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Prolonged atrazine exposure beginning *in utero* and adult uterine morphology in mice

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Abstract

Through drinking water, humans are commonly exposed to atrazine, a herbicide that acts as an endocrine and metabolic disruptor. It interferes with steroidogenesis, including promoting oestrogen production and altering cell metabolism. However, its precise impact on uterine development remains unknown. This study aimed to determine the effect of prolonged atrazine exposure on the uterus. Pregnant mice ($n = 5$/group) received 5 mg/kg body weight/day atrazine or DMSO in drinking water from gestational day 9.5 until weaning. Offspring continued to be exposed until 3 or 6 months of age ($n = 5$–9/group), when uteri were collected for morphological and molecular analyses and steroid quantification. Endometrial hyperplasia and leiomyoma were evident in the uteri of atrazine-exposed mice. Uterine oestrogen concentration, oestrogen receptor expression, and localisation were similar between groups, at both ages ($P > 0.1$). The expression and localisation of key epithelial-to-mesenchymal transition (EMT) genes and proteins, critical for tumourigenesis, remained unchanged between treatments, at both ages ($P > 0.1$). Hence, oestrogen-mediated changes to established EMT markers do not appear to underlie abnormal uterine morphology evident in atrazine exposure mice. This is the first report of abnormal uterine morphology following prolonged atrazine exposure starting *in utero*, it is likely that the abnormalities identified would negatively affect female fertility, although mechanisms remain unknown and require further study.

Introduction

Atrazine is a selective herbicide, widely used for the control of broadleaf weeds in crops and turf.[1] Despite being banned in the European Union since 2003, due to the ubiquitous contamination of water supplies, annually more than 36,000 tonnes of atrazine are still used in the USA and 3000 tonnes in Australia.[2,3] As a consequence, concentrations ranging between 0.02 µg/l and 2.4 mg/l have been detected in Australian waterways and drinking water[2,4] and levels ranging between 0.01 and 191 µg/l in waterways and drinking water are reported in the USA.[5–8] This raises concerns about the potential impacts of human and animal exposures. In humans, atrazine is present in bodily fluids including urine, breast milk, follicular and seminal fluids (all < 1.04 µg/kg).[9] Epidemiologic studies indicate that exposure is associated with an increased incidence of preterm birth, small for gestational age infants,[10,11] early onset and precocious puberty,[12,13] and extended menstrual cycles.[14,15] However, the specific impact of atrazine exposure on the development and function of the female reproductive tract remains unclear as the current literature is limited to adult atrazine exposure, and consists of conflicting findings regarding the potential toxic effects of atrazine.[16–19]

Atrazine is an endocrine-disrupting chemical (EDC). EDCs interfere with normal hormone function through a variety of mechanisms, including direct binding to androgen or oestrogen receptors to mimic the natural hormone activity.[20] They can also act antagonistically, binding to hormone receptors but not activating them.[21,22] While atrazine has a relatively low binding affinity for androgen and oestrogen receptors, it can interfere with hormone synthesis pathways.[23] This activity includes inhibiting phosphodiesterase, which increases aromatase levels allowing enhanced conversion of androgens to oestrogens, thus increasing oestrogen availability, as well as acting to decrease 5α-reductase expression, which in turn reduces the conversion of testosterone to the more potent androgen dihydrotesterone.[23–25] In addition, *in vitro* studies show that atrazine can bind directly to steroidogenic factor 1 (SF-1) and exert effects across the entire steroidogenic pathway. One example of this is increasing the interactions between SF-1 and the CYP19 promoter ArPII, which further drives increases in oestrogen levels.[26] Importantly, elevated local oestrogen levels can have adverse effects on reproductive physiology, including increasing the risk of cancer in endocrine sensitive tissues.[27–29]

Reproductive dysfunction following atrazine exposure has been investigated in mice and rats, with variable outcomes reported. High doses of atrazine administered by gavage (50–300 mg/kg/day) suppress the pulsatile secretion of gonadotropin-releasing hormone in female rats, disrupting normal reproduction by reducing or delaying the LH surge.[30] The same group subsequently showed this influence upon the hypothalamic–pituitary–gonadal axis may contribute to premature reproductive senescence in atrazine-treated animals, as evidenced by a constant state of oestrus and consistent oestrogen levels.[19] In contrast, another study reported that dietary atrazine supplementation (30–1460 ppm) resulted in no effect on endocrine or reproductive end points in females.[31] Conflicting reports, such as these, maybe due to the differences in dose, administration route, length in administrations, and age of the animals studied.

Despite evidence demonstrating the ability of atrazine to alter reproductive hormones, no studies have investigated the effects of prolonged exposure on the uterus, an oestrogen-sensitive organ. One potential impact of atrazine could be an increased predisposition to endometrial cancer, as a consequence of altered oestrogen levels. Endometrial cancer occurs in the inner lining of the uterus and is the most common invasive gynaecologic malignancy in developed countries.[32] It typically affects postmenopausal women, but its incidence is rising in women of reproductive age, the reasons for which are unclear.[33] It is often preceded by endometrial hyperplasia; a proliferative process within the endometrial glands that leads to an increase in the glandular–stromal ratio.[34] This process can be associated with unopposed oestrogen stimulation, though endocrine, genetic, and inflammatory factors are all likely to contribute to initiation and progression.[35] Incomplete understanding of the precise aetiology and molecular regulators have hampered advances in detection and therapies.

To improve our understanding of the potential impacts of atrazine exposure on the uterus, this proof of principle study investigated the effect of prolonged atrazine exposure, beginning in gestation, on uterine morphology in mice using a high dose (10-fold higher than the no-observed-effect level (NOEL) is used to calculate the acceptable daily intake (ADI) of atrazine in Australian drinking water).[36] This dose is more comparable than dosages used in the majority of previous studies to environmental atrazine concentrations and was selected based on our previous study that identified detrimental effects on the reproductive and metabolic phenotype of male mice.[37] Pregnant dams were exposed to atrazine (5 mg/kg body weight/day) in their drinking water, beginning during gestation before sexual differentiation and ovarian and uterine development in the fetus. In addition, offspring were continually exposed after birth and throughout postnatal life. The effect of atrazine exposure on uterine morphology, and expression of epithelial-to-mesenchymal transition (EMT) markers, in reproductively young (3 months) and older (6 months) female offspring was subsequently characterised. It was hypothesised that prolonged atrazine exposure would induce uterine abnormalities consistent with precancerous lesions, which may predispose to uterine cancers.

