenocarcinoma (Fig.6a). In addition, the RPL8, RPL3, SPCS2, SEC61A1, SRP9, RPN1, DDOST, PDIA4, ATP2A3, TNNC2, ATP2A1 RNA encoding the proteins showed altered

expression in the genome of patients diagnosed with prostate adenocarcinoma (PRAD) (Fig.6b). The interaction network indicates direct interaction among RNP1, SEC61A1, PDIA4, DDOST, SPCS2, SRP9, RPL8, RPL3 and direct interaction among ATP2A1, TNNC2, ATP2A3 (Fig.6c).

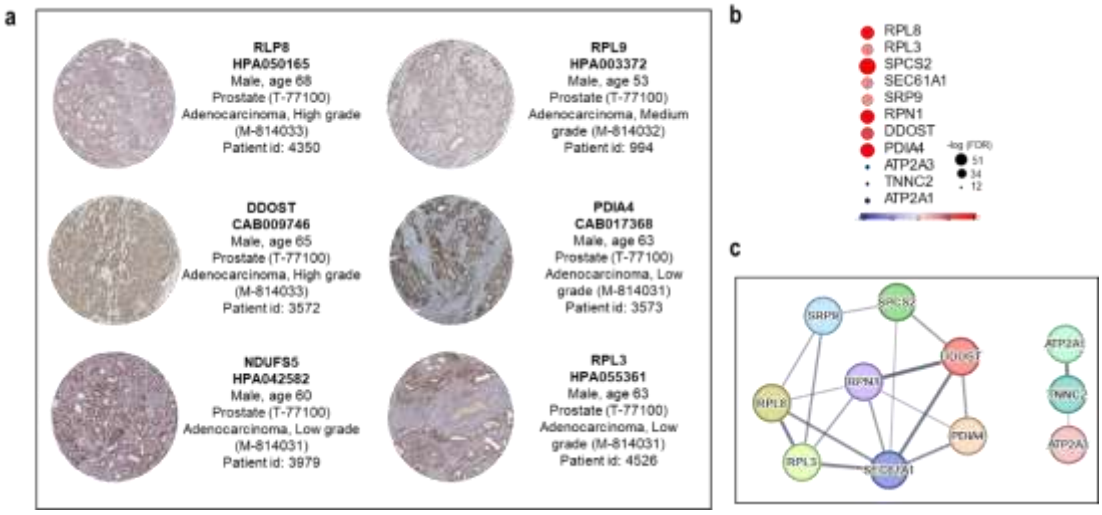

Figure 6: a. Histological photomicrograph showing the positive reaction (by immunohistochemistry) of RPL8, DDOST, NDUFS5, RPL9, PDIA4, RPL3 proteins in prostate adenocarcinoma. The data was obtained in "*The human protein Atlas"* (HPA) database **b.** Heatmap representing the deregulated expression of RPL8, RPL3, SPCS2, SEC61A1, SRP9, RPN1, DDOST, PDIA4, ATP2A3, TNNC2, ATP2A1 RNA encoding the proteins in PRAD. The heatmap was made considering $\log_2$(fold change) with data collected in "*The Cancer Genomic Atlas" (*TCGA) database **c.** IPP showing interaction among RNP1, SEC61A1, PDIA4, DDOST, SPCS2, SRP9, RPL8, RPL3 and interaction among ATP2A1, TNNC2, ATP2A3. The IPP was constructed in string database.

The results obtained for the downregulated proteins pointed that among the DAP there is proteins related to apoptosis as Bax, Bcl2I14 and Casp8, cell cycle as Cdkn2b, Jak1 and Kras, extracellular matrix degradation as Mmp2, Tcn2 and Fgf2 (Fig.7a). On the other hand, the interference of the treatment on downregulated proteins (Cdkn2b, Gpnmb, Cd44, Dpp4, Krt19, Krt80, Jak1, Nfkb2, Fgf2, Vcan, Fbln2, Fbln5, Tnc and Mmp2) on VP of senile offspring exposed to high dose also point to Prostate morphogenesis; Cell differentiation (proteins highlighted in yellow); Regulation of cell adhesion; Regulation of cell proliferation; Response to fibroblast Growth factor and Matrix extracellular (Fig.7b, Fig. 8a-b and S Fig3).

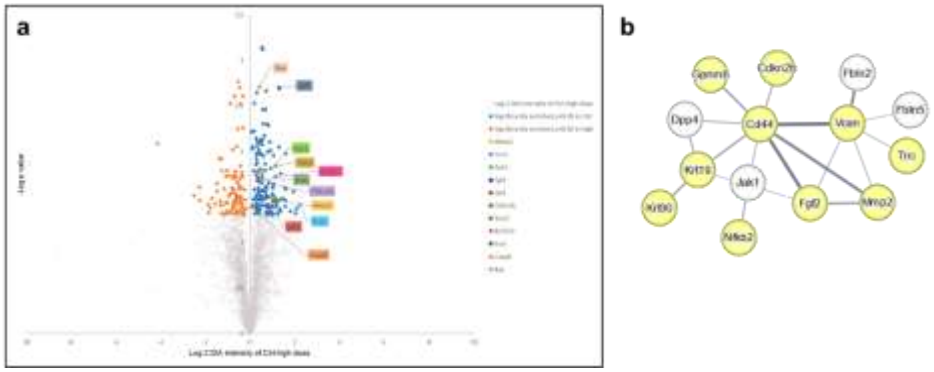

Figure 7: a. Vulcano plot highlighting the main target proteins differentially abundant (Mmp2, Tcn2, Itpr2, Fgf2, Jak1, Cdkn2b, Stat3, Bcl2l14, Kras, Casp8 and Bax) on the proteome of the senile offspring (PND540). The graph was plotted using log10 of pValue <0,05 and log2 DIA intensity considering the difference between ctrl vs. high dose group. **b.** The IPP network shows interaction among the downregulated proteins on VP of senile offspring of mother exposed to highest dose of the mixture (200mg/kg/day). The IPP network highlights proteins involved in biological processes such as Prostate morphogenesis by Tnc, Mmp2 and Cd44 protein; Cell differentiation by Gpnmb, Cdkn2b, Cd44, Vcan, Tnc, Krt19, Krl80, Nfkb2, Fgf2 and Mmp2 protein; Regulation of cell adhesion by Gpnmb, Dpp4, Cd44, Jak1, Mmp2, Tnc and Fbln2 protein; Regulation of cell proliferation by Gpnmb, Cdkn2b, Cd44, Dpp4, Fgf2, Mmp2, Tnc and Fbln5 protein, Response to fibroblast Growth factor by Cd44, Fgf2 and Tnc protein and Matrix extracellular organization by Fbln2, Fbln5 Mmp2 and Nfkb2 protein.

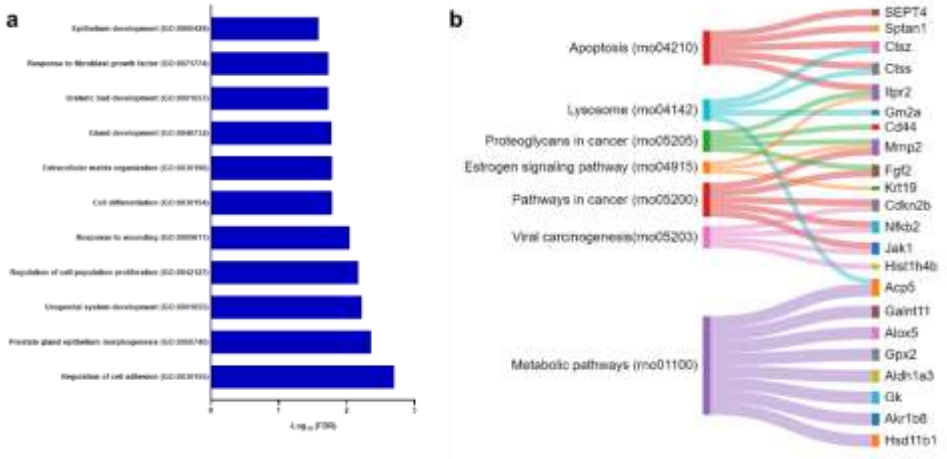

Figure 8: a. Bar chart showing the top 10 biological processes enriched by downregulated proteins. The graph was plotted using -log10 of FDR. **b.** Alluvial graph showing the main

pathways influenced by the downregulation of the proteins in the proteomic profile of the senile offspring exposed to the highest dose of the treatment (200mg/kg/day). In the graph it is possible to identify the altered pathways are apoptosis, pathways in cancer, metabolic pathway, Estrogen signaling, lysosome, proteoglycans in cancer and viral carcinogenesis (KEGG database). The pathways were selected according to FDR.

There was no change in the activity of metalloproteinases when the experimental groups were compared (Fig. 9a), however, when the abundance of proteins was measured, there was a significant reduction in the abundance of Mmp2 in both treatment groups; increased on the quantity of Tcn in the group exposed to the lowest dose (20µg/kg/day) and significant reduction in the group exposed to the highest dose (200mg/kg/day). On the other hand, the cell proliferation index measured by PCNA quantity showed a significant increase in both treatment groups (Fig. 9b).

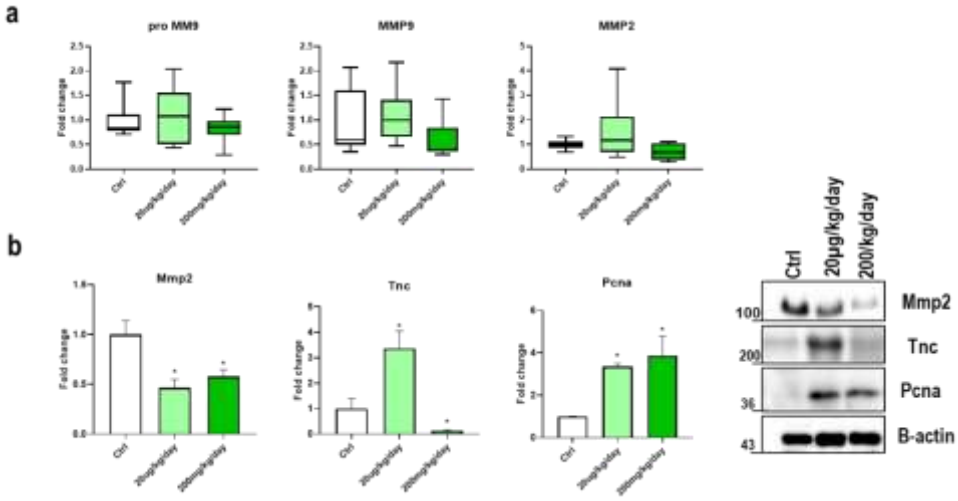

Figure 9: a. Representative graph showing the activity of metalloproteinases 9 and 2 on VP of senile offspring of mother exposed in the comparison between control vs. low dose group (20µg/kg/day) and control vs. high dose group (200mg/kg/day). **b.** Representative graphs showing the abundance of the proteins Mmp2, Tnc and PCNA on VP of senile offspring (PND540) in the comparison between control vs. low dose group (20µg/kg/day) and control vs. high dose group (200mg/kg/day). The data obtained for the metaloproteinases was analyzed by median followed by [Q1-Q3] in the comparation two by two. The abundance of the proteins was analyzed by mean ± SEM in the comparation two by two. Asterisks represent statistical difference by Many-Whitney test: *p <0,05.

