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Developmental low-dose exposure to bisphenol A induces chronic inflammation, bone marrow fibrosis and reduces bone stiffness in female rat offspring only

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ABSTRACT

Background: Developmental exposure to low doses of the endocrine disruptor bisphenol A (BPA) is known to alter bone tissue in young rodents, although how bone tissue is affected in aged animals is not well known. We have recently shown that low-dose developmental exposure to BPA increases procollagen type I N-terminal propeptide (P1NP) levels, a peptide formed during type 1 collagen synthesis, in plasma of 5-week-old female rat offspring while male offspring showed reduced bone size.

Objective: To analyze offspring bone phenotype at 52 weeks of age and clarify whether the BPA-induced increase in P1NP levels at 5 weeks is an early sign of bone marrow fibrosis development.

Methods: As in our 5-week study, pregnant Fischer 344 rats were exposed to BPA via drinking water corresponding to 0.5 μg/kg BW/day (BPA0.5), which is in the range of human daily exposure, or 50 μg/kg BW/day (BPA50) from gestational day 3.5 until postnatal day 22. Controls were given only vehicle. The offspring were sacrificed at 52 weeks of age. Bone effects were analyzed using peripheral quantitative and micro-computed tomography (microCT), 3-point bending test, plasma markers and histological examination.

Results: Compared to a smaller bone size at 5 weeks, at the age of 52 weeks, femur size in male offspring had been normalized in developmentally BPA-exposed rats. The 52-week-old female offspring showed, like the 5-week-old siblings, higher plasma P1NP levels compared to controls but no general increasing bone growth or strength. However, 2 out of 14 BPA-exposed female offspring bones developed extremely thick cortices later in life, discovered by systematic *in vivo* microCT scanning during the study. This was not observed in male offspring or in female controls. Biomechanical testing revealed that both doses of developmental BPA exposure reduced femur stiffness only in female offspring. In addition, histological analysis showed an increased number of fibrotic lesions only in the bone marrow of female rat offspring developmentally exposed to BPA. In line with this, plasma markers of inflammation, Tnf (in BPA0.5) and Timp1 (in BPA50) were increased exclusively in female offspring.

Conclusions: Developmental BPA exposure at an environmentally relevant concentration resulted in female-specific effects on bone as well as on plasma biomarkers of collagen synthesis and inflammation. Even a dose approximately eight times lower than the current temporary EFSA human tolerable daily intake of 4 μg/kg BW/day, appeared to induce bone stiffness reduction, bone marrow fibrosis and chronic inflammation in female rat offspring later in life.

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1. Introduction

The number of studies describing associations between human disease and exposure to synthetic compounds with endocrine-disrupting properties is mounting (Vandenberg et al., 2009; Rubin, 2011; Gore et al., 2015; Seachrist et al., 2016). Bisphenol A (BPA), an endocrine-disrupting chemical (EDC), is used in the production of polycarbonate plastics and epoxy resins. It has been observed that these types of plastic, used in for example water pipes and food packaging, leak BPA, which is why it can be detected in the urine in the great majority of humans examined (Vandenberg et al., 2007; Calafat et al., 2008; Guidry et al., 2015; Ye et al., 2015). Exposure to EDCs (including BPA) has been shown to correlate with non-monotonic dose responses, which means that it is relevant to study exposure also to lower EDC doses (Vandenberg et al., 2012, 2013). Studies on low-dose effects are relevant in relation to the chronic low-level exposure in the general human population. When investigating low-dose effects it is important that exposure starts early in development to mimic the chronic low-dose exposure during a sensitive window. The fetus is known to be very vulnerable to perturbation by for example early-life malnutrition, stress, and exposure to environmental chemicals, particularly EDCs that can interfere with the endocrine system (Bern, 1992; Newbold et al., 2009; Howard, 2018). Often, developmental effects are not manifested until years or decades later. Indeed, the developmental