Materials and methods

Animals, treatments, and tissue collection

Female C57BL6J mice (WEHI, Melbourne, Australia) were housed under conventional conditions, with *ad libitum* soy-free food and water and held in a 12-hour light and dark cycle. Six-week-old pregnant females were housed individually and randomly assigned a treatment group ($n = 5$/group). Atrazine-treated mice were exposed to 5 mg/kg body weight of atrazine per day in drinking water from gestational day 9.5 of pregnancy onwards (before sexual differentiation and ovarian and uterine development), and through lactation, while control mice were exposed to 0.5% DMSO v/v in drinking water. The chosen dose is 10-fold higher than the NOEL determined by the Australian Government.[36] Oral administration of this dose is known to expose mouse fetuses to a similar *in utero* concentration to that measured in maternal plasma.[38] Pups were weaned at 4 weeks of age and only female pups were utilised for this study. Littermates were split within each treatment, weighed weekly, and maintained until 3 or 6 months of age ($n = 5$–9/age/treatment group). Food and water consumption were monitored for six females from each treatment group for 5 weeks. At 3 or 6 months of age, adult female offspring were weighed before being humanely culled by cervical dislocation, anogenital distance measured (6-month-old only), as well as vaginal cytology smear was collected to confirm the stage of the oestrous cycle. At post-mortem, body weight was recorded, the uteri were immediately dissected out (and weighed at 6 months of age only), with one horn snap-frozen at −80°C, and the other was fixed in neutral buffered formalin (10% v/v) for 24 h before paraffin embedding. The liver and fat (perigonadal and retroperitoneal) were removed and weighed. Liver and total fat weights were calculated by individual body weight (g/g).

Histological analysis of uteri

Paraffin sections (5 μm, three–five sections per animal, five–six animals per group) were dewaxed in Histolene (Grale HDS, Trajan 11031) and rehydrated through 70% and 100% graded ethanols before staining with Harris haematoxylin (Amber Scientific, HH-500) for 15 min. After thorough rinsing with tap water, sections were moved through acid alcohol and lithium carbonate solution before staining with Eosin (Amber Scientific) for 2 min. Sections were thoroughly rinsed in water before rapid dehydration through 100%- and 70%-graded ethanols and clearing with Histolene. Sections were mounted with DPX (Merck, 100579) and coverslips were applied. Morphological analysis was performed as previously described.[39] Briefly, atypical endometrial hyperplasia was defined by irregularly shaped glands with little stroma in between individual glands, and more than one layer of glandular epithelial cells surrounding the gland lumen. Cystic endometrial hyperplasia was characterised by large, dilated spaces lined by columnar or flattened epithelial cells. Leiomyoma was defined by the presence of a large tumour with spindle-shaped leiomyoma cells with elongated nuclei. Gland abnormalities were identified as unusual clustering of small glands or an uneven distribution of glands across the uteri cross sections.

Immunofluorescence

Immunofluorescence staining was performed as previously described.[40] Paraffin sections (5 μm, three–five sections per animal, five–six animals per group) were dewaxed in Histolene and rehydrated through graded ethanols, as above. Heat-mediated antigen retrieval was performed with citrate buffer. Serum block was formed with 10% donkey serum (Sigma) in 3% bovine serum albumin (BSA; Sigma A9418) in 0.1 M Tris, 150 mM NaCl (TN buffer). Primary E-cadherin antibody (R&D systems AF748) was applied at 1:1000 in 1% BSA in TN buffer, overnight

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at 4°C. A secondary antibody (Donkey anti-goat Alexa Fluor 488 (A11055); ThermoFisher Scientific) was then applied at 1:500 in 1% BSA in TN buffer for 1h at room temperature. DAPI (Dako 3005ONW) was applied at 1:1000 in PBS for 10 min at room temperature. Sections were mounted with FluorSave Reagent (Calbiochem 345789) and coverslips were applied. Sections were imaged using Eclipse Ti-E inverted confocal microscope (Nikon). Images were collected from random fields of view across the whole uterus cross section using 20× objective and utilised for localisation analysis. E-cadherin was expected to localise to the uterine epithelium.

Immunohistochemistry

Immunohistochemical staining was performed as previously described.[41] Paraffin sections (5 µm) were dewaxed in Histolene (Grale HDS, Trajan, 11031) and rehydrated through graded ethanols (70% and 100%). Heat-mediated antigen retrieval was performed with citrate buffer. Endogenous peroxide block was performed for 30 min at room temperature using 0.3% H_2O_2 (Amber Scientific) in dH_2O. Serum block was formed with 10% goat serum (Sigma, G9023) in 3% BSA in TN buffer. Primary antibody was added overnight at 4°C (Oestrogen receptor 1 (Esr1; Ab32063) at 1:500, Zinc finger protein1 (Snail) CST#3879 at 1:400) in 1% BSA in TN buffer. Secondary antibody (Goat anti-rabbit biotinylated BA-1000 (Vector)) was applied at 1:500 in 1% BSA in TN buffer for 30 min at room temperature, followed by Vectastain ABC system (Vector Labs, PK-6100) for 30 min. Diaminobenzidine (DAB; Agilent, K346811-2) was added (˜50 µl for 3–5 min) and sections were immersed in water when brown staining developed as seen under a light microscope. Sections were counterstained with Harris haematoxylin (Amber Scientific, HH-500), dehydrated through graded ethanols (70% and 100%), and cleared with Histolene. Sections were mounted with DPX (Merck) and coverslips were applied. Images were collected using Provis AX70 Widefield (Olympus) microscope. Immunohistochemical stain scoring was completed by blinded scorers with 0 representative of no positive DAB staining, and 3 representatives of intense positive DAB staining.[42,43]

Quantitative real-time polymerase chain reaction (RT-qPCR)

RNA was isolated from a piece of the whole uterus using the RNeasy Mini kit (Qiagen, 74104) according to the manufacturer's instructions. RNA concentration and purity were assessed using a NanoDrop spectrophotometry. RNA (500 ng) was reverse transcribed and cDNA was made using Superscript III First-strand synthesis kit (Invitrogen, 18080051). Established EMT markers[44] were investigated to determine if atrazine exposure promotes a mesenchymal, tumour-like phenotype that may contribute to the development of abnormal morphology. Hormone receptor genes were also investigated to determine if the abnormal morphology was hormone-driven. The primer sequences used for all genes are listed in Table 1. Real-time qPCR was performed using QuantiNova SYBR Green (Qiagen, 208052) with oligo primer pairs (Sigma). Expression levels were normalised to housekeeping 18 s and analysed using comparative cycle threshold (ΔΔCT) method as previously described.[45] Relative quantification (RQ) was calculated relative to the average ΔΔCT value for the control animals at each age group.

