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ORIGINAL ARTICLE

Maternal Phthalate Exposure Alters Prostate Proteome in Rat Offspring: Linking Omics Insights to Prostate Cancer Risk in Humans

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Background. Phthalates are compounds used as plasticizers to increase the flexibility of plastics and are considered endocrine disruptors. Some studies suggest that the origin of prostate cancer (PCa) may be associated with disturbances during embryo-fetal development. Previous data showed that perinatal exposure to the same phthalate mixture (PM) used here increased the incidence of adenocarcinomas in the prostates of aged rats. Building on our earlier work, this study identifies proteins altered in the prostate proteome by exposure to a PM during gestation and lactation in rats, focusing on proteins in the human secretome and their correlation with PCa.

Methods. Pregnant SD rats were divided into three groups and treated from gestational day (GD)10 to postnatal day (PND)21. On PND22 the differentially abundant proteins in the offspring's prostate were compared with the predicted secreted proteins in humans. Then, the abundance of selected proteins was compared among groups and enriched. Finally, a protein-protein interaction network was obtained. The resulting data were cross-referenced with data for PCa and some targets were validated by RT-qPCR and Western blot.

Results. Perinatal exposure to PM affected the endoplasmic reticulum, decreasing the amount of certain proteins crucial for protein folding and secretion, impairing secretion of several proteins important for proper prostate development. Furthermore, *in silico* analysis revealed that several proteins in the rat proteome are also altered in patients with PCa.

Conclusions. Our results suggest that early exposure to phthalates may modulate protein secretion, creating a microenvironment that impairs tissue development and increases susceptibility to oncogenesis.

Key Words: Phthalate mixture, Early exposure, DOHaD, Secretome, Proteome, Prostate.

Introduction

The increased use of plastic items is one of the most urgent contemporary socio-environmental issues. In 2021 alone, 390.7 million tons of plastic were produced (1). In the field of toxicology, phthalates are a family of chemical compounds used as plasticizers to increase the flexibility of plastic polymers. However, these plasticizers are harmful to all forms of life, including humans (2). Phthalates are found in toys, packaging films, blood product storage systems, adhesives, vinyl tiles, sealants, auto care products, and personal care items (3). The non-covalent binding of phthalates in products allows them to be released into

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the environment, contaminating soil (0.03–1,280 mg/kg), drinking water (0.16–170 μg/dm³), air samples (<0.4–65 ng/m³), sewage (0.0004–58.3 g/kg), and dust samples (2.38–4.1 g/kg) (4) Humans can be exposed to phthalates through direct skin contact with contaminated items, consumption of food and beverages, and inhalation (5).

The problem is exacerbated by the fact that phthalates are classified as endocrine-disrupting chemicals (EDCs) (6). EDCs are chemical substances that disrupt homeostasis or endogenous hormonal function by influencing the production, release, transport, metabolism, binding, action, or elimination of hormones that regulate or maintain normal growth and development (7). Phthalates are responsible for developmental disruptions in hormone-dependent structures, primarily in the reproductive system. They have been associated with early puberty, altered levels of sexual steroid hormones, irregular menstrual cycles, and endometriosis (8). It has been established that phthalates can cross the placental barrier and alter embryonic-fetal development, triggering significant consequences for the offspring (9,10).

The mechanism by which external agents interfere in the placenta-embryo-fetus cycle is directly related to the Developmental Origins of Health and Disease (DOHaD) concept. This compiles epidemiological, clinical, experimental, and translational evidence to establish how exposure to environmental stressors during the prenatal, perinatal, and postnatal periods influences health and disease plasticity during different windows of developmental biology (11–13). Environmental insults have a more significant effect during early development, a period of high susceptibility due to rapid DNA synthesis, epigenetic phenomena standardization, and chromatin organization necessary for homeostatic tissue development (14–17). In addition, the first thousand days of life, which include lactation, may involve maternal exposure to EDCs that alter the quality of breast milk and compromise immune system development in offspring (18,19). Phthalates have been linked to the DOHaD concept, and their significance throughout key stages of gestation and postnatal development is becoming more widely recognized (20,21).

Several studies have demonstrated that constant exposure to environmental chemicals with anti-androgenic and estrogenic actions increases the incidence of reproductive disorders (20,22–25). The prostate gland, an integral component of the male genital system, is critical for reproductive success, particularly in maintaining sperm viability. Nonetheless, it may be affected by benign prostatic hyperplasia and cancer throughout life (26). William Gardner's manuscript "The Prenatal Origins of Prostate Cancer" historically linked the development of PCa to factors present during embryonic development (27), placing it within the DOHaD context. The prostate develops in a series of stages during the prenatal and neonatal periods, as well as during puberty (28,29). These processes are primarily regulated by androgens and growth factors (30). This reinforces the idea that exposure to endocrine disruptors such as phthalates can interfere with the essential stages in gland development and maturation. While there are differences between human and rodent prostates, substantial evidence of molecular pathway similarities and biological responses makes rats a useful animal model for studying the impact of phthalates on prostate development (31).

The DOHaD hypothesis postulates that the incidence and prevalence of chronic diseases, as well as their long-term consequences result from early exposure to multiple stressful conditions. Previous studies have shown an increase in adenocarcinomas in the prostates of aged rats exposed to the same mixture used in this study (32). Thus, we aim to evaluate the effects of a PM on the proteomic profile of the prostate during early life and adulthood, correlating it with data from the human secretome and PCa. We hypothesize that disturbances in the maternal-fetal environment caused by exposure to the PM may influence prostate development by modulating important processes mediated by secreted proteins, thereby increasing susceptibility to prostate disorders later in life.