The data obtained from the CTD demonstrated the interaction of phthalate diesters as DBP, DEP, DEHP, DnHP, and monestes as MBzP and other types of phthalates with the up and downregulated proteins DAP in this investigation. Which reinforces that although the DAP identified in this investigation did not show a strong interaction network among themselves, they can be modulated by the different phthalates present in the mixture (Table1).

Table 1: Table showing the relationship of DAP on VP of senile offspring exposed to highest dose of the mixture (200mg/kg/day) with different types of phthalates.

Phthalates	Upregulated	Downregulated
Dibutyl Phthalate	ACSBG1, ATP2A2, ATP2A3, BCAT2, CISD1, CMTM6, CPT1A, CYB5B, DHRS3, DNAJC19, HSPB6, LDB3, LRRC59, MDH1, MGST3, MRPS21, MYH4, NDUFA6, NDUFS5, OSTC, PCP4, PRMT3, RGD1304884, RIDA, RNFT1, RPN1, RPS11, RPS27L, SEC61A1, SGPP1, SH3GL2, SLC30A7, SUCLG1, TST, VDAC1	AKR1B8, ALDH1A3, ARHGAP45, ARHGDIB, BIN2, CD44, CLDN23, CLU, CSNK1G2, EXOSC8, FILIP1L, FMNL3, GALNT11, GK, GPNMB, HIST1H4B, HSD11B1, KRT19, LYZ2, MARCKSL1, MMP2, NFKB2, OTUD7B, POLE, RAP2C, RRP9, RTN1, SESTD1, SIRPA, STK10, TMSB4X, TOR3A, VCAN
Diethyl phthalate	PCP4	RTN1
Diethylhexyl phthalate	AGPAT1, ANGPTL1, ATP2A1, ATP2A2, BCAT2, CPT1A, CRAT, CYB5B, DHRS3, FKBP11, GALE, HSPB6, KCNK1, LDB3, MDH1, MGST3, MYH1, PCP4, RPS27L, SGPP1, SULT1D1, TF, TNNC2, TUBA1C	AKR1B8, ALDH1A3, ARHGAP45, ARHGDIB, CD44, CDKN2B, CLU, DPP4, FBLN5, HSD11B1, KRIT1, KRT19, LYZ2, MMP2, PAG1, PPL, RTN1, SCPEP1, TNC, TOR3A
Di-n-hexyl phthalate	-	HSD11B1
Monobenzyl phthalate	-	CDKN2B
Phthalic acid	ABHD13, LRRC59, OSTC, RPL3, TST	ALDH1A3, GALNT11, KRT19, PCOLCE, SESTD1

Similar results were obtained in the analysis on the proteome profile in the senile offspring exposed to the highest dose (200mg/kg/day) (Fig. 10a). The results showed that the treatment also was able to interfere with protein abundance (Fig. 10b). However, the lower treatment dose expressed a more DAP profile than that was observed at the higher dose group (Fig. 10c). The lower dose of exposure upregulated 164 proteins and downregulated 90 proteins. The IPP network of the upregulated protein (Prom1, Krt5, Akr1b1, Lgals3, Aldoc, Gsta1, Bdh2, Ctsl, Cdc42, Plec, Sfn, Gsta3,

Cbr1, Lamb2, Idua, Col5a2, Lum, Plg, Abi3bp, Col14a1, Dpt, Dcn, Serping1, Lgals1, Timp1, Cpb2, Klkb1, F13a1, Ceacam1, Ncam1, Apoe, Cfh, Fabp5, Sirt1, Cfb, Timp2, Mb, Ace, Car2, Arsb, Adh1, Slc2a4, Sord, Gstp1, Dtymk, Hmgcs2, Sult1a1, Ahsg, Mfge8, Anxa3, LOC103689965, Zak, Alb, Apoh, Dcn, Hspa2, Naprt1, Gsta4, Serpina1, Nqo1, Ephx2, Serpinb6, Hspa12a, Fv1, C6, Rnasel, Actb, Gnb1, Kng1, H2afx, Serpinb1a, Cd163, Ube2d2, Mep1b, Apoa4, Glul, Msra) was separated into six protein clusters with more expressive protein interaction (Fig. 11a-f).

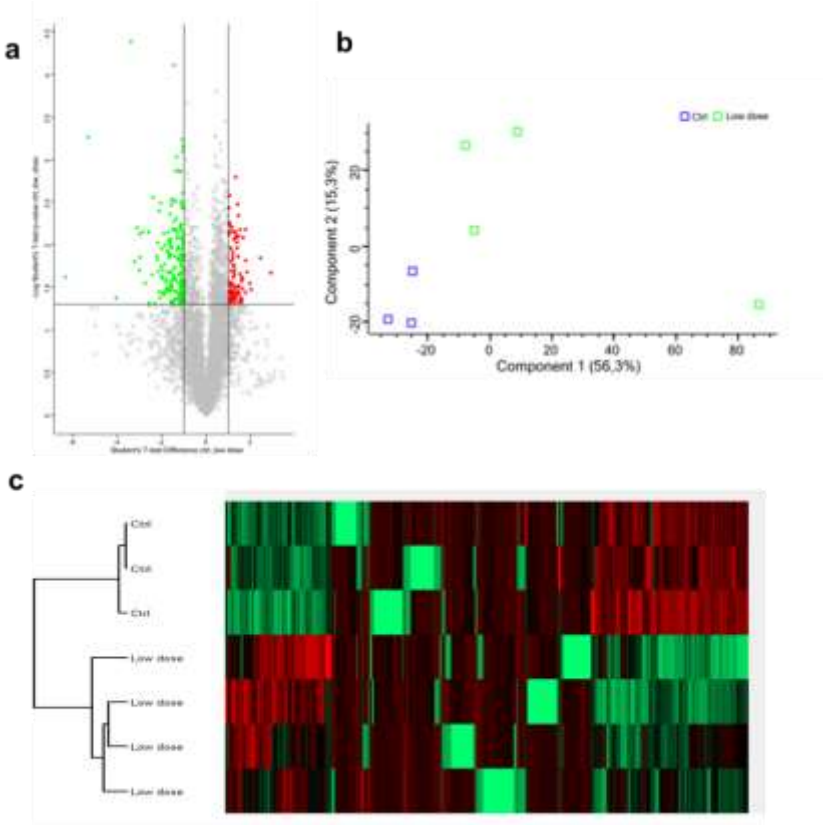

Figure 10: Representative figures highlighting the pattern of protein abundance on VP of senile offspring (PND540) of mother exposed to lower dose of the treatment (20µg/kg/day) in the comparison between control vs. low dose group. **a.** Vulcan plot showing the pattern of the DAP according to the log p-Value. **b.** PCA plot showing the cluster of samples by treatment group. **c.** Heatmap showing the differentiation of protein abundance profile in each sample and treatment group (p < value).

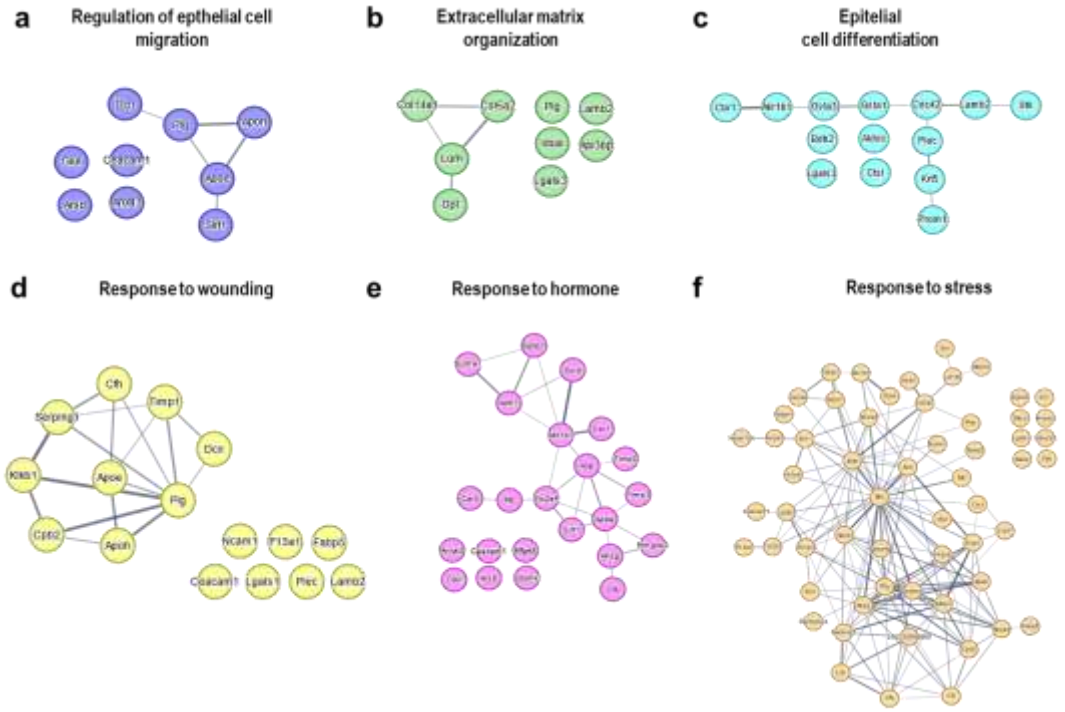

Figure 11: The IPP network shows clusters of upregulated proteins in the offspring exposed to highest dose of the phthalate mixture (20µg/kg/day). **a.** The dark blue cluster is composed by the interaction of proteins that are involved in regulation of epithelial cell migration (Prom1, Krt5, Akr1b1, Lgals3, Aldoc, Gsta1, Bdh2, Ctsl, Cdc42, Plec, Sfn, Gsta3, Cbr1, Lamb2). **b.** The green cluster is composed by the interaction of proteins responsible to keep the extracellular matrix organization (Idua, Col5a2, Lum, Lgals3, Plg, Abi3bp, Col14a1, Lamb2, Dpt). **c.** The light blue cluster is composed of protein associated with epithelial cell differentiation (Prom1, Krt5, Akr1b1, Lgals3, Aldoc, Gsta1, Bdh2, Ctsl, Cdc42, Plec, Sfn, Gsta3, Cbr1, Lamb2). **d.** The yellow cluster is composed of proteins which are involved in response to wounding (Apoh, Dcn, Serping1, Lgals1, Timp1, Cpb2, Klkb1, F13a1, Plg, Plec, Ceacam1, Ncam1, Apoe, Cfh, Fabp5, Lamb2). **e.** The pink cluster is composed of protein that are related to response to hormone (Sirt1, Cfb, Timp2, Mb, Ace, Akr1b1, Car2, Timp1, Arsb, Adh1, Slc2a4, Sord, Gstp1, Ctsl, Dtymk, Hmgcs2, Sult1a1, Ceacam1, Apoe, Ahsg, Mfge8, Anxa3, Cbr1). **f.** The orange cluster is compost by a lot of proteins involved in response to stress (Sirt1, Cfb, LOC103689965, Zak, Alb, Apoh, Dcn, Mb, Hspa2, Serping1, Ace, Naprt1, Gsta4, Serpina1, Akr1b1, Car2, Lgals1, Timp1, Lgals3, Cpb2, Aldoc, Nqo1, Klkb1, F13a1, Slc2a4, Sord, Plg, Ephx2, Serpinb6, Hspa12a, Gstp1, Ctsl, Fv1, Hmgcs2, C6, Cdc42, Rnasel, Plec, Sfn, Actb, Gnb1, Ceacam1, Ncam1, Apoe, Ahsg, Kng1, H2afx, Serpinb1a, Cfh, Cd163, Ube2d2, Anxa3, Mep1b, Fabp5, Lamb2, Apoa4, Glul, Msra).