Liquid chromatography–tandem Mass spectrometry (LS-MS/MS) to measure steroid concentration in uteri

Pieces of whole uterus tissue samples ($n = 5$–8 per group) were prepared and analysed as previously described.[46] Briefly, frozen tissue was allowed to thaw on ice in 5 ml glass tubes. Buffer containing 0.5% BSA w/v, 5 mM EDTA in PBS was added and the tissue was homogenised on ice for 20 s on the highest setting using an IKA T10 basic disperser (IKA Werke, Germany). Homogenates were centrifuged at 1500 *g* for 10 min at 4°C and the supernatant was transferred to a clean tube for immediate analysis by LC-MS/MS. Samples were transferred to clean, glass tubes and extracted with 1 ml of hexane:ethyl acetate (3:2). Phase separation was allowed to occur in extracted samples for 1h at 4°C, followed by 30 min at −80°C to freeze the lower aqueous layer. The upper layer containing the steroids of interest was moved to a clean glass tube and evaporated overnight at 37°C, before dried samples were resuspended in 1.2 ml 20% methanol in PBS. Samples were thoroughly mixed and loaded into autosampler vials of which 1 ml was injected into a C8 column (Shimadzu) for analysis. Steroid concentrations were calculated as concentration per mass (mg) of tissue used. Hormones measured include testosterone (T), dihydrotestosterone (DHT), 5α-androstane-3α (3α-diol), 5α-androstane-3β (3β-diol), 17β-oestradiol (E2), and oestrone (E1). The limit of quantification (LOQ) was set against calibration standards for each hormone to allow calculation of hormone concentrations in ng/ml or pg/ml. LOQ for E2 was 5 pg/ml and for E1 3 pg/ml.

Statistical analyses

Data normality was tested with Shapiro–Wilk normality test. Parametric data (average birth weight, post-mortem body weight, relative organ weight) were analysed using unpaired *t*-tests. Non-parametric data (survival to weaning, gene expression, protein expression, and uterine steroid concentration) were analysed using Mann–Whitney test. Non-parametric statistical analyses were performed using GraphPad Prism 7. Sex ratio was compared with an expected 50:50 ratio as well as between groups by a corrected χ^2 procedure and was double-checked by binominal analysis. Normal data are represented as mean ± standard error of the mean (SEM), while the median is presented for non-parametric data. Alpha value was set at $P < 0.05$.

Results

Body and organ weights unchanged by prolonged atrazine exposure

Atrazine had no effect on the sex ratio of pups compared to control (Control $n = 18$ males, $n = 10$ female, Atrazine $n = 18$ males, $n = 18$ females, $P > 0.1$), nor their average birth weight (Control 3.8 ± 0.4 g, $n = 28$, atrazine 4.3 ± 0.2 g, $n = 36$, $P > 0.1$) or survival to weaning (Control $n = 27/28$, 96.4%; atrazine $n = 34/36$, 94%; $P > 0.1$). All animals survived post-weaning, and food and water intake measured for 5 weeks were comparable between treatment groups. Average water intake for control and atrazine treatment were 25.9 ± 0.6 ml/week and 25.7 ± 0.8 ml/week, respectively, while average food intake were 27.6 ± 0.9 g/week and 27.9 ± 0.9 g/week, respectively.

Body weights at collection were comparable between treatment groups at both ages (Fig. 1a). Fat and liver weights relative to body weight were also similar between treatment groups at both ages

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Table 1. Primer sequences utilised for gene expression analysis

Gene	Forward 5′-3′	Reverse 5′-3′	Reference
18S	GTAACCCGTTGAACCCCATT	CCATCCAATCGGTAGTAGCG	DOI: 10.1038/srep42736
E cadherin	CGTCCATGTGTGTGACTGTG	GCTCTTTGACCACCGTTCTC	DOI: 10.1172/JCI58815
Oestrogen receptor 1 (Esr1)	GCTCCTAACTTGCTCCTGGAC	CAGCAACATGTCAAAGATCTCC	DOI: 10.1073/pnas.1620903114
Tumour protein 53 (p53)	ACAGGACCCTGTCACCGAGAC	GACCTCCGTCATGTGCTGTGA	DOI: 10.1158/0008-5472.CAN-08-1274
Phosphatase and tensin homolog (Pten)	TGGATTCGACTTAGACTTAGA	GTTTGATAAGTTCTAGCTGTG	DOI: 10.3389/fimmu.2018.00688
Zinc finger protein 1 (Snail)	CACACGCTGCCTTGTGTCT	GGTCAGCAAAAGCACGTT	DOI: 10.1158/1535-7163.MCT-15-0677
Transforming growth factor beta 1 (Tgfb1)	GAGAAGAACTGCTGTGTGCG	GTGTCCAGGCTCCAAATATAG	DOI: 10.3389/fimmu.2018.00688
Twist family protein 1 (Twist1)	CTGCCCTCGGACAAGCTGAG	CTAGTGGGACGCGGACATGG	DOI: 10.1158/1535-7163.MCT-15-0677

(Fig. 1b, 1c). Relative uteri weights and anogenital distance were only available for 6-month-old animals and no differences were observed between atrazine and control for either (Fig. 1d, 1e).