Material and Methods

Animals and Experimental Design

The proteomic profile used in this study was obtained through the experimental design described in detail by Aquino AM, et al. (32). Briefly, male ($n = 10$) and female ($n = 40$) Sprague-Dawley rats (120 d old) were kept in the animal facility of the Department of Structural and Functional Biology of the Institute of Biosciences (UNESP). The rats were raised under regulated conditions of light (12 h of light/12 h of dark) and temperature (23–25°C), with free access to water and food.

After confirming pregnancy, the pregnant rats were divided into three experimental groups ($n = 10$/group): control group (CTR): vehicle-corn oil; T1: 20 μg/kg/day of the PM and T2: 200 mg/kg/day of the PM. The lowest dose was selected to mimic daily human exposure levels based on the amount of bis (2-ethylhexyl) phthalate (DEHP). The higher dose was selected to compare our results with available single phthalate studies as described by Aquino AM, et al (32). The mixture included the phthalate amounts specified by Zhou, Gao and Flaws. Zhou and Flaws calculated the quantities of the phthalate combination based on the metabolites found in the urine of pregnant women. The percentages were: 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) (33).

Pregnant and lactating female rats were treated daily from gestational day (GD) 10 to postnatal day (PND) 21. On PND22 and PND120, the male rats were weighted and euthanized (pentobarbital anesthesia followed by heart puncture), and their ventral prostates (VP) (n = 3/group) were collected for molecular analysis. The experimental design is represented in the Supplementary Figure 1. This study followed the Brazilian Council on Animal Experimentation Control (CONCEA) guidelines and was authorized by the Ethical Committee on Animal Use at the Institute of Biosciences of São Paulo State University (Protocol: 1040/CEUA). All animal experiments were conducted according to the ARRIVE guidelines.

Global Protein Expression by Mass Spectrometry (LC-MS/MS)

Proteomic analysis was performed as described by Aquino AM, et al (32). For this analysis, VP samples (300 mg; n = 3/group and age) were homogenized on ice for 5 min in 1,000 μL of extraction buffer (8 M urea, 100 μl, 1 M Tris, 100 mM PHSF, 1% protease inhibitor, and 65 mM DTT). The homogenate was then 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 assays (34). Proteolytic cleavage of proteins, as well as the identification and analysis of protein expression followed the steps previously described (32).

Integrative Analysis of the Proteome of Rats Exposed with the Secretome Predicted for Humans

The differentially expressed proteins (DEP) in the proteomes of offspring rats exposed to phthalates during pregnancy and lactation at PND22 and PND120 (Supplementary Table 1) were cross-referenced with the predicted secreted protein data available for humans on the "The Human Protein Atlas" platform (HPA) (https://www.proteinatlas.org/humanproteome/blood+protein/secretome / Supplementary Table 2). All proteins predicted in the human secretome (Supplementary Table 3, 2771 genes) were collected and cross-referenced with the DEP data obtained from the animal proteome. Subsequently, a comparison of common expression between ages and treatments was performed using the Venny 2.1 platform (https://bioinfogp.cnb.csic.es/tools/venny/).

Enrichment Analysis of Differentially Expressed Proteins (DEP)

The DEPs from the mouse proteome and the human secretome were evaluated using a protein-protein interaction network, with a minimum confidence level of 0.700 (high confidence). The data were filtered using a full string network, and are considered evidence from text mining, experiments, databases, co-expression, neighborhood, gene fusion and co-occurrence. The network was collected from the String database (https://string-db.org/). DEP pathway enrichment analyses were conducted using the EnrichR database (https://maayanlab.cloud/Enrichr/) with a p <0.05. Ontological terminology was drawn from biological processes and pathways were sourced from the Reactome and KEGG Pathways databases. Key enriched pathways were selected based on the lowest p and the greatest biological relevance to the prostate. The paths were represented using alluvial diagrams on the SankeyMATIC platform (https://sankeymatic.com/build/), whereas the ontological words were represented by graphs from the enrichR database (https://maayanlab.cloud/Enrichr/).

In silico Validation of Potential Secretome Biomarkers for PCa

After obtaining the secretome profile potentially modulated by the phthalate treatment analyzed in this study, a differential gene expression analysis was performed between normal prostate tissues from the Genotype-Tissue Expression (GTEX) database and tissues with prostate adenocarcinoma from the same database and The Cancer Genome Atlas (TCGA) comparing the expression of each gene in the two tissue types using the XENA browser platform (https://xenabrowser.net/).

RT-qPCR

The targets highlighted in the proteome and secretome analyses described above were experimentally validated by RT-qPCR. Briefly, molecular analyses of total RNA extraction from samples (PND22 and PND120) were performed following the TRIzol® protocol (*Invitrogen* Life Technologies, USA). Total RNA was extracted from VP samples (n = 3/group) in both groups using Trizol Reagent (Thermo Fisher Scientific) according to the product datasheet. The primer sequences are shown in Supplementary Table 4. The synthesis of cDNA was performed using a high-capacity cDNA Archive Kit (Thermo Fisher Scientific) according to the manufacturer's guidelines. Aliquots of cDNA from each sample were mixed with Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA). Expression levels of mRNAs were measured by RT-qPCR using the QuantStudio™ 12K Flex Real-Time PCR System (Thermo Fisher Scientific). All qPCRs performed complied with the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines. The relative quantification of each mRNA was carried out using the Livak et al. approach (35). The ratio obtained between the informative gene and the reference gene (gadph) to mRNAs was used to standardize the results obtained for all samples.