The DAP on VP of senile offspring exposed to lower dose (20µg/kg/day) showed a strong interaction network, and the analysis of the major proteins clusters demonstrated the Biological Process (BP) (GO) influenced by the deregulation of these proteins were epithelial cell migration, extracellular matrix organization, epithelial cell differentiation, response to wounding, response to stress, response to hormone and to stress (fig.12a-b). This significant result observed in the descendants exposed to the lower dose indicates a greater accumulation of tissue stress, which may be related to the long-term dose response effect. The pathways enrichment (KEEG) analysis indicates that the relationship among the targets with deregulation in pathways as Chemical carcinogenesis, Cytocromo P450 metabolism and pathways in cancer (Fig. 12b).

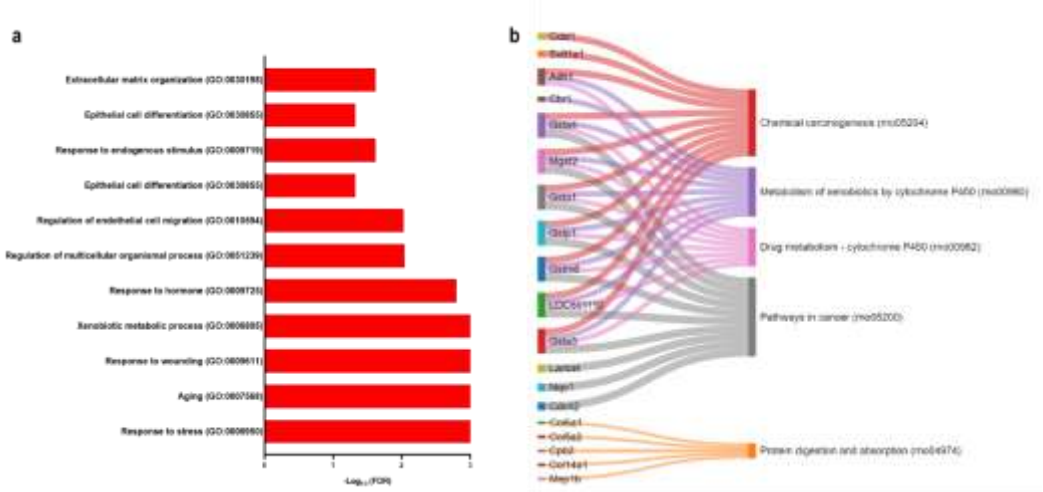

Figure 12. a. Bar chart showing the top 11 biological processes enriched by upregulated proteins on VP of senile offspring exposed to lower dose of the treatment (20µg/kg/day). The graph was plotted using -log10 of FDR. **b.** Alluvial graph showing the main pathways influenced by the upregulation of the proteins in the proteomic profile of the animals exposed to the lower dose of the treatment (20µg/kg/day). In the graph it is possible to identify the altered pathways Chemical carcinogenesis, Metabolism of xenobiotics by cytochrome P540, Drug metabolism – cytochrome P450, pathways in cancer and protein digestion and absorption (KEGG database). The pathways were selected according to FDR.

Regarding downregulated proteins, the IPP network was separated into three clusters with more expressive protein interaction (Fig.13a-c). Enrichment analysis

pointed an interesting interference of these proteins in BP such as Transport and intracellular transport (Fig.13a and d), Response to endoplasmic reticulum stress (Fig13b and d) and lipid and steroid hormone biosynthetic process and primary metabolic process (Fig.13c and d), which may be is related to the ability of phthalates to bind to specific receptors that block or reduce the hormone synthase chain and triggering different biological response. Interestingly at least ten of these proteins are involved in the protein processing in endoplasmic reticulum pathway (rno04151).

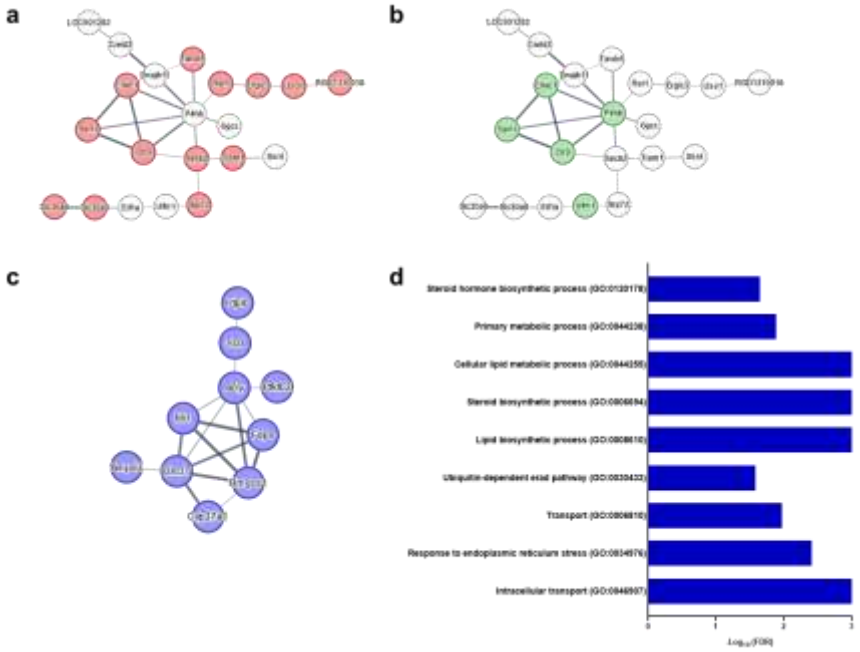

Figure 13: The IPP network shows interaction among the downregulated proteins in the senile offspring exposed to lower dose of the mixture (20µg/kg/day). a. The IPP network highlights proteins involved in Transport and intracellular transport (Srp72, Uso1, Slc30a6, Sec62, RGD1310016, Erlec1, Sel1l, Ergic3, Os9, Tram1), Response to endoplasmic reticulum stress (Erlec1, Sel1l, Os9, P4hb, Ufm1), Primary metabolic process and Steroid biosynthetic process (Ptplb, Pfkfb2, Idi1, Cyp27a1, Acly, Hmgcs1, Ampd3, Dhcr7, Fdps, Scd). **b.** Bar chart showing the top 10 biological processes enriched by downregulated proteins. The graph is plotted using -log10 of FDR.

Unlike what was observed in the offspring exposed to the highest dose, the integrative analysis of the data available in the CTD shows fewer proteins that have interaction of different type of phthalate (Table 2); however, these data can be analyzed

in two ways, either the result obtained in this investigation may contribute to update the data available on phthalate-target interaction or the altered proteins in this treatment group may be related to a side effect and not necessarily to the direct binding of phthalates.

Table2: Table showing the relationship of DAP on PV of senile offspring exposed to lower dose of the mixture (200mg/kg/day) with different types of phthalates.

Phthalates	Upregulated protein	Downregulated protein
Dibutyl Phthalate	ABI3BP, ACTG1, ADH1, AEBP1, AKR1B1, ALCAM, ALDOC, ANXA3, APOE, ARSB, CADM3, CBR1, COL6A1, CRYL1, CSRP1, CTSL, CUTC, DCLK1, DCN, DDAH1, DES, DTYMK, EHD2, FABP5	ACLY, AQP1, COA3, COPZ2, CPEB2, CTCF, CYP27A1, DDX39A, DHCR7, DNAJA1, EIF1A, FDPS, FKBP5, FURIN, GGCX, GRSF1, GTPBP4, HACD2, HMGCS1, IDI1, MCM6, MFSD6, NT5DC2, SSR4
Diethyl phthalate	ALDOC, CADM3, FABP5, GSTA3, HSPA12A, RAB3B, TIMP1	DHCR7, FKBP5, HMGCS1
Diethylhexyl phthalate	AASS, ACTBL2, ACTG1, ADH1, AEBP1, ALDOC, ANGPTL1, ANXA3, APOA4, APOE, BDH2, C6, CADM3, CKMT2, CPB2, CSRP1, CTSC, CTSL, DCLK1, DCN, EHD2, ENO2, EPHX2, FABP5	ACLY, AQP1, BRI3BP, CDKN2B, CEP170B, CPEB2, CYP27A1, DDX39A, DHCR7, DNAJA1, DNAJB11, FDPS, FKBP5, FURIN, HACD2, HMGCS1, HTATIP2, IDI1, MCM6, NT5DC2, P4HB, PERP, RER1, SLC7A5
Di-n-hexyl phthalate	SIRT1	-
Monobenzyl phthalate	CD163	CDKN2B
Phthalic acid	ALB, ALDOC, ANXA3, CFH, CTSL, GSTA5, GSTP1, HDGFL3, NCALD, NCAM1, SORD, TBC1D25	DHCR7, FDPS, FURIN, HMGCS1, IDI1, SSR4, TRAM1, UBAP2

Histopathological analysis showed that the prostate of animals in DPN540 presented simple cylindrical epithelium, varying in some regions to cylindrical pseudostratified (Fig.14a). Most animals, regardless of the experimental group, presented acini with epithelial atrophy, which is considered an adaptive change related to prostate senility (Fig. 14b; Fig.15).

The animals in both treatment groups (200µg/kg/day and 200mg/kg/day) presented inflammatory foci, associated or not with regions of inflammatory reactive atypia (ARI; Fig. 14c). Although the treated groups showed a higher incidence of regions of epithelial atypia (EA; fig.14d) and low-grade dysplasia (LGD; fig.14e) compared to the control group, this difference was not significant (Fig.14 and fig.16).

Among the proliferative changes were observed the adaptive modification, such as focal hyperplasia (FH; Fig.14d; detail), the benign tumors (adenoma), found only in one senile offspring of mother exposed to the low dose of the treatment (20µg/kg/day) (aden; Fig.15e), the premalignant lesions (PIN; Fig.14d) and the potentially malignant ones, such as adenocarcinomas in situ (Ais; Fig.15a-c), for some authors, also considered premalignant with high potential for malignancy. Focal hyperplasia had a higher incidence on senile offspring of mother exposed to the high dose (200mg/kg/day) of the treatment (p=0.07) when compared to control (Fig.16). On the other hand, lesions with higher malignancy potential (PIN and Ais) were observed significantly more incident in the T2 group when compared to the other groups (Fig.16).

It was observed a significant increase ($p < 0.05$) in the incidence of Prostatic Intraepithelial Neoplasia (PIN) in senile offspring of mother exposed to the low dose of the treatment (20µg/kg/day) when compared to control. In addition, a relevant percentage increase in the incidence of Adenocarcinomas in situ was observed in the same group. Also, an increasing trend (#, $p = 0.07$) of Focal Hyperplasias was seen in senile offspring of mother exposed to the high dose of the treatment (200mg/kg/day) (Fig.12).