Prolonged atrazine exposure alters uterine morphology

Cystic endometrial hyperplasia (Fig. 2a) was absent at 3 months of age in both treatment groups, and present only at 6 months of age (40% (2/5) controls, 67% (4/6) atrazine) (Table 2). Atypical endometrial hyperplasia (Fig. 2b) was observed most in atrazine-exposed animals at both ages (3 months: 33% (2/6) controls, 67% (4/6) atrazine, 6 months: 0% (0/5) controls, 33% (2/6) atrazine (Table 2). Leiomyoma was observed in one atrazine-exposed mouse at 6 months of age (Fig. 2c). Leiomyoma was absent for all other treatment groups and ages (Table 2). Gland abnormalities (Fig. 2d) were present in most groups 3 months: 0% (0/6) controls, 17% (1/6) atrazine, 6 months: 20% (1/5) controls, 33% (2/6) atrazine (Table 2).

Atrazine exposure does not influence steroid concentrations or oestrogen responsiveness

Local uterine oestradiol 17β (E2) (LOQ 5 pg/mg) and oestrone (E1) (LOQ 3 pg/mg) concentrations were determined by LS-MS/MS and revealed no changes with atrazine exposure at 3 or 6 months of age, compared to controls ($P > 0.1$; Fig. 3a, 3b). Oestradiol 17β concentrations remain unchanged in the 6-month-old cohort when separated by oestrous cycle stage at the time of collection ($P > 0.1$; Supplementary Figure 1). Levels of T (LOQ 0.03 ng/mg), DHT (LOQ 0.1 ng/mg), 3α-diol (LOQ 0.1 ng/mg), and 3β-diol (LOQ 0.1 ng/mg) were all below the detection level of the assay.

Expression of oestrogen receptor alpha (*Esr1*) mRNA was similarly unchanged in response to atrazine exposure at 3 and 6 months of age versus age-matched controls (Fig. 3c; $P > 0.1$). Oestrogen receptor-alpha protein (ERα) localised to the mouse endometrial epithelial and stromal compartments (Fig. 3d–3e), though ERα protein levels remained unchanged between treatment groups, at both ages (Fig. 3f–3h; $P > 0.1$).

Atrazine exposure does not alter markers of epithelial-to-mesenchymal transition in the mouse uterus

Since abnormal uterine morphology was observed in atrazine-exposed animals, and one animal developed leiomyoma, EMT markers were investigated to determine if atrazine exposure promotes a mesenchymal, tumour-like phenotype that may contribute to the development of abnormal morphology. *E-cadherin* mRNA levels were unchanged between groups, at both ages ($P > 0.1$; Fig. 4a). E-cadherin protein localised to the endometrial luminal and glandular epithelium and staining patterns were unchanged between treatment groups, at both ages ($P > 0.1$; Fig. 4b). Snail family zinc finger 1 (*Snail)* mRNA levels (Fig. 4c) and Snail protein localisation (Fig. 4d) remain unchanged following atrazine exposure versus control at both 3 and 6 months of age ($P > 0.1$). Similarly, relative mRNA expression levels of *p53*, phosphatase and tensin homolog (*Pten)*, transforming growth factor beta 1 (*Tgf-β1*), and twist family protein 1 (*Twist1*) all remain unchanged in response to prolonged atrazine exposure at 3 and 6 months of age ($P > 0.1$; Fig. 5).

Discussion

This study identified prolonged atrazine exposure, beginning *in utero* influences adult mouse uterine morphology. Several precancerous lesions were present in atrazine-exposed females at 3 and 6 months of age, including cystic endometrial hyperplasia, atypical endometrial hyperplasia, and one case of leiomyoma. These morphological abnormalities were not associated with gene or protein expression changes in oestrogen or its receptor, or key regulators of EMT. Thus, the mechanisms driving the development of atrazine-induced changes in the uterus, remain unknown and may potentially be due to the effect of atrazine on cell metabolism. While previous literature demonstrates atrazine can have adverse effects on male reproductive potential,[20,47] this study provides the first evidence that changes in the uterine morphology are evident in atrazine exposure mice.

The present study raises the possibility that exposure to the environmental toxicant, atrazine, maybe a possible contributing factor to endometrial hyperplasia and carcinoma. Interestingly, atypical endometrial hyperplasia was observed following atrazine exposure as early as 3 months of age, while cystic endometrial hyperplasia and leiomyoma were evident at 6 months, both representing ages of normal reproductive capacity in mice. Even in an established mouse model of endometrial hyperplasia and carcinoma, characterised by heterozygous loss of the tumour suppressor, *Pten,* mice develop lesions from 4 months of age.[48] Therefore, atrazine in the current model may have quite a pronounced effect on endometrial hyperplasia and leiomyoma incidence. While reproductively older, the 6-month-old cohort of mice are still fertile, therefore, it would be interesting for future investigations to study an older cohort of mice at ˜12–18 months of prolonged atrazine exposure, in order to more accurately define the long-term consequences.

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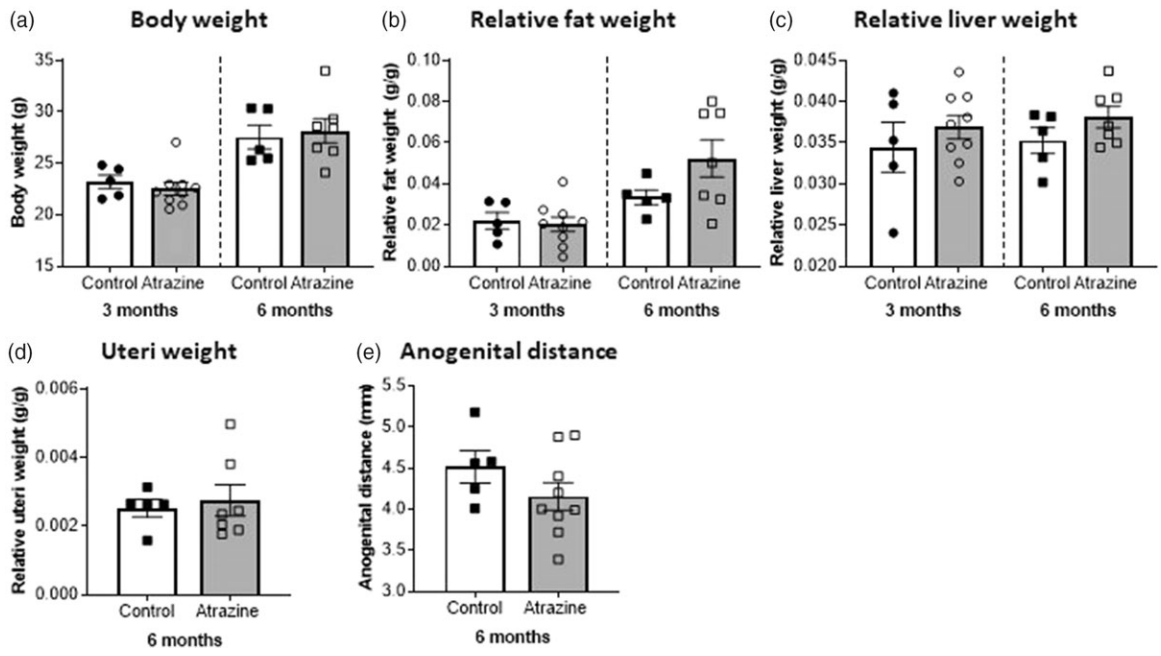