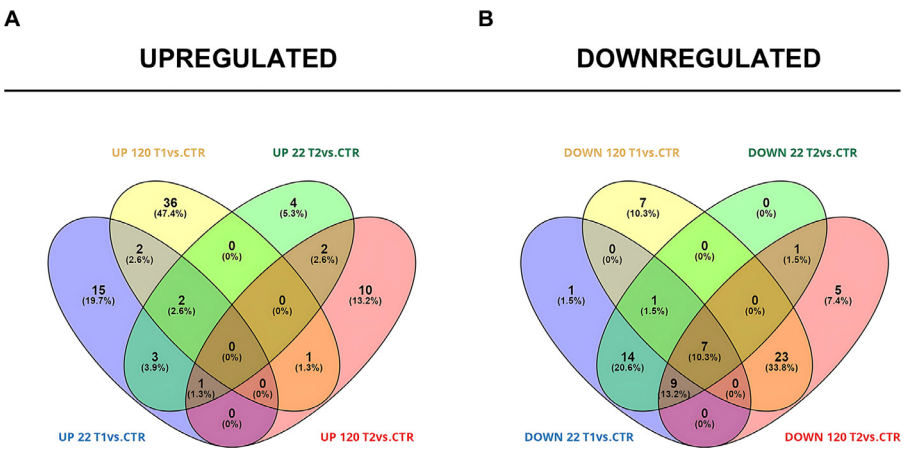

Figure 1. Overlapping up/down proteins across treatments. The Venn diagrams represent the differentially abundant proteins that intersect with the proteins presented in the human secretome and their correspondence in the treated groups (T1 and T2). The Venn diagrams also show the three comparisons between the up- and downregulated profiles at PND22 and PND120. T1: 20 μg/kg/d T2: 200 mg/kg/d.

Western Blotting

To quantify protein expression, Bradford assays were performed using the Quick Start™ Bradford Protein Assay kit (Bio-Rad, Hercules, CA, USA), and each sample was measured in duplicate (34). Subsequently, 70 μg/mL of protein (n = 3/group and age) was resolved through electrophoresis in a 10% polyacrylamide gel under reducing conditions (SDS-PAGE). The proteins were transblotted to a nitrocellulose membrane (Amersham, Little Chalfont, UK), blocked with 5% non-fat milk for 1 h, and incubated overnight at 4°C with specific primary antibodies (Supplementary Table 5). Afterwards, the membranes were washed with TBS-T and incubated with HRP-conjugated secondary antibodies at room temperature for 1.5 h (Supplementary Table 5). The reactions were developed using an ECL kit (Amersham, USA) and images of the bands were captured using a CCD camera (GBOX system - Syngene, Cambridge, UK). Quantitative analyses of the bands were performed using ImageJ software (National Institutes of Health, USA). The expression levels of the targets were normalized to β-actin and the results were expressed as a fold change with a mean ± SD.

Statistical Analysis

After the Shapiro-Wilk normality test, a one-way Anova test was performed for parametric data and the Kruskal-Wallis test for non-parametric data; both were followed by a Dunn's test. Data were presented as the mean ± SD. Differences were considered significant when $^{*}p < 0.05$; $^{**}p < 0.01$, $^{***}p < 0.001$ between the treatment and control groups.

Results

Proteomic analysis of the T1 group revealed that PM exposure resulted in the upregulation of 283 proteins and the downregulation of 269 proteins on PND22, and the upregulation of 323 proteins and the downregulation of 332 proteins on PND120 in the prostate compared to the control group (Figure 1). In the T2 group, the PM exposure led to 124 upregulated proteins and 323 downregulated proteins on PND22, and 98 upregulated proteins and 323 downregulated proteins on PND120 in the prostate compared to the control group (Figure 1).

Integrative analyses of the differentially expressed proteins in the T1 and T2 groups at two different ages, combined with proteins from the human secretome, revealed the following: in the T1 group, 23 proteins were upregulated and 32 were downregulated on PND22; meanwhile 41 proteins were upregulated and 38 were downregulated on PND120 in the prostate compared to the control group (Supplementary Figure 2). In the T2 group, 12 proteins were upregulated and 32 proteins were downregulated on PND22. On PND120, 14 proteins were upregulated and 45 proteins were downregulated in the prostate compared to the control group (Supplementary Figure 2).

Overall, the PM had the most significant effect on decreasing protein expression. Several common downregulated proteins were observed across the different treatment groups and ages. For example, seven proteins were downregulated in all groups and ages, 31 were downregulated on PND22 regardless of the doses administered, and 30 were downregulated on PND120 also regardless of the dose. For this reason, clusters with downregulated and differentially abundant proteins were primarily selected for further analysis.

Specifically, the cluster of 31 proteins downregulated by phthalates at both doses on PND22 was selected for further investigation. This cluster was chosen because the same set of 31 proteins downregulated by phthalates at both doses on PND22 also showed divergent expression profiles on PND120, including sustained downregulation in some proteins and upregulation in others, suggesting

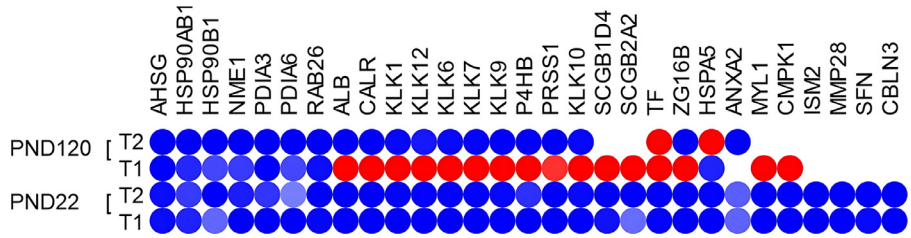

Figure 2. Heatmap of differential protein abundance. Heatmap graph shows the main proteins and their abundance in the different treatment and age groups. The blue circles represent proteins that are downregulated in the treatment groups compared to the control group, and the red circles represent proteins that are upregulated in the treatment groups compared to the control group. PND22 = postnatal day 22; PND120 = postnatal day 120; T1:20 μg/kg/d T2: 200 mg/kg/d. Downregulated <p 0.95> Upregulated.