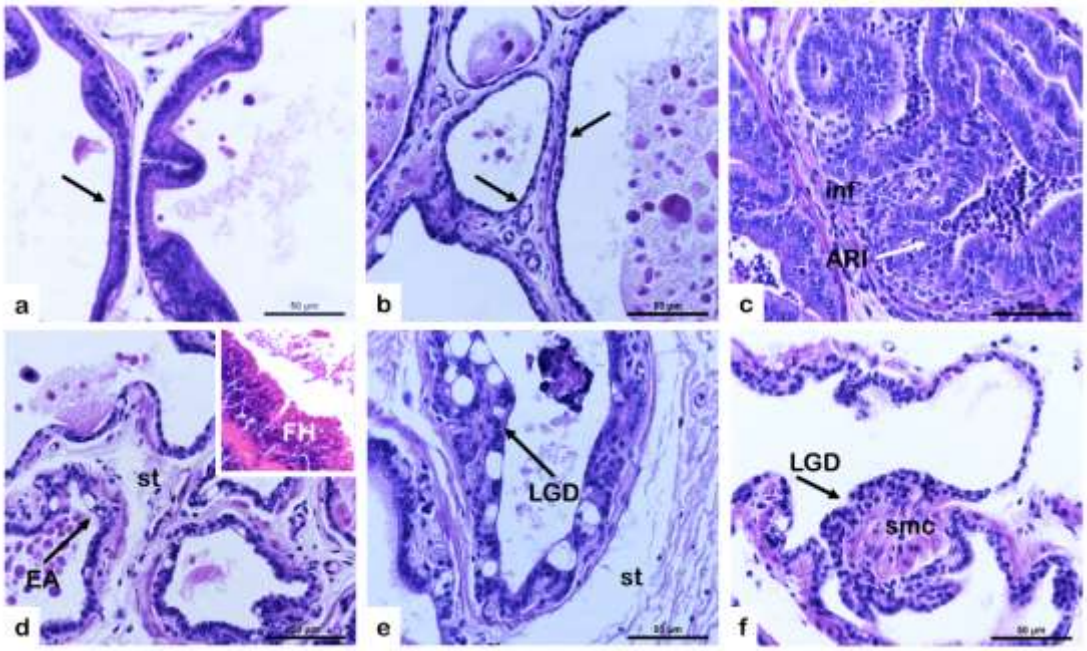

Figure 14: Histological sections of the prostate of senile offspring of mother exposed (DPN540) exposed to the different treatment. The figures are representative of the different histopathological changes observed in the different groups. a: Control group - the figure demonstrates the arrangement of the prostatic epithelium (arrow); b: low dose (200µg/kg/day): the black arrows point to acini with regions of epithelial atrophy; control: inflammatory focus (inf) associated with stratification of the epithelium that projects into the lumen of the acinus, called inflammatory reactive atypia (ARI); d: low dose (200µg/kg/day) T1: acini with epithelial atypia (EA) presenting cells with mucinous inclusions, and detail pointing to a region of focal epithelial hyperplasia (HF; 400x); e high dose (200mg/kg/day): region of mucinous inclusions with stratification, characterized as low-grade dysplasia (LVD); f: Group T2: region of low-grade epithelial dysplasia (LVD) with projection of smooth muscle cells into the stroma (smc). Abbreviations: st: stroma.

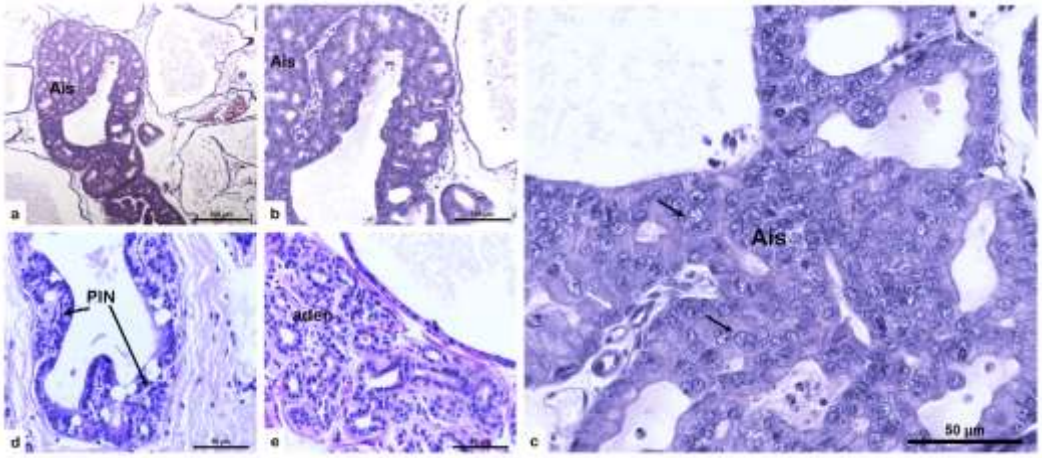

Figure 15: Histological sections of the prostate of senile offspring (DPN540) exposed to the different experimental groups. The figures are representative of the different histopathological changes observed in the different groups. a-c: Group 200mg/kg/day - the figures show regions of adenocarcinoma in situ with stratified epithelium forming micro-acini that project into the lumen of the acinus, presenting cells with loss of polarity, cellular and nuclear pleomorphism (black arrows) and mitosis figures (white arrow); d: 200µg/kg/day: presence of prostatic intraepithelial neoplasia (PIN): regions of epithelium with stratification, cribriform pattern and loss of polarity with cellular atypia; e: Group T1: region of ademoma (aden) with glandular hyperplasia and benign pattern of proliferation.

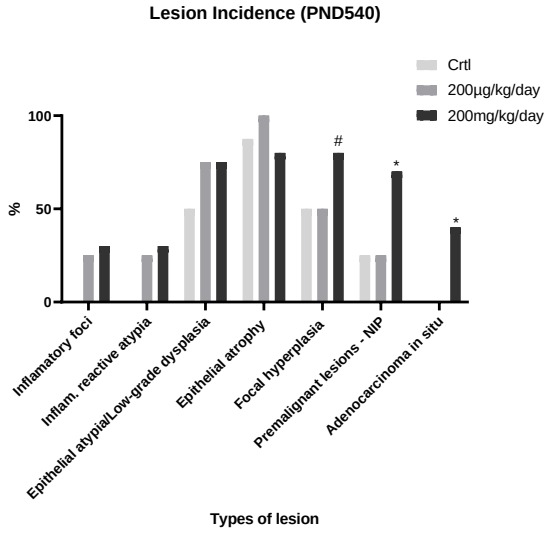

Figure 16: Bar graph related the percentage incidence of prostatic lesions in the VP of offspring in PND540 (n=8). It is possible to observe the classification in the different experimental groups. The prostatic disorders identified were epithelial atrophy, Inflammatory focus, inflammatory reactive atypia, epithelial atypia, focal epithelial hyperplasia, Low-grad dysplasia, adenocarcinoma in situ, prostatic intraepithelial neoplasia (NIP) and adenoma (aden). *Represents statistically significant $p<0.05$ and # significant tendency observed in the senile offspring exposed to 200 mg/kg/day.

Results of the cell tissue experiment

Based on the previous data published by Cavalca et al., 2021 with LNCaP and PNT2 prostate cell exposed to the same phthalate mixture used in this investigation, the viability test was performed to confirm the time and concentration used for DU145 cell. The test pointed that at 72 hours, the dose closest to the IC50 was 20000umol/L, for the other concentrations tested no significant difference was observed. Exposure to DMSO did not show exacerbated changes when compared to the control group (fig. 17).

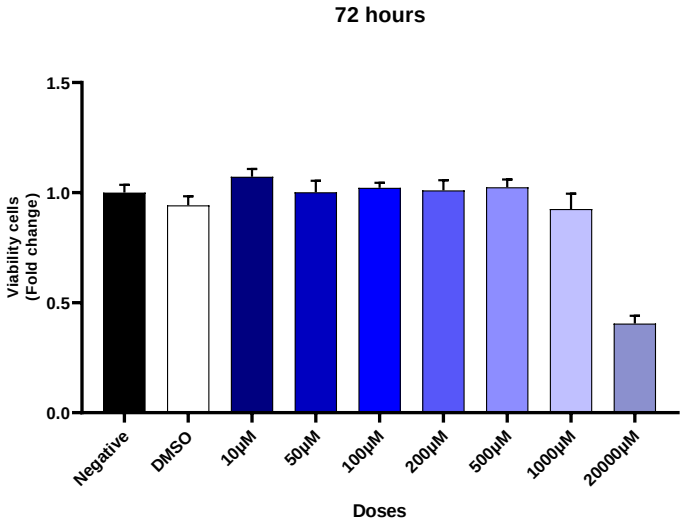

Figure 17. Viability patterns of DU145 cells after the cells being exposed to decreasing doses of the monoester phthalate mixture for 72 h. Cell viability is expressed as fold change. Negative control cells were treated with DMEM. DMSO, cells were treated with dimethyl sulfoxide added to the medium at a concentration of 0.05% at a dose of 1000µmol/L.

The DAP proteins in the proteome of cells exposed to the mixture of phthalates at concentration 1000µm/L shows a proteomic profile different from that obtained in the different groups of the *in vivo* experiment. However, the results obtained in *in vitro* investigation may present an interesting pathway for the initiation of the changes that was observed in the *in vivo* model.

The number of the DAP was higher considering the comparison of the data obtained to *in vitro* model, which is due to the *in vitro* model presenting cleaner data (Fig. 18a-c), free of possible contaminants from adjacent tissues or blood, for example. A similar point is that cells exposed to the treatment showed a higher number of downregulated proteins, which is a feature that has been observed not in these experimental model as well as in complementary data from another investigation with young animals. This leads to the hypothesis that exposure to phthalate monoesters and diesters downregulates the abundance of proteins essential for the control of tissue physiology and differentiation and may be directly related to a possible pathological condition (Fig. 3c and 10c; 18c).

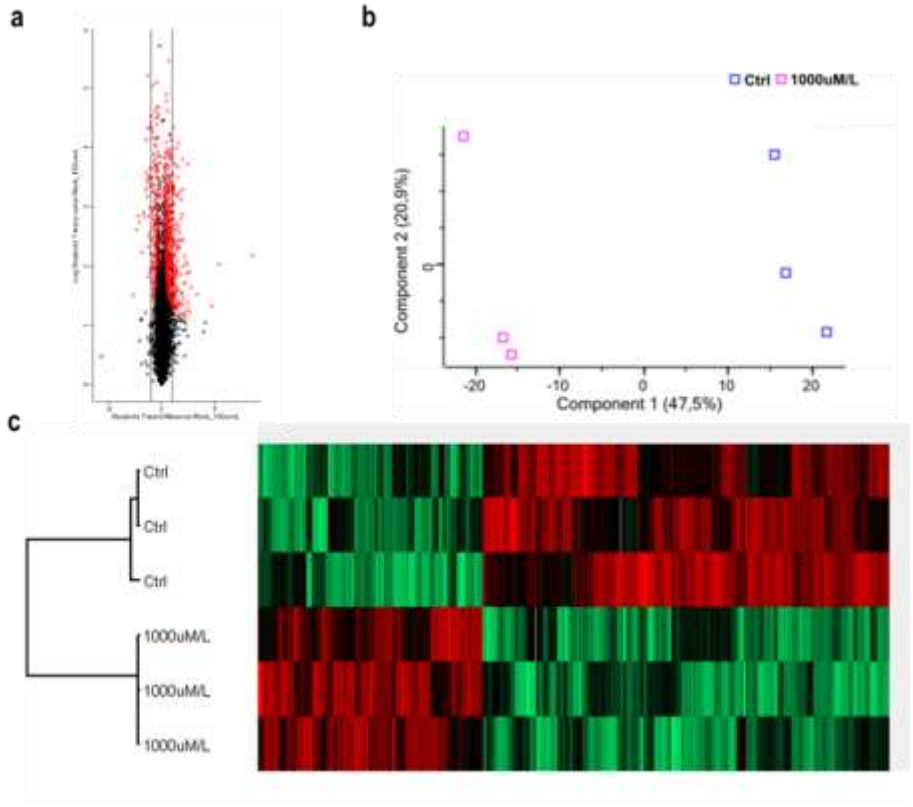

Figure 18: Representative figures highlighting the pattern of protein abundance on prostate cancer cell - DU145 treated with 1000µM/L of the monoester phthalate mixture in comparison between the cell underexposed. **a.** Vulcan plot showing the pattern of protein abundance according to the log of p-Value. **b.** PCA plot showing the separation of samples by treatment group. **c.** Heatmap showing the differentiation of protein abundance profile in each sample and treatment group (p < value).