Fig. 1. Body weight and relative organ weights, as well as anogenital distance, are similar between atrazine-exposed (5 mg/kg bw/day from gestation day 9.5 until 3 or 6 months of age) animals and unexposed control female mice. (a) Body weight, (b) relative fat weight, (c) relative liver weight, (d) relative uteri weights, and (e) anogenital distance. Individual data points and means are presented, $n = 5$–9 animals/group, from $n > 3$ litters. Differences within cohort age groups are shown ($P < 0.05$).

Existing literature reports atrazine exposure reduces uterine weights and delays vaginal opening, indicating delayed sexual maturity.[49–52] However, these studies gave only short-term (single dose, of 7–23 consecutive days) atrazine exposure at high doses (50–300 mg/kg), to adolescent or adult aged rodents, and with atrazine often administered via gavage. The current study uses a more environmentally relevant dose and delivery method of atrazine with consistent exposure throughout embryonic and postnatal development.

The morphological abnormalities observed following long-term atrazine exposure in the current study are common precursors of endometrial carcinoma and can be induced by sustained exogenous oestradiol supplementation in a rodent model.[53] Specifically, atypical hyperplasia is apparent 6–8 weeks after exogenous oestradiol supplementation begins, with the lesions becoming precancerous by 10 weeks. Therefore, the abnormalities observed with atrazine exposure in the current study may develop into more severe precancerous lesions after 6 months of age.

In order to evaluate an oestrogen-independent mechanism responsible for the prevalence of precancerous lesions and the leiomyoma found in mice exposed to atrazine, regulators of EMT were investigated. EMT is a hallmark of tumour development, however, no differences were observed for E-cadherin or Snail, and similarly, tumour suppressor's *p53* and *Pten*, and EMT genes *Tgfβ1* and *Twist1* were also unchanged, suggesting atrazine is not driving uterine EMT and activation of the PI3K-Akt pathway to cause the precancerous lesions observed. Instead, it is possible that atrazine increases proliferation, since *in vitro* data demonstrates enhanced proliferation in prostate cancer cell lines following atrazine exposure, driven by activation of Stat3.[54] But, conflicting results were reported in human hepatoma cells, whereby atrazine exposure caused the opposite effect, inducing cell cycle arrest at S phase.[55] The precise impacts of atrazine on endometrial proliferation remain to be determined and may be improved through the analysis of EMT genes in only those areas in which abnormalities are evident, instead of analysing tissues collected from all animals; with and without morphological abnormalities. Alternatively, it is possible that uterine cell metabolism may be altered in response to atrazine. Atrazine exposure is known to cause mitochondrial dysfunction *in vitro* in muscle and liver[55,56] and this is indeed its mechanism of action in plant cells.[57] Further investigation into the effects of atrazine on the mitochondria and metabolic activity in mouse uterus may therefore be warranted in future studies. A major consideration in interpreting these results is the supra-environmental dose of atrazine administered to mice in this study. Indeed, these data, as well as comparisons with the literature regarding the impacts of atrazine exposure on offspring development must be taken with caution, due to high variations in doses, as well as the method, age, and duration of atrazine administration utilised across different studies. This ranges from 5 mg/kg/day in the current study (closer to environmental levels), up to 200 mg/kg.[20] Regardless, one consistency is that all doses reported exceed what can be considered environmentally relevant, since current data suggests drinking water levels of atrazine and its active metabolites range between 0.01 and 5 ng/l.[5] Therefore, now that the current preliminary study has identified changes in uteri morphology end point markers, future investigations should examine the potential impacts of environmentally relevant doses of atrazine on offspring development, as well as chronic exposure over multiple generations.

Despite this limitation, the data from this study are supported by findings following exposure to other EDCs, which act by similar steroid pathways. It is well-known that bisphenol A (BPA) and diethylstilbestrol (DES) both act via the oestrogen pathway and are detrimental to the female reproductive system. Gestational or neonatal BPA exposure accelerates folliculogenesis in some species, including sheep and rat,[58,59] culminating in premature reproductive senescence. BPA exposure is also known to negatively influence oocyte quality and viability.[59–62] Moreover, BPA can impair the hormone responsiveness of the uterus, causing a