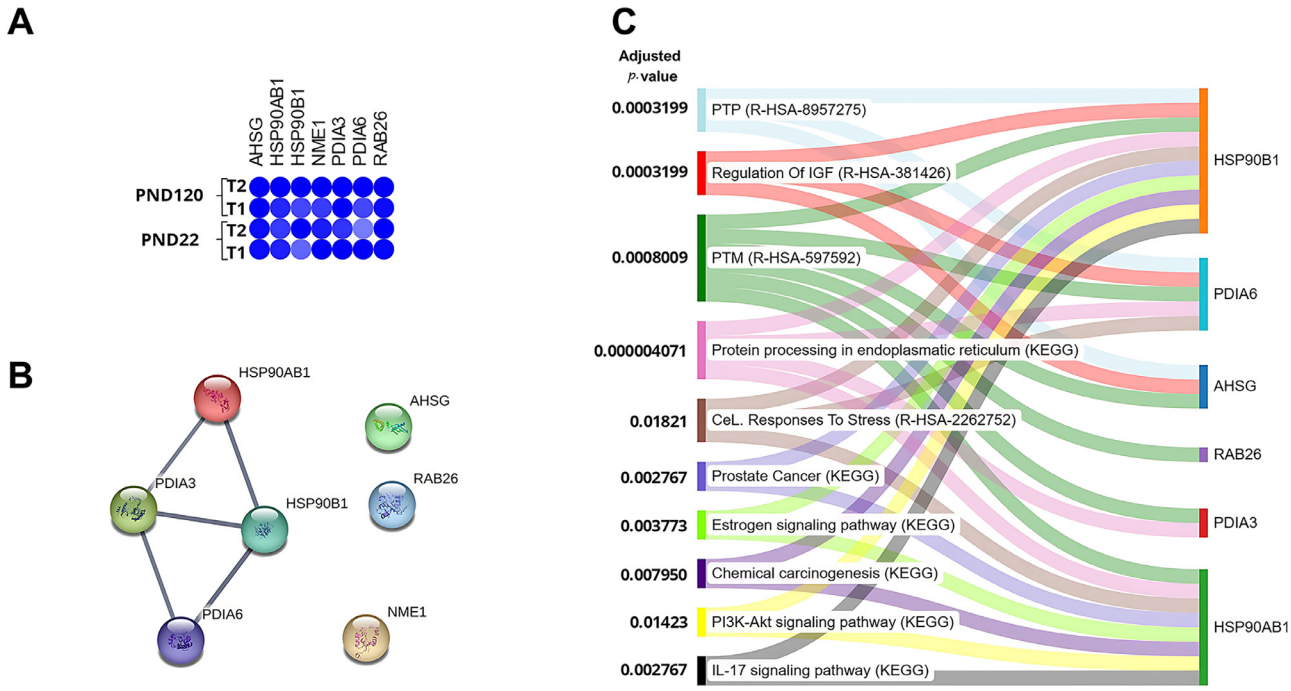

Figure 3. Functional and interaction analysis of cluster 1. Representative graphics of the selected cluster and the pathways associated with DEP on PND22 and PND120. A. Heat map of downregulated proteins at all ages and treatments. B. Protein-protein interaction network (PPI). Proteins are represented as nodes in the network, connected by lines whose thickness indicates a confidence level greater than 0.7. C. An alluvial graph showing the main pathways affected by the DEPs, together with their associated proteins and modified p values, using the REACTOME and KEGG databases, generated with the sankeyMATIC tool. D. A bar graph and an ENRICHR tool reference table depict the key biological processes associated with the cluster of seven proteins.

a potential dose- and time-dependent regulatory pattern (Figure 2).

First, complementary analyses were carried out on the seven proteins (AHSG, HSP90AB1, HSP90B1, NME1, PDIA3, PDIA6, and RAB26) that were downregulated across all ages and phthalate treatments (Figure 3A). We identified the interaction of the proteins HSP90AB1, HSP90B1, PDIA3, and PDIA6 (Figure 3B). The main enriched pathways associated with these proteins were related to post-translational phosphorylation (PTP), regulation of IGF transport and absorption, post-translational modifications (PTM), protein processing in the endoplasmic reticulum, cellular response to stress, cancer prostate, estrogen signaling pathway, chemical carcinogenesis, PI3K-Akt signaling pathway, and IL-17 signaling pathway (Figure 3C). The HSP90B1 protein was responsible for enriching 10 of these pathways, PDIA6 enriched five pathways, AHSG enriched three pathways, HSP90AB1 enriched eight pathways, PDIA3 influenced two pathways and RAB26 only influenced one. The main biological processes (GO biological processes) related to this cluster are represented in Figure 3D.