After some filtering the data shows that the upregulated proteins are involved in regulation of cell process, regulation signaling, regulation of cell communication and response to stress. In addition, it was possible observed classical biomarkers for cell cycle by TP53; Oxidative stress by CAT and autophagy by SQTM1 (p62) upregulated in the proteomic profile of cell exposed to 1000µM/L of mixture of monoesters of phthalate (Fig. 19a and 20). In addition, there are other biomarkers involved in transcriptional activity and cell death pathways as JUN and BCAR1, an intersecting target involved in cell adhesion cell migration and cell branching. Both JUN and BCAR1 are described for

being involved in cancer condition (Fig.20). Despite having no statistically significant change, targets such as PCNA, ATG7, BCL2L3 was highlighted on the volcano plot because they are targets that has interacting with targets differentially abundant or they were used for validation by WB as PCNA, for example (Fig.20). The IPP network of downregulated proteins pointed these proteins are related with catalytic activity, oxidoreductase activity and metabolic pathways (Fig.19b).

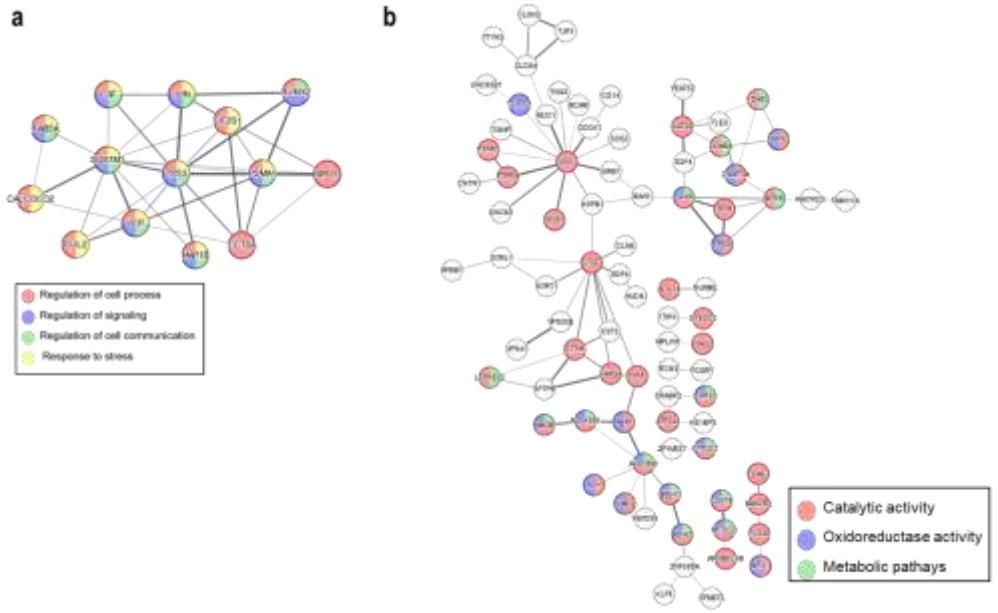

Figure 19: The IPP network showing the interaction of the upregulated (a) and downregulated (b) DAP identify on the proteome of cell exposed to 1000µM/L of mixture of monoesters of phthalate for 72 hours in comparation with unexposed cell.

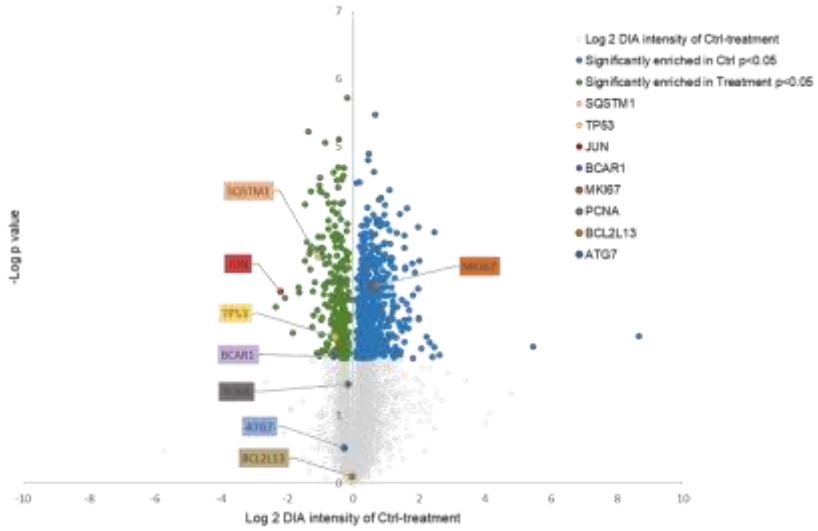

Figure 20: Vulcano plot highlighting the main target proteins DAP (SQTM1, JUN, TP53, BCAR1, MKI67) on the proteome of the of cell exposed to 1000µM/L of mixture of monoesters of phthalate for 72 hours in comparation with unexposed cell. The graph was plotted using log10 of pValue <0,05 and log2 DIA intensity considering the difference between ctrl and high dose group.

The results of the validation by WB prove that PCNA, an important marker of cell proliferation, does not express alteration when the experimental groups were compared even when the cell was submitted to autophagy blocking (exposition to ConA + 1000µM/L of mixture of monoesters of phthalate) (Fig 21a). On the other hand, the results about CASPASE, to show cell death status, pointed that there is a significant decrease on the full length CAPSAPSE 3 when compared to control group (Fig 21b), however, the Cleaved-CASPASE 3 does not follow the alteration observed in full length CASPASE 3 when the experimental groups were compared (Fig 21c).

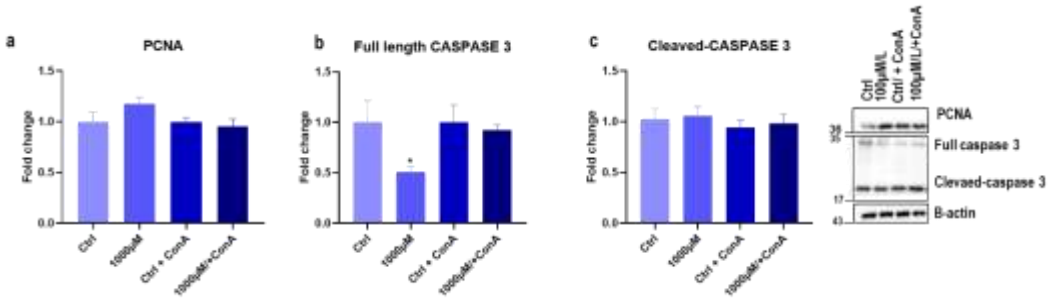

Figure 21: Representative bar graph showing the quantity of the PCNA (a), Full length CASPASE 3 (b) and Cleaved CASPASE 3 (c) proteins on cells exposed to 1000µM/L of mixture of monoesters of phthalate for 72 hours (treatment condition) in the comparation with unexposed cell. Similar results were obtained when the cells were submitted to autophagy blocking under treatment with the monoesters of phthalate for 72 hours. The ConA was added to the medium for 2 hours in 20µmol concentration after 70 hours of treatment. On the right side it is shown the representative figures of protein abundance. The experiment was performed in triplicate experimental. The experimental groups were compared two by two to perform the statistical analysis used was T test, followed by the Mann-Whitney test. *p<0.05.

The result about P62 and VMP_1 abundance, markers for autophagy process, showed that was an increase of P62 in the cell exposed only to the treatment and the same results were not the same when the cell were exposed to treatment + ConA (Fig 22a). However, when the P62 abundance of the treatment group was normalized by the results obtained when the cells were exposed to treatment + ConA (Fig 22a). Therefore, when the autophagy process was blocked by ConA exposure for two hours and the data was normalized by these results, it was possible to observe a decreased on P62 quantity on the treatment group (Fig 22b). On the other opposite side, the results obtained for VMP_1 quantity showed decrease in the treatment group and increased when the results were normalized by ConA exposition (Fig 22c-d). Together these results pointed out that phthalate can exposure blocked autophagy.

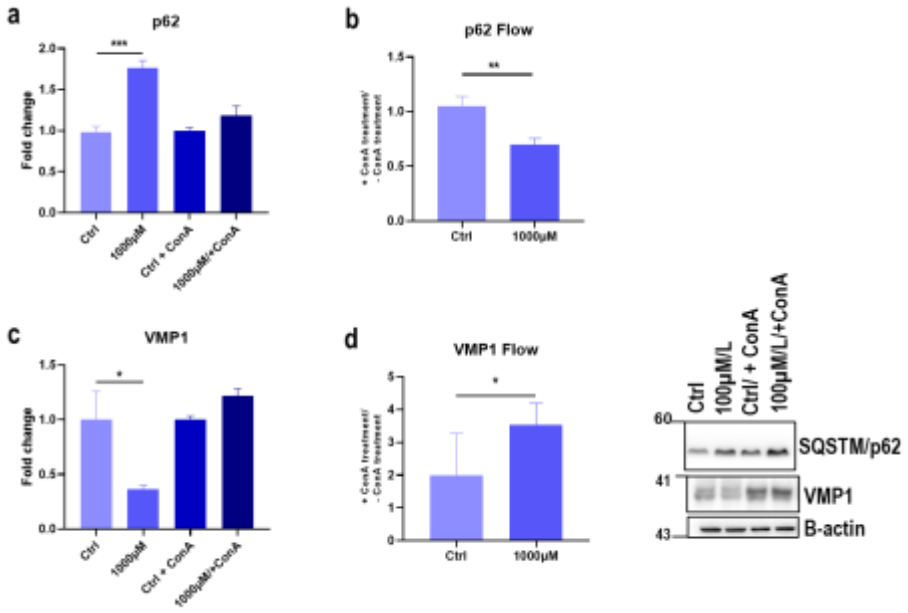

Figure 22: Representative bar graph showing the quantity of P62 (a) and VMP_1 (c) proteins on cells exposed to 1000µM/L of mixture of monoesters of phthalate for 72 hours (treatment condition) in the comparation with unexposed cell and when the cells were submitted to autophagy blocking under treatment with the monoesters of phthalate for 72 hours. The ConA was added to the medium for 2 hours in 20µmol concentration after 70 hours of treatment. The P62 (b) and VMP_1 (d) quantity was normalized by the quantity measured when cells were exposed to treatment and autophagy process was blocked by ConA exposure. On the right side it is shown the representative figures of protein abundance. The experiment was performed in triplicate experimental. The experimental groups were compared two by two to perform the statistical analysis used was T test, followed by the Mann-Whitney test. *$p<0.05$.

The result about LC3, another important marker for autophagy process, showed that there was not significant variation on the LC3 I and II on the cell exposed to the treatment, as well as in cell exposed to the treatment and the autophagy process was blocked (Fig 23a-b).