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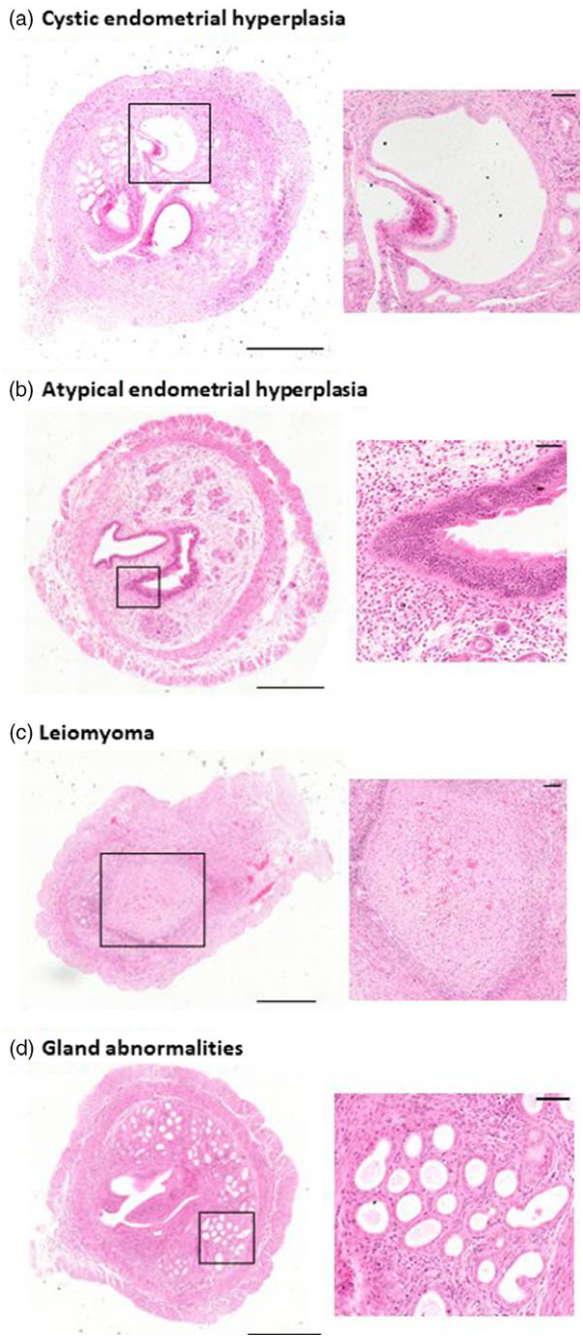

Fig. 2. Representative H&E images of the morphological differences assessed in uteri from control animals, or following prolonged atrazine exposure (5 mg/kg bw/day from gestation day 9.5 until 3 or 6 months of age). (a) Cystic endometrial hyperplasia, (b) atypical endometrial hyperplasia, (c) leiomyoma, (d) gland abnormalities. Scale bars are 500 or 50 μm. Boxes depict a high magnification region for each abnormality.

reduced endometrial proliferation in response to hormone stimulation in rats, or decreased progesterone receptor expression following oestrogen supplementation in a non-human primate model.[58]

Additionally, mouse studies demonstrate BPA exposure during early postnatal life is associated with abnormal adult female reproductive tract morphology including cystic ovaries, cystic endometrial hyperplasia, atypical endometrial hyperplasia, stromal polyps, and leiomyoma.[39] Conversely, neonatal exposure to DES is associated with a 100% penetrance of uterine fibroids by 16 months of age in rodents.[63] It has been postulated this may occur due to increased accumulation of DNA damage, and reduced ability of myometrial stem cells to undergo DNA repair, predisposing these cells to mutations that drive fibroid development *in vivo*.[64] Although the possibility of atrazine acting via this mechanism was not investigated in the current study, this hypothesis is also supported by human data showing prenatal exposure to DES increased the risk of developing uterine fibroids in women over 35, by 13%.[65] As atrazine has been proposed to behave similarly to EDCs like DES or BPA, the abnormalities observed in the current study may arise via similar mechanisms, hence mutations and DNA damage should be investigated in future studies.

It should be noted that control animals unexposed to atrazine in the current study have low incidences of both cystic endometrial hyperplasia, and atypical endometrial hyperplasia. While abnormalities such as these may not be expected in the control group, previous studies utilising other mouse strains (largely CD1) have reported incidences of these abnormalities in wild-type animals.[66–68] Therefore, it is possible these abnormalities are able to arise in otherwise healthy animals.

Evidently, EDCs have particularly pronounced effects when exposure occurs during early development. During this time, circulating hormones are typically bound by alpha-fetoprotein, leaving chemicals with oestrogenic capabilities like BPA, DES, or atrazine to influence development,[37,63,69] including early uterine development and subsequently predisposing exposed individuals to lifelong consequences. Although differences in steroid hormones were not detected in the current study, this may be attributed to the fact that whole uterine tissue was analysed, not only the endometrium, which could be addressed in future studies.

Ultimately, the long-term consequences of atrazine-induced uterine abnormalities may have on fertility remain unknown and should be addressed further by performing breeding studies. Since this model of atrazine exposure spanned from *in utero*, to postnatal life, it is possible that atrazine may influence epigenetic reprogramming during fetal development. In fact, this has already been demonstrated in the male germline, and is therefore likely to also occur in embryonic females exposed during sex determination.[70–72] This presents the possibility of atrazine culminating in multigenerational effects on offspring. There is literature to suggest other EDCs (including BPA and DES) alter EZH2 expression, influencing DNA methylation during epigenetic reprogramming, ultimately leading to tumourigenesis later in life.[73] Importantly, atrazine may exhibit multigenerational impacts on overall health, as evidenced by the accumulation of epigenetic disease inheritance across generations following atrazine exposure, despite the initial generation that was exposed not experiencing any increase in disease burden.[72]

Conclusions

This study demonstrates for the first time uterine abnormalities in mice following prolonged atrazine exposure beginning *in utero* until maturity (3 and 6 months of age). The potential mechanisms driving these abnormalities remain unclear, although the current data suggest that these changes may be oestrogen-independent, although additional end point measures and time points are required to conclusively collude atrazine acts via oestrogen-independent mechanisms. Furthermore, the current study demonstrated actions may be distinct from EMT and changes to key tumour suppressors in the endometrium. A large extent of variability was observed in response to atrazine exposure, both in the gross

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Table 2. Number and percentage of females with gross uterine morphological differences following prolonged atrazine exposure (5 mg/kg bw/day) or unexposed females from gestation day 9.5 until 3 or 6 months of age

Age	Group	Cystic endometrial hyperplasia	Atypical hyperplasia	Leiomyoma	Gland abnormalities
3 months	Control	0/6	2/6	0/6	0/6
		(0%)	(33%)	(0%)	(0%)
	Atrazine	0/6	4/6	0/6	1/6
		(0%)	(67%)	(0%)	(17%)
6 months	Control	2/5	0/5	0/5	1/5
		(40%)	(0%)	(0%)	(20%)
	Atrazine	4/6	2/6	1/6	2/6
		(67%)	(33%)	(17%)	(33%)