We subsequently analyzed the second cluster through the protein-protein interaction network. Three primary interaction clusters were used: cluster 1 (HSPA5, ANXA2, CALR, P4HB, ALB, and TF), cluster 2 (KLK7 and KLK10), and cluster 3 (SCGB2A1, SCGB2A2, SCGB1D2) (Figures 4A and 4B). Enrichment analysis re-

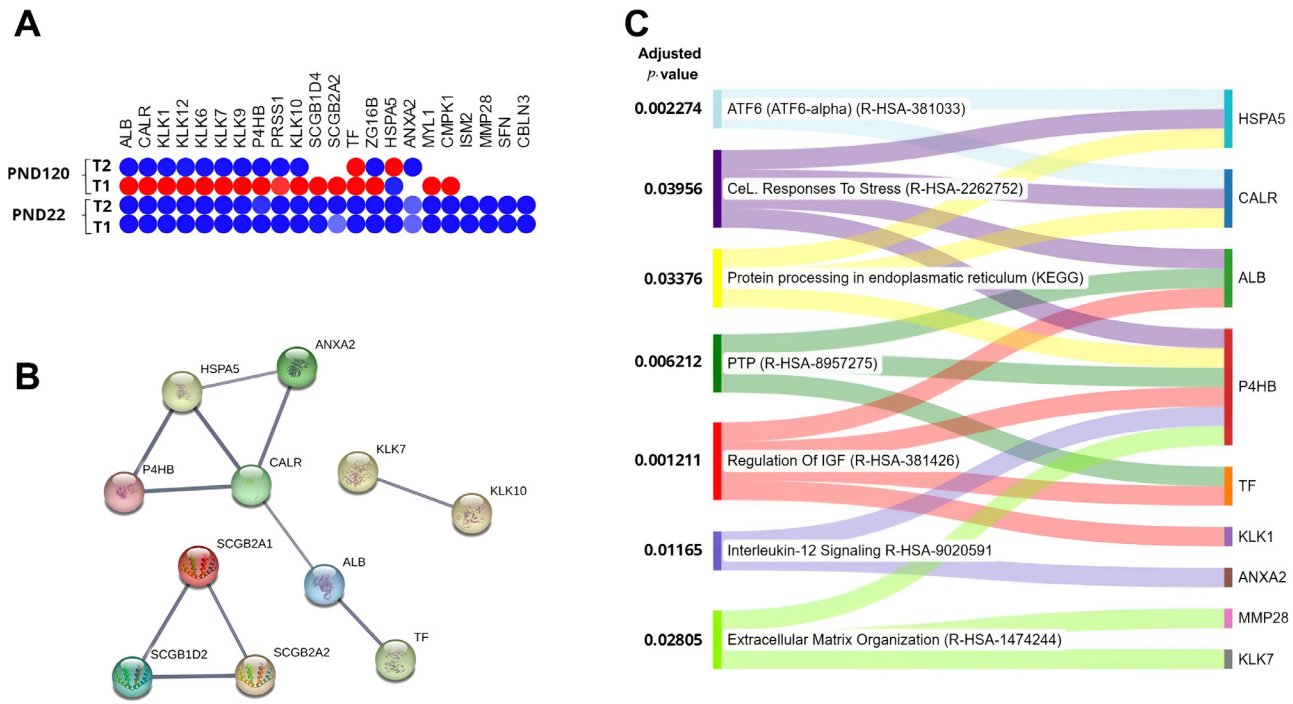

Figure 4. Functional and interaction analysis of cluster 2. Representative graphics of the selected cluster and the pathways associated with DEPs at PND22 and PND120. A. Heat map of downregulated proteins at all ages and treatments. B. Protein-protein interaction network. Proteins are represented as nodes in the network connected by lines, whose thickness indicates a confidence level greater than 0.7. C. An alluvial graph showing the principal pathways impacted by proteins, together with their associated proteins and modified *p* values, using the REACTOME and KEGG databases, generated with the sankeyMATIC tool. D. A bar graph and an ENRICHR platform reference table depict the key biological processes associated with the cluster of 24 proteins.

vealed that the main pathways influenced by the proteins were related to the ATF6 response, the cellular stress response, protein processing in the endoplasmic reticulum, post-translational phosphorylation, regulation of IGF transport and absorption, IL-12 signaling, and extracellular matrix organization (Figure 4C). Our data demonstrate that HSPA5, CALR, and ALB enriched three routes, P4HB enriched six pathways, while TF, KLK1, ANXA2, MMP28, and KLK7 enriched one pathway each.

After performing enrichment analyses, we crossed the targets with the gene expression of patients with PCa. The data indicate a higher expression of *Hsp90b1, Hsp90ab1, Nme1, Pdia3, Pdia6, Hspa5, Calr, Klk12, P4hb, Cmpk1, Scgb1d2*, and *Zg16b* in the patients with PCa compared to patients without PCa. In contrast, expression of genes such as *Prss1, Ism2*, and *Alb* was lower in PCa samples than in samples without PCa (Figure 5).

The expression of *Hsp90ab1* and *Hsp90b1* was similar to control tissues on PND22 and PND120 when some proteins and mRNAs associated with the dysregulated pathways and PCa were studied (Figures 6A–6D).

Since the enrichment analysis revealed altered targets associated with estrogen signaling pathways and prostate cancer, we tested the abundance of the androgen receptor (AR) and the estrogen receptor (ERα) by Western blot. On PND22, the T2 group exhibited increased AR and ERα protein levels compared to the control group (Figure 6). On PND120, the PM did not alter the protein levels of AR and ERα compared to the control group (Figure 6).

Discussion

The novelty of this study lies in the investigation of both the immediate and long-term consequences of maternal exposure to an environmentally and occupationally relevant PM on the prostate. Our focus was on elucidating the proteomic profile of the prostate and comparing it with the human secretome to evaluate the effects of phthalates on secreted proteins. We assessed and developed relationships between prostate diseases in rats and humans and identified potential biomarkers of PCa. This approach identified common molecules and pathways associated with PCa, offering insights into predictive markers and how phthalates act as disruptors with long-term consequences for prostate health as individuals age. Our results revealed the immediate effects of the PM exposure in early life, suggesting a predisposition to prostate disorders later in life, with the effects persisting in adulthood. Specifically, the proteomic profile of rat prostates exposed to phthalates showed gene dysregulation similar to that observed in the secretome of human patients with PCa. Our study demonstrates that maternal exposure to phthalates dysregulates the prostate microen-