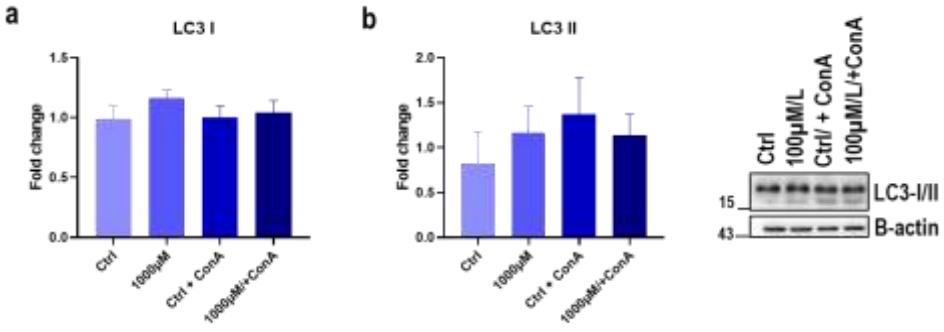

Figure 23: Representative bar graph showing the quantity of LC3 I (a) and LC3 II (b) proteins on cells exposed to 1000µM/L of mixture of monoesters of phthalate for 72 hours (treatment condition) in the comparation with unexposed cell. Similar results were obtained when the cells were submitted to autophagy blocking under treatment with the monoesters of phthalate for 72 hours. The ConA was added to the medium for 2 hours in 20µmol concentration after 70 hours of treatment. On the right side it is shown the representative figures of protein abundance. The experiment was performed in triplicate experimental. The experimental groups were compared two by two to perform the statistical analysis used was T test, followed by the Mann-Whitney test. *p<0.05.

When the markers p62, Vmp$_1$ and LC3 were investigated in the *in vivo* model (Fig 24a-c), the results showed that the p62 was increased in the senile offspring of mother exposed to low dose (20µg/kg/day) of the treatment (Fig 24a) and both Vmp$_1$ and LC3 I were decreased in the offspring of mother exposed to high dose (200mg/kg/day) when compared to control group (Fig 24b-c).

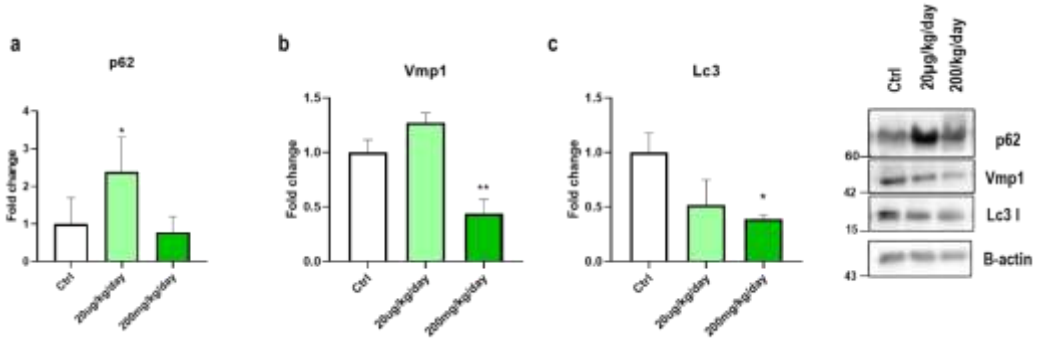

Figure 24: Representative bar graph showing the quantity of p62 (a) Vmp$_1$ (b) and LC3 I on VP of senile offspring of mother exposed to the treatment in the comparison between control vs. lower dose group (20µg/kg/day) and control vs. highest dose group (200mg/kg/day). On the right side it

is shown the representative figures of protein abundance. The experiment was performed in triplicate experimental. The experimental groups were compared two by two to perform the statistical analysis used was T test, followed by the Mann-Whitney test. *p<0.05.

The Pp70, AMPβ and mTOR, were investigated to check if the phthalate can interfere in the activators of autophagy pathways. The treatment was able to increase Pp70 and decrease mTOR abundance (Fig. 25a-b). In addiction the small variation observed in Pp70 abundance on the cell exposed to treatment + ConA, showed that the combination was not able to significantly increase the quantity of the proteins, which was the same for mTOR. The AMPβ abundance was similar in all the experimental groups (Fig. 25c). On the other hand, the TP53, a marker that was DAP on the proteome results and it is directly involved in the pathways as cell cycle arrest, apoptosis, and DNA repair, was increase in the cell exposed to the treatment (Fig. 25d).

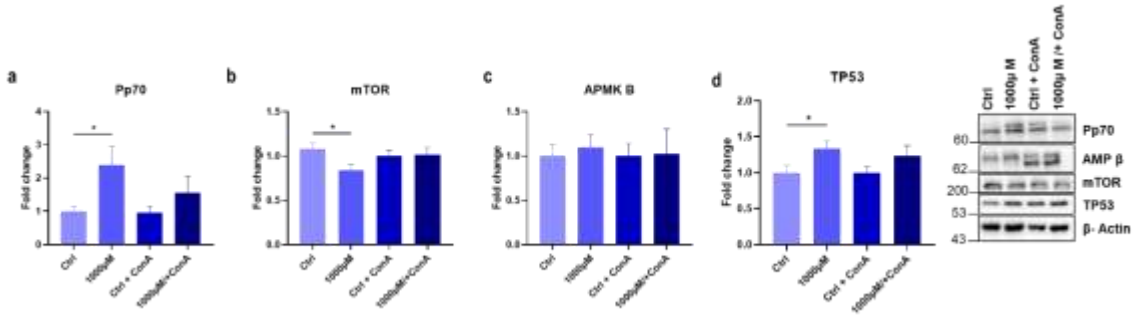

Figure 25: Representative bar graph showing the quantity of Pp70 (a) mTOR (b) AMPK (c) and TP53 (d) protein on cells exposed to 1000µM/L of mixture of monoesters of phthalate for 72 hours (treatment condition) in the comparation with unexposed cell. Similar results were obtained when the cells were submitted to autophagy blocking under treatment with the monoesters of phthalate for 72 hours. The ConA was added to the medium for 2 hours in 20µmol concentration after 70 hours of treatment. On the right side it is shown the representative figures of protein abundance. The experiment was performed in triplicate experimental. The experimental groups were compared two by two to perform the statistical analysis used was T test, followed by the Mann-Whitney test. *p<0.05.

The mTOR is associated with the initiation process of autophagy by blocking the beginning of this process. Despite the results for mTOR pointing out an interesting decrease in the treatment group, the results showed that the treatment was not able to interfere in the ULK1 and ATG101 quantity. Together with other proteins both ULK1 and

ATG101 are responsible for triggering the initiation process of autophagy (Fig 26a and d). In addition, potential targets as ATG14, BECLIN-1 and BCL2 also do not presented significant variation between the treatment and when the cell was exposed autophagy blocking (Fig 26b, c, e and f).

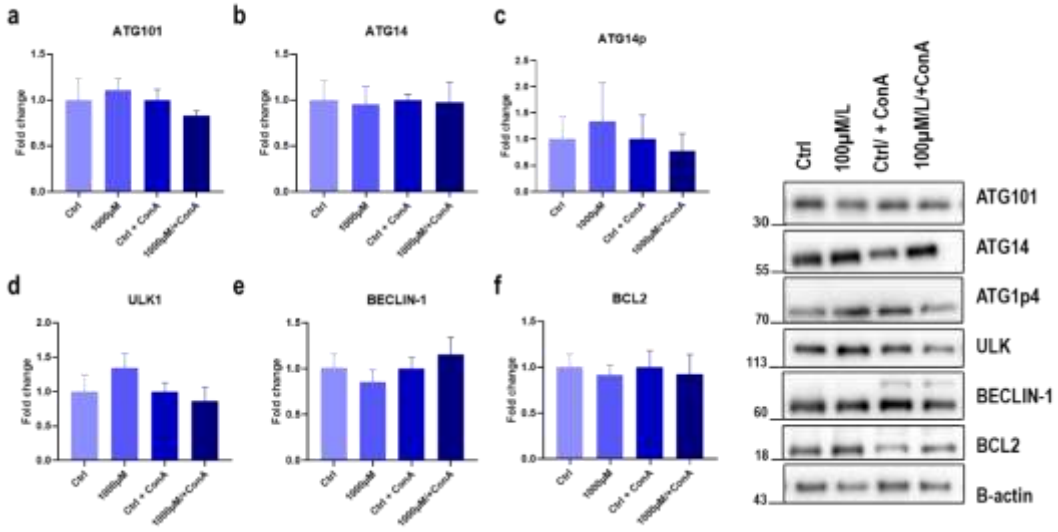

Figure 26: Representative bar graph showing the abundance of the ATG101 (a) ATG14 (b) ATG14P (c) ULK (d) BECLIN-1 (e) and BCL2 (f) protein on cells exposed to 1000µM/L of mixture of monoesters of phthalate for 72 hours (treatment condition) in the comparation with unexposed cell. Similar results were obtained when the cells were submitted to autophagy blocking under treatment with the monoesters of phthalate for 72 hours. The ConA was added to the medium for 2 hours in 20µmol concentration after 70 hours of treatment. On the right side it is shown the representative figures of protein abundance. The experiment was performed in triplicate experimental. The experimental groups were compared two by two to perform the statistical analysis used was T test, followed by the Mann-Whitney test. *p<0.05.

The immunofluorescence labeling of p62/SQMT1 and LC3 has not yet been quantified, but according to the representative images it is possible to observe that when compared to the control group the exposed cells express greater release of the markers into the cytoplasm. When the cells are exposed to the autophagy blocker (ConA), it is possible to observe that there is a visual reduction in the labeling for LC3 and although

there is release of p62 to the cytoplasm there is more labeling of the protein in the nuclear membrane, which may be associated with a seems to be more retained (fig.27).

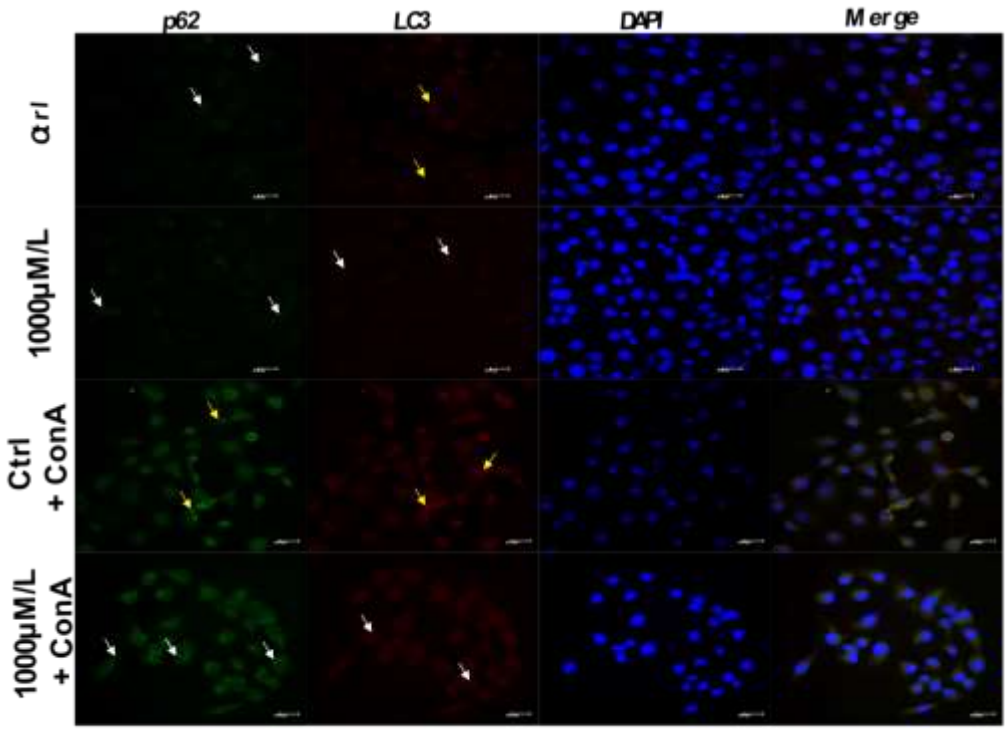

Figure 27: Representative fluorescence photomicrography of the prostate cancer cells (DU145) untreated (Ctrl), exposed to 1000µM/L, untreated and exposed to 20µmM/L of ConA and exposed to 1000µM/L + 20 µmM/L of ConA for 72 hours. The figure showed positive reaction for p62 and LC3 in the different treatment groups. The yellow arrows represent the dot formation, and the white arrows point to the distribution of the label in the cytoplasm and nuclear membrane of the cell. The photomicrographs are in 63x.