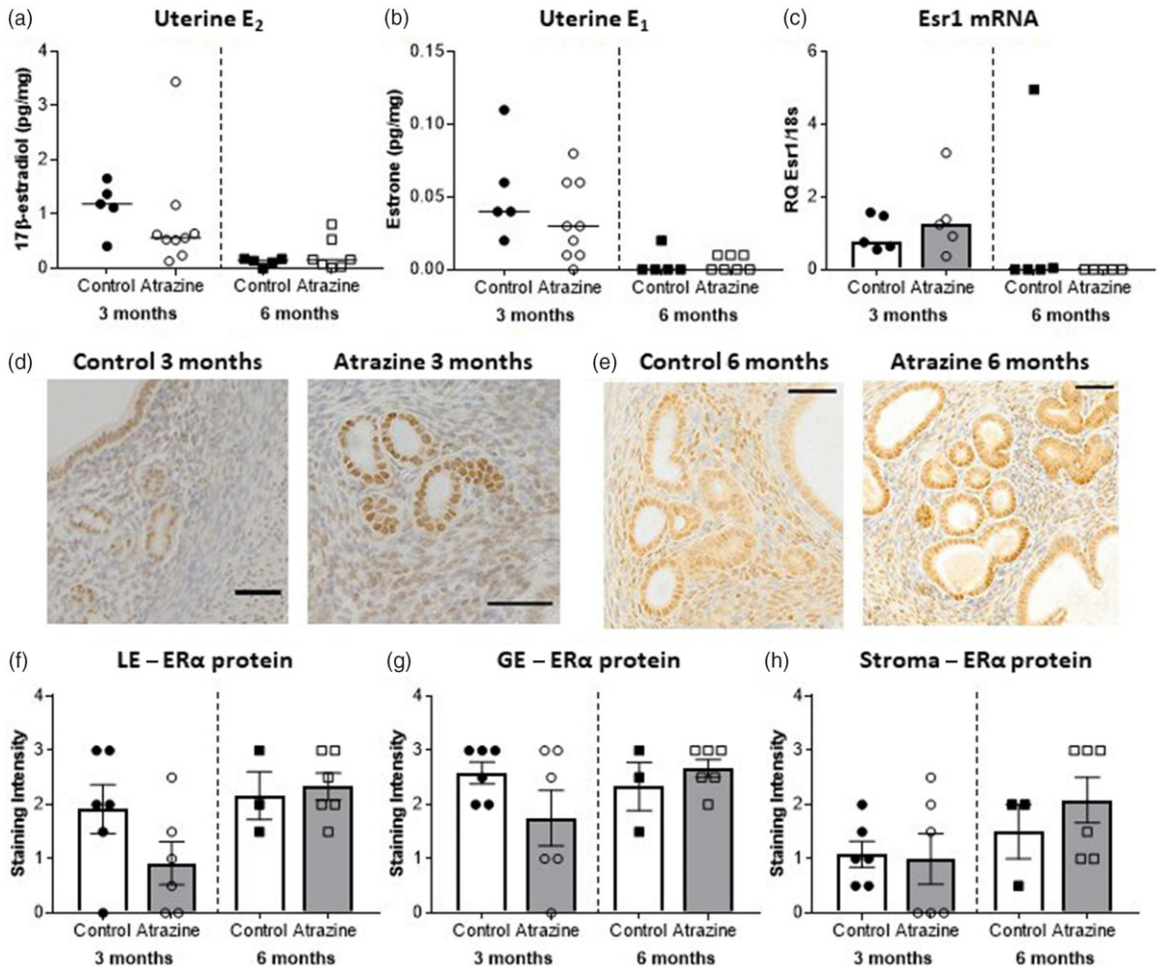

Fig. 3. Oestrogen hormone concentration and receptor expression levels are unchanged despite prolonged atrazine exposure (5 mg/kg bw/day) from gestation day 9.5 until 3 or 6 months of age compared with unexposed control females. (a) Local uterine 17β-oestradiol concentration, (b) oestrone concentration, and (c) oestrogen receptor alpha expression is unchanged ($P > 0.05$) after 3 or 6 months of atrazine exposure compared with unexposed females. (d–e) Localisation of oestrogen receptor alpha does not change with prolonged atrazine exposure, nor does relative protein expression levels in uterine compartments (f–h). (a–c) Individual data points are presented with median. (f–h) Data are presented as mean ± SEM. For each cohort age, $n = 3$–9 animals/group, from $n > 3$ litters ($P < 0.05$). Scale bars are 50 μm.

morphological abnormalities and oestrogen hormone and receptor levels, suggesting that larger sample sizes and an older group of mice would be beneficial to future studies. It is also a possibility that different oestrous cycle stages at the time of tissue collection contributed to the variability observed. This should be addressed in future studies by oestrous cycle analysis. Ultimately, atrazine has the potential to contribute to reproductive tract abnormalities and due to the abundance of atrazine present in ground and drinking water exposed to humans, this relationship should be investigated further.