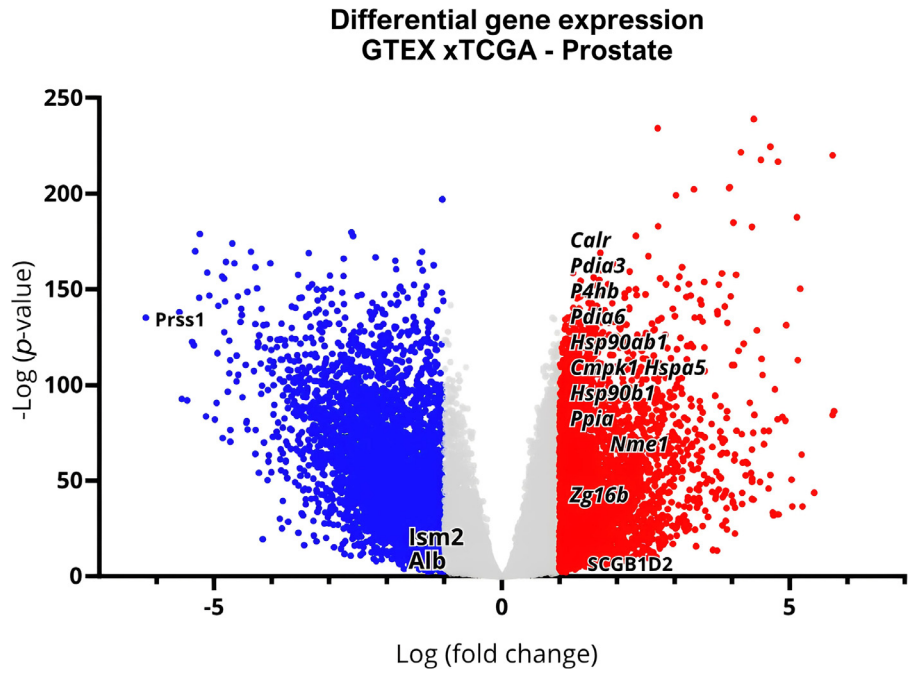

Figure 5. Differential gene expression in PCA. Graph of differential gene expression between prostate adenocarcinoma tissues from the TCGA platform (blue) and normal prostate tissues from the GTEX platform (green). The analysis was performed using the Xena browser tool. FC >1.0 and p <0.05.

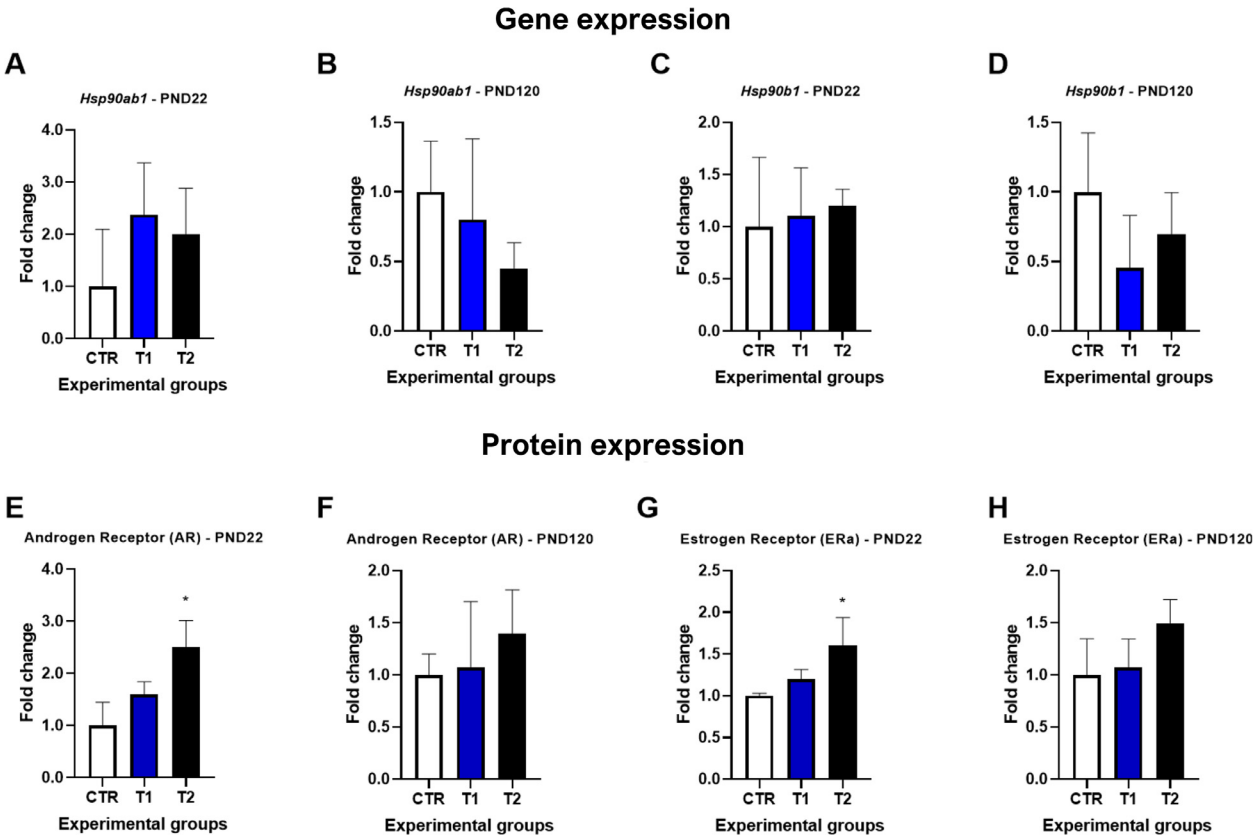

Figure 6. Validation of gene expression and protein levels. Representative graphs showing the expression of the *Hsp90ab1* – PND22 A. *Hsp90ab1* – PND120; B. and *Hsp90b1* – PND22 C. *Hsp90b1* – PND120 D. targets obtained by RT-qPCR. B) Representative graphs of protein expression of the androgen receptor (AR) at PND22; E. AR at PND120; F. ERa at PND22; G. and ERa at PND120; H. performed by Western blotting. Data are expressed as mean ± SD (n = 4–5/group). * Significative difference when p <0.05.

vironment, with significant implications for gland health and potential long-term consequences.