When comparing the results obtained in the proteomic profile of the *in vitro* model with the *in vivo* model, it is observed that in the offspring of mothers exposed to the highest dose of treatment, classical proteins of the autophagic pathway such as Sqsmt1 (p62), Atg7 and Atg5 are not differentially altered (Fig 27a), however, the ATG101 protein is downregulated in the treatment group and the data measured by WB indicate that there was a reduction in the p62, VMP1 and LC3 quantity (Fig 27 a-c). On the other hand, the proteomic profile of the offspring of the mother exposed to the lower dose

shows that Agt7 and VMP_1 are upregulated in the treatment group (Fig 27b). The result of WB validation shows that there is an increase in p62, however, VMP_1 and LC3 did not show significant changes (Fig 27 a-b).

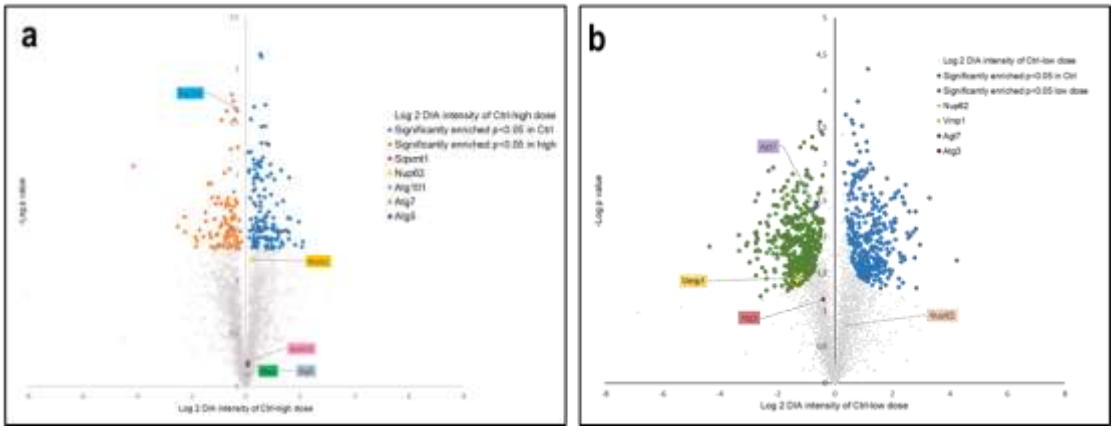

Figure 27: a. Vulcano plot highlighting the proteins identified in the proteome profile of VP of the offspring of mother exposed to the high dose (200mg/kg/day) in comparation with control group. **b**. Vulcano plot highlighting the proteins identified in the proteome profile of VP of the offspring of mother exposed to the low dose (20µg/kg/day). The graph was plotted using log10 of pValue <0,05 and log2 DIA intensity considering the difference between ctrl and high dose group.

Except for the proteins FAM129A and MGEA5 (Fig. 28c), which is downregulated in the *in vitro* model and appears upregulated in the *in vivo* model, the proteins Ppl, Rab27a, Rpn1, Hsd17b11, Gltp, Agrn, Fam129b and Flot2 appear commonly upregulated in both experimental models (Fig. 28 a-c). According to string database, these DPA have no direct interaction (Fig 28d). However, the GLTP, RAB27A, RPN1 targets were described in the CTD to have their expression modulated by Dibutyl phthalate and PPL to have the expression modulated by Diethylhxy phthalate. On the other hand, HSD17B11 is modulated by both diesters described above.

According to data extracted from *Enrichr* platform, RPN1, FAM129 and HSD17B11 proteins are related to hormonal and inflammatory pathways, FAM129A, GLTP, FLOT2 proteins (Fig 29b). In addition, all targets were detected in human prostate adenocarcinoma samples (Fig 29a).

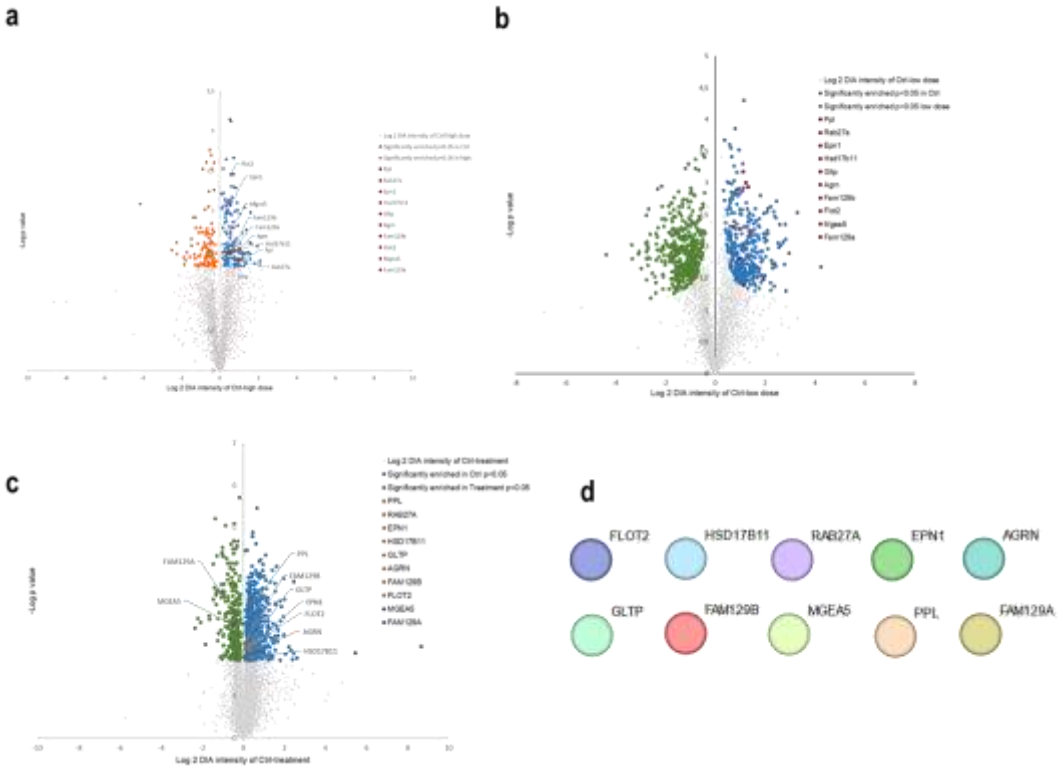

Figure 28: a. Vulcano plot highlighting the proteins identified in the proteome profile of VP of the offspring of mother exposed to the high dose (200mg/kg/day) in comparation with control group. **b**. Vulcano plot highlighting the proteins identified in the proteome profile of the offspring of mother exposed to the low dose (20µg/kg/day). **c.** Vulcano plot highlighting the main target proteins differentially abundant (SQTM1, JUN, TP53, BCAR1, MKI67) on the proteome of the of cell exposed to 1000µM/L of mixture of monoesters of phthalate for 72 hours in the comparation with unexposed cell. The graph was plotted using log10 of pValue <0,05 and log2 DIA intensity considering the difference between ctrl and high dose group. **d.** The IPP networked pointed out that this group of proteins do not have direct interaction.

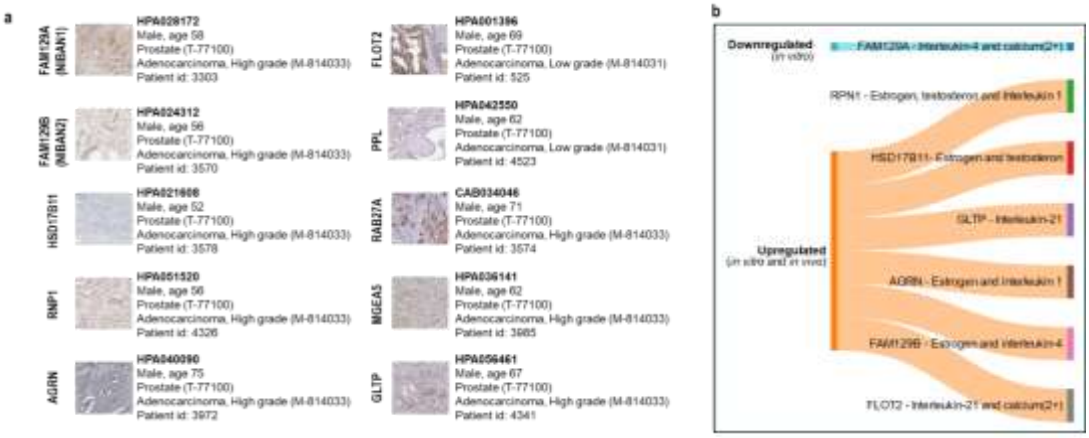

Figure 29: a. Histological photomicrograph showing the positive reaction (by immunohistochemistry) of FAM129A (NIBAN1), FAM129B (NIBAN2), HSD17B11, RNP1, AGRN, FLOT2, PPL, RAB27A, MGEA5, GLTP proteins in prostate adenocarcinoma. The data was obtained in "*The human protein Atlas"* (HPA). **b.** Alluvial graph showing correlated the protein with the hormonal, inflammatory or calcium pathways. These pathways are influenced by the upregulation of the proteins. The data was performed with data extracted of *Enrichr* database and using the pValue as parameter ($p<0,05$).

Anexo I

Protocolo CEUA

unesp
UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"

Comissão de Ética no Uso de Animais
Instituto de Biociências de Botucatu

CERTIFICADO

Certificamos que a proposta intitulada "Efeito da exposição perinatal a uma mistura de ftalatos sobre o envelhecimento e incidência de lesões prostáticas e mamárias", protocolada sob o CEUA nº 9748091019 (ID 000012), sob a responsabilidade de **Wellerson Rodrigo Scarano** *e equipe; Luis Fernando Barbisan; Luis Antonio Justulin Junior* - que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa científica ou ensino - está de acordo com os preceitos da Lei 11.794 de 8 de outubro de 2008, com o Decreto 6.899 de 15 de julho de 2009, bem como com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi **aprovada** pela Comissão de Ética no Uso de Animais do Universidade Estadual Paulista (IBB/UNESP) na reunião de 25/11/2019.