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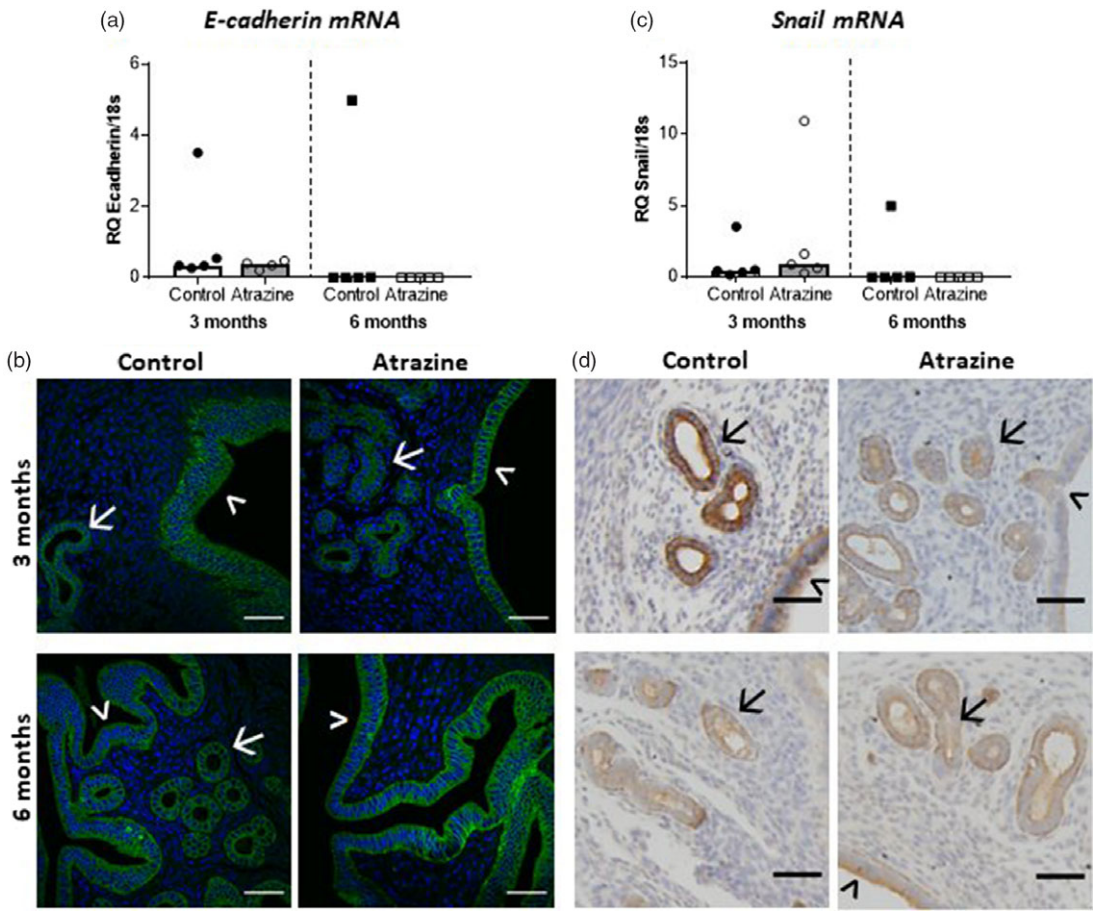

Fig. 4. Epithelial-to-mesenchymal transition factors are unchanged in uteri of atrazine exposed (5 mg/kg bw/day from gestation day 9.5 until 3 or 6 months of age) compared with unexposed control female mice. (a) E-cadherin mRNA levels are unchanged after 3 or 6 months of prolonged atrazine exposure and (b) protein localisation is unchanged at both time points. Similarly, (c) Snail mRNA and (d) Snail protein localisation is unchanged. Individual data points and means are presented, $n = 3$–5 animals/group, from $n > 3$ litters, within each age cohort ($P < 0.05$). Scale bars are 50 µm. à Glandular epithelium, >Luminal epithelium.

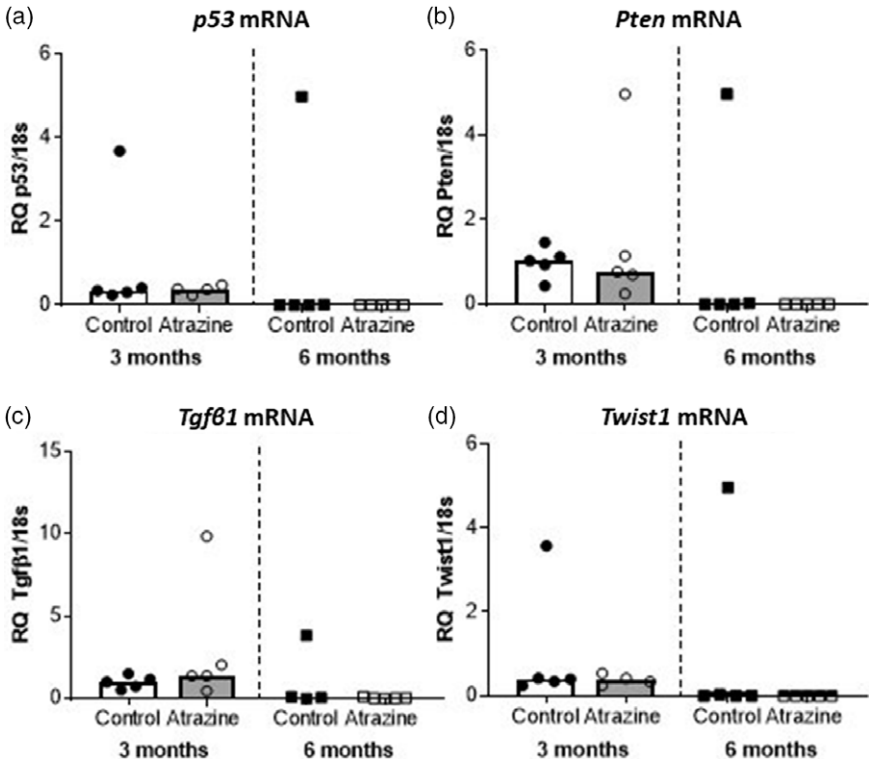

Fig. 5. (a) Tumour suppressors' *p53* and (b) *Pten*, and epithelial-to-mesenchymal transition genes (c) *Tgfβ1* and (d) *Twist1* mRNA levels are unchanged with prolonged atrazine exposure (5 mg/kg bw/day from gestation day 9.5 until 3 or 6 months of age) compared with unexposed control female mice. Individual data points and means are presented, $n = 3$–5 animals/group, from $n > 3$ litters. Significance within age cohorts ($P < 0.05$).

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Conflicts of interest. None.

Ethical standards. The authors assert that all procedures contributing to this work comply with the ethical standards of the National Health and Medical Research Council (NHMRC) Australian Code of Practice for the Care and Use of Animals for Scientific Purposes[74] and has been approved by the University of Melbourne Animal Ethics Committee (AEC 1513481.5).

Authors' contributions. M.J.G. and A.L.W. undertook all the laboratory studies, analysed the data, drafted the manuscript, and prepared the figures. A.P.H. and B.J.F. undertook the animal study. A.P.H., B.J.F., E.O.S., and J.M.S. collected the tissues. M.P.G. and K.J.H. were responsible for the experimental design and supervised the study. All authors reviewed and approved the final manuscript.

Consent for publication. All authors consent to publication.

Availability of supporting data. All data is included within the manuscript and supplementary files.

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