Recent research from our group demonstrated that perinatal exposure to an environmentally relevant mixture of phthalates altered the expression of miRNAs in the prostate of male offspring and in the ovaries of female offspring (36,37). These phthalate-induced miRNA alterations led to mRNA expression changes in the prostate microenvironment, making it more susceptible to PCa development in young rats (36). No significant changes were observed in body weight, prostate weight, or prostate lesions in adult animals (36) in PM-treated rats compared to controls. However, using the same experimental protocol, Aquino AM, et al (32). observed a significant increase in the incidence of carcinomas *in situ* in the prostate of aging offspring (PND540) treated with 200 mg/kg/d of the PM during gestation and lactation compared to the control group (200 mg/kg: 37.5 vs. control: 0%). Thus, targets associated with prostate oncogenesis were considered primary candidates for our analysis.

Interestingly, we found that phthalate exposure predominantly led to decreased protein expression compared to controls. The results were dose-dependent, with a persistent downregulation of proteins on PND22 and PND120 in the T2 group. However, the T1 group showed an inversion in protein expression between PND22 and PND120. Many studies have explored the relationship between maternal exposure to phthalates and both immediate and long-term consequences, often focusing on epigenetic mechanisms (20,36,38). These effects are likely linked to epigenetic changes resulting from maternal exposure to phthalates. On PND22, we observed a direct effect of phthalates on protein expression in early life, regardless of dose, which may influence prostate morphogenesis. On PND120, we noted that the same 31 proteins exhibited a dose-dependent effect, either maintaining or reversing their profiles. These proteins were selected for further analysis to understand the early-life impacts of phthalates and their long-term consequences.

The first cluster, which was associated with the downregulation of proteins across both ages and phthalate treatments included the following proteins: AHSG, HSP90AB1, HSP90B1, NME1, PDIA3, PDIA6, and RAB26. These protein-enriched pathways were related to post-translational modifications, particularly those involving chaperone activity (HSPs), which regulate phosphorylation, acetylation, and s-nitrosylation (39). The proteins responsible for protein folding in the endoplasmic reticulum (ER), such as HSP90AB1, HSP90B1, PDIA3, and PDIA6, were downregulated in both the T1 and T2 groups at both ages compared to controls. The literature indicates that protein folding is critical for ER quality control (40) and that chaperones play a decisive role in the process of protein folding by repairing misfolded proteins (41). However, changes to this coordinated process can disrupt cellular homeostasis, leading to negative effects such as apoptosis (42). The association between PDIA3 and PDIA6 is important to support protein folding (40). Our data suggest that phthalate exposure leads to decreased responses to ER stress, phosphoprotease activity, protein processing in the ER, cellular responses to stress, and estrogen signaling pathways. Thus, both environmental and occupational phthalate exposure appears to impact post-translational processes, protein function, and hormonal signals, all of which could affect prostate homeostasis in adulthood and aging.

HSPs have several pathological and physiological functions. They bind and act to promote the protein folding, trafficking, assembly, and signal transduction. In addition, these chaperones are necessary for the maturation of steroid receptors, which require the organization of the molecular chaperones and associated proteins into complexes that improve the interaction of the receptors with their steroid targets. In this sense, the Hsp90 subgroup maintains the receptors in a structurally ephemeral state, suitable for the sequential series of reactions that they undergo during maturation. This suggests that Hsp90 is the central protein in the steroid receptor maturation pathway (43–45). Exposure to phthalates has already been associated with decreased levels of HSPs in the duodenum, jejunum (46) and liver (47). Our experiments suggest that phthalate exposure decreases HSPs in all treatment and age groups, which may lead to a lower steroid receptor responsiveness. These alterations could be a compensatory cellular response to reduced chaperone activity, possibly involving transcriptional or post-transcriptional regulatory mechanisms aimed at preserving hormone activity. Alternatively, changes in receptor levels could indicate an accumulation of incorrectly folded or nonfunctional receptors as a result of insufficient chaperone assistance, rather than an increase in functional receptors, which are important for the development and maintenance of prostate health.

Regarding hormonal responsiveness, we observed an increase in the expression of sexual hormone receptors, such as AR and ERα, in T2 at PND22. This process may compensate for the low responsiveness of the receptor, due to the chaperones. This may create an environment susceptible to an exaggerated hormonal response at other life stages. Previous studies have suggested that increased estrogen in early life may be associated with PCa initiation and progression in aging rats (48–50). Initiation and progression of PCa are strongly linked to androgen levels and AR expression (51). A growing body of evidence suggests that estrogen receptors (ERs) are promising targets for PCa therapies. ERα and ERβ expression levels have been associated with higher Gleason scores and poorer survival rates in patients with PCa (52,53).

The second cluster of proteins was associated with cellular response to stress, protein phosphorylation, protein procession in the ER and extracellular matrix organization, and regulation of IGF and IL12 signaling. We pro-

pose that the phthalate-induced downregulation of these proteins impacts prostate differentiation in post-weaning animals by contributing to delayed tissue maturation. The prostate, a male exocrine gland that secretes seminal fluid components, undergoes complex developmental processes, including organ specification, epithelial budding, branching morphogenesis, canalization, and cytodifferentiation (54). These processes rely on an adequate supply of epithelial cells and an extracellular matrix. Disruptions in prostate structure and function due to various stressors can potentially lead to prostate disease.