We certify that the proposal "Effect of perinatal exposure to a phthalate mixture on aging and incidence of prostate and mammary lesions", utilizing 224 Heterogenics rats (males and females), protocol number CEUA 9748091019 (ID 000012), under the responsibility of **Wellerson Rodrigo Scarano** *and team; Luis Fernando Barbisan; Luis Antonio Justulin Junior* - which involves the production, maintenance and/or use of animals belonging to the phylum Chordata, subphylum Vertebrata (except human beings), for scientific research purposes or teaching - is in accordance with Law 11.794 of October 8, 2008, Decree 6899 of July 15, 2009, as well as with the rules issued by the National Council for Control of Animal Experimentation (CONCEA), and was **approved** by the Ethic Committee on Animal Use of the São Paulo State University (IBB/UNESP) in the meeting of 11/25/2019.

Finalidade da Proposta: Pesquisa (Acadêmica)

Vigência da Proposta: de 11/2019 a 11/2021 Área: Ciências Biomédicas

Origem:	CEMIB - UNICAMP						
Espécie:	Ratos heterogênicos	sexo:	Machos e Fêmeas	idade:	90 a 120 dias	N:	224
Linhagem:	Sprague Dawley			Peso:	200 a 250 g		

Local do experimento: Biotério do Departamento de Morfologia - IBB/UNESP

Botucatu, 22 de setembro de 2021

Profa. Dra. Ana Carolina Inhasz Kiss
Coordenadora da Comissão de Ética no Uso de Animais
Universidade Estadual Paulista

Prof. Assoc. Luis Fernando Barbisan
Vice-Coordenador da Comissão de Ética no Uso de Animais
Universidade Estadual Paulista

Rua Prof. Dr. Antônio Celso Wagner Zanin, 250 - Distrito de Rubião Junior - Botucatu/SP - CEP 18618-689 - tel: 55 (14) 3880-0851
Horário de atendimento: 2ª a 6ª das 8h às 11h30 - 14h às 18h - e-mail: ceua.ibb@unesp.br
CEUA N 9748091019

Anexo II

Artigo científico publicado

Chemosphere 341 (2023) 140020

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Chemosphere

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

Integrated transcriptome and proteome analysis indicates potential biomarkers of prostate cancer in offspring of pregnant rats exposed to a phthalate mixture during gestation and lactation

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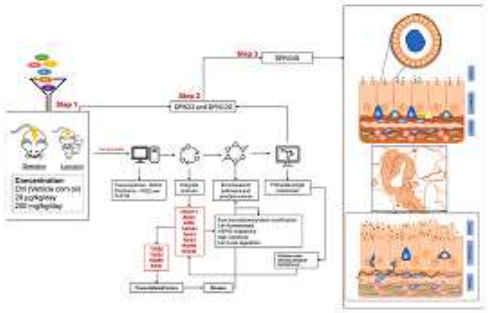
[d] Department of Comparative Biosciences, University of Illinois at Urbana-Champaign, Urbana, IL, USA

HIGHLIGHTS

- Phthalate mixture was able to alter gene expression and protein abundance in the prostate of rats exposed during perinatal period
- Many of the deregulated targets in pre-puberty remained altered in adulthood
- Enrichment analysis showed that deregulated genes and proteins are related to developmental and oncogenic pathways
- Aged animals treated with the phthalate mixture during perinatal period presented increase in the incidence of adenocarcinomas *in situ*
- Phthalate mixture exposure during the prostate critical developmental window was able to increase the susceptibility to carcinogenesis in the senescence

GRAPHICAL ABSTRACT



ARTICLE INFO

Handling Editor: Jian-Ying Hu

Keywords:
Phthalate mixture

ABSTRACT

As the second leading cause of death for cancer among men worldwide, prostate cancer (PCa) prevention and detection remain a critical challenge. One aspect of PCa research is the identification of common environmental agents that may increase the risk of initiation and progression of PCa. Endocrine disrupting chemicals (EDCs) are strong candidates for risk factors, partially because they alter essential pathways for prostate gland development

Abbreviations: PCa, Prostate Cancer; EDCs, Endocrine Disrupting Chemicals; DOHaD, Developmental Origin of Health and Disease; GD, Gestational day; PND, Post-Natal day; DEHP, Bis (2-ethylhexyl) phthalate; DEP, Diethylphthalate; DBP, Di-n-butylphthalate; DiBP, Diisobutylphthalate; BBzP, Bbutylbenzylphthalate; DiNP, Diisononylphthalate; VP, Ventral prostate; DER, Differentially Expressed mRNA; DAP, Differentially Expressed Protein.

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Anexo III

Artigo científico publicado

OXFORD SOT | Society of Toxicology
academic.oup.com/toxsci

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https://doi.org/10.1093/toxsci/kfac064
Advance Access Publication Date: 28 June 2022
Research article

Multigenerational Effects of an Environmentally Relevant Phthalate Mixture on Reproductive Parameters and Ovarian miRNA Expression in Female Rats

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ABSTRACT

Phthalates are endocrine-disrupting chemicals used in many consumer products. Our laboratory previously developed an environmentally relevant phthalate mixture consisting of 6 phthalates and found that it disrupted female fertility in mice. However, it was unknown if maternal exposure to the mixture affects reproductive parameters and ovarian post-transcription in the F1 and F2 generation of female rats. Thus, we tested the hypothesis that maternal exposure to the phthalate mixture affects folliculogenesis, steroidogenesis, and ovarian microRNA (miRNA) in the F1 and F2 generations of female rats. Pregnant female rats were divided into 4 groups and orally dosed daily from gestational day 10 to postnatal day 21 with corn oil (control group), 20 μg/kg/day, 200 μg/kg/day, or 200 mg/kg/day of the phthalate mixture. Maternal exposure to the phthalate mixture impaired folliculogenesis in the F1 and F2 generations of female rats and affected steroidogenesis in the F1 generation of female rats compared to control. Further, the phthalate mixture altered ovarian expression of some genes related to the cell cycle and steroidogenesis compared to control in the F1 and F2 generations of female rats. The mixture also increased ovarian expression of rno-mir-184 that is involved with the oocyte maturation process. Collectively, our data show that maternal exposure to the phthalate mixture affects folliculogenesis and steroidogenesis in the F1 and F2 generations of female rats and alters ovarian miRNA expression in the F1 generation of female rats.

Key words: phthalates; ovary; steroidogenesis; endocrine-disrupting chemicals; miRNA.

Phthalates are chemical plasticizers that modify thermal and mechanical properties of a given polymer. These chemicals are added to polymer solutions to promote their plasticity and flexibility (Godwin, 2011). In addition, some phthalates are used to help dissolve other materials (CDC, 2021; Godwin, 2011). Phthalates are present in hundreds of products such as vinyl flooring, lubricating oils, and personal-care products (soaps, shampoos, nail polish, hair sprays). Some phthalates are in polyvinyl chloride plastics, which are used to make products such as plastic packaging, garden hoses, and medical tubing (CDC, 2021). Humans are often exposed to phthalates mainly via ingestion, but exposure also occurs by inhalation and dermal absorption.

Previous studies have shown that exposure to a mixture of phthalates can cause adverse reproductive outcomes in

Anexo IV

Atividades completares desenvolvidas durante o doutorado

Atividades de formação complementar

Estágio de pesquisa no Exterior pelo programa BEPE, no “Proteomic autophagy Lab”, na Universidade de Fribourg, Suíça

Estágio no laboratório de “Sertoli cell and gamente Biology lab”, na Universidade de Porto, Portugal.

Filiações

Membra da Sociedade Brasileira de Biologia Celular

Membra da Sociedade DOHaD Brasil

Membra do grupo DOHaD Jovem

Atividades de Ensino

1. Estágio Docência na Disciplina Embriologia
2. Estágio Docência/PAADES na Disciplina Citologia/Histologia/Embriologia
3. Estágio Docência na Disciplina "Morfologia Animal e Humana"
4. Estágio Docência na Disciplina "Módulo Célula

Atividades de ensino em curso de extensão

1. Participação no curso de Férias do Instituto de Biociências de Botucatu

2. Integrante do grupo de teatro “bando de siriema”

Coorientação de projetos de Iniciação Científica

1. Análise da expressão de enzimas antioxidantes e citoquinas inflamatórias na próstata ventral de ratos expostos a uma mistura de ftalatos em desenvolvimento perinatal

2. Avaliação da expressão genética dos receptores e proteínas ligados à esteroidogênese na glândula adrenal de ratos expostos a uma mistura de ftalatos durante a gravidez e a lactação.
3. Exposição materna a diferentes ftalatos combinados: efeitos transgeracionais relacionados com o desenvolvimento da próstata.
4. Aspectos morfológicos e moleculares da adrenalina de ratos senescentes expostos a uma combinação de ftalatos durante a organogênese da glândula.

Participações em eventos científicos

1. 34 participações em eventos acadêmico-científicos
2. 11 apresentações de trabalho em eventos-científicos e 18 coautorias de trabalhos apresentados em eventos-científicos
3. 8 Cursos de capacitação ou formação complementar
3. Palestra proferida no evento intitulado “Mesa redonda Vivências de Formação Junto ao PIBID Ciências Biológicas da UNIFAL-MG”

Premiação e homenagem

1. Repercussions of maternal exposure to a combination of phthalates on the offspring prostate: modulation of protein kinases and transcriptional factors - Menção honrosa.
2. Homenagem ao I Prêmio UNESP: Destaque de Jovens Mulheres Cientistas, Instituto de Biociências de Botucatu - Homenagem.
3. miRNAs diferentemente expressos na próstata de animais expostos a uma mistura de ftalatos durante a gestação e lactação e os seus descendentes - Menção honrosa.
4. Proteomic profile in the prostate of rats exposed to a phthalate mixture during the perinatal period: Transgenerational approach - Menção honrosa.
5. Interference pf plasticizers mixture on autophagy in prostate cancer cells and on extracellular matrix degradation in prostate of senile rats exposed during gestation and breast-feeding by maternal route - Menção honrosa.

6. Integrative analysis of the influence of plasticizers on the prostate proteome of offspring exposed during pregnancy and lactation with the human proteome - Menção honrosa.
7. 2,4-dichlorophenoxyacetic acid (2,4-d) exposure during postnatal development modulates the effects of western diet on mice prostate - Menção honrosa.
8. The western diet induces structural and molecular damage in the prostate of mice exposed after prepuberty - Menção honrosa.

Participação em banca de Trabalho de Conclusão de Curso e outras bancas de avaliação

Influência da terapia hormonal com testosterona sobre a remodelação tecidual prostática em ratos adultos saudáveis, Universidade do Oeste Paulista.

Banca examinadora do Congresso de Iniciação Científica da UNESP

Envolvimento em atividades administrativas

1. Representação discente no conselho do programa de pós-graduação em Biologia Geral e Aplicada
2. Representação discente no conselho do departamento de morfologia do Instituto de Biociências de Botucatu
3. Representação discente na CONGRAGAÇÃO do Instituto de Biociências de Botucatu

Parecer para revistas cientificas

Desde 2021 Reproductive Toxicology (5)

Desde 2021 Journal of Pharmacy and Pharmacology (2)

Organização de eventos

III Simpósio Bases da Carcinogênese: da célula às repercussões clínicas.

Simpósio Anual do Programa de pós-graduação em Biologia Geral e Aplicada.

Pint of science Brasil. 2019. (Festival).

Curso de Férias "Virando a Célula do Avesso"- Difundindo e Popularizando a Ciência na UNESP: Interação entre Pós-Graduação e Ensino Básico.

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