The kallikreins (KLK1, KLK6, KLK7, KLK9, KLK10, KLK12, and PRSS1) play essential roles in the regulation of bioactive molecules, hormones, growth factors, and matrix formation during early development by organizing and degrading the extracellular matrix in various organs. However, they can also influence cancer progression, including PCa (55). KLK activity is modulated by post-translational modifications, a mechanism that is enriched in our analysis and linked to the action of phthalates (56,57). KLKs are also associated with angiogenesis, matrix hydrolysis, and cellular migration, all of which play a role in neoplastic progression (58,59). KLK1 activates the epidermal growth factor receptor (EGFR) and ERK1/2 signaling cascade involved in cell proliferation (60) and regulates the IGF pathway by degrading IGF-binding proteins, thus increasing IGF availability and proliferation signals (59).

Our results suggest that reduced production and secretion of this protein cluster could alter pathways related to extracellular matrix organization, IGF regulation, and post-translational modifications, which are associated with androgen receptor signaling pathways. Downregulation of these pathways early in life disrupts prostate development and, may lead to negative effects in adulthood, particularly in the T2 group. In contrast, the T1 group exhibited an upregulation of these pathways in adulthood, which may act as a compensatory mechanism to maintain prostate maturation and reproductive health. However, environmental and occupational exposures may create a microenvironment susceptible to prostate disorders as individuals age, as suggested by Aquino AM, et al. (13,32).

The secretoglobins (SCGB2A1 and SCGB1D4) regulate steroid levels in tissue and may enhance hormonal action. Dysregulated SCGB pathways were linked to altered binding of androgens and other steroids, which are critical for prostate morphogenesis. SCGBs are highly expressed in the prostate when stimulated by androgens and function to bind steroid hormones in prostate acini (61). SCGB2A1 has been shown to be a target of prostate reprogramming by the endocrine disruptor bisphenol A (BPA), a plasticizer (62). SCGB2A1 also binds estramustine, a chemotherapeutic agent used to treat PCa, and increases its uptake in prostate tissues (63). In our study, the T1 group exhibited a switch in SCGB expression shifting from downregulation in early life to upregulation in adulthood, which may represent a compensatory mechanism for reproductive success. The T2 group maintained these proteins downregulated across ages, which could negatively affect fertility and overwhelm androgen responses in adulthood, potentially serving as a predictive factor for prostate disorders.

When comparing our results with human PCa data, we identified several commonly dysregulated proteins, such as PDIA3, PDIA6, CALR, KLK12, and SCGB1D2. Differential expression analysis showed that several of these proteins are overexpressed in PCa. Although RT-qPCR validation in our model did not reveal significant differences between treatment groups due to post-transcriptional modifications. This is a known mode of action of phthalates that modulates histones and miRNAs, leading to epigenetic changes during development (36,64). Our integrative analysis of the secretome of humans and the proteome of rats exposed to phthalates during pregnancy and lactation highlights the critical role of maternal influence on “omic” patterns during organogenesis and prostate development. These findings may offer potential biomarkers for prostate diseases before their onset.

In this study, we investigated the relationship between dysregulated biomarkers in the prostates of young and adult animals exposed to daily exposure and occupational levels of phthalates by comparing them to the human secretome. Although the evidence is promising and suggests an oncogenic potential, caution should be exercised when extrapolating these findings to humans. Rats serve as an excellent model for such studies, but some discrepancies may arise.

Therefore, our study reinforces the translational link between preclinical and clinical data in the search for early biomarkers of prostate disorders associated with maternal exposure to plastic pollutants. Here, we integrated two distinct biological layers - the tissue proteome and the secretome - and, although we did not directly assess their interdependence, we recognize a likely functional connection between altered intracellular proteins and secreted biomarkers identified in PCa datasets. Future investigations should aim to characterize the secretome more comprehensively and strengthen the correlation between preclinical findings and clinical relevance. Furthermore, while epigenetic mechanisms were beyond the scope of this study, we acknowledge their critical role in the context of the DOHaD framework. Future research should explore how environmental toxicants influence epigenetic regulation during gestation and lactation, and how these early exposures may influence long-term prostate health and cancer susceptibility in offspring.

The comparison between proteomic data from rodents and the human secretome represents a promising approach, but it still requires direct validation. In addition, while the correlation between DEPs and gene expression levels of these targets in PCa is valid, it is somewhat limited because it involves comparing different layers of molecular regula-

tion (proteomic vs. transcriptomic). Finally, to strengthen these inferences, it would be important to investigate potential epigenetic mechanisms. Our study paves the way for future preclinical and clinical analyses, further emphasizing the importance of the intrauterine and lactational environments, as well as exposure to plastic waste and their effects on prostate health throughout life.

Conclusion

Gestational and lactational exposure to an environmentally relevant mixture of phthalates affects proteostasis in the endoplasmic reticulum. This exposure reduces the presence of several proteins involved in the process of protein folding and secretion, and influences post-translational modifications. This investigation revealed how the investigation of metabolic pathways and biomarkers can predict prostate diseases and articulate consequences at the beginning and throughout life, making the prostate vulnerable to the development of PCa.

Ethical Consent

The animal procedures were approved by the Institute of Biosciences/UNESP Ethics Committee for Animal Experimentation (Protocol: 1040/CEUA), and complied with the ethical principles of animal research and the Brazilian legislation established by the Brazilian Council of Control in Animal Experimentation.

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Conflict of Interest

None of the authors declare any conflicts of interest.

Supplementary Materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.arcmed.2025.103297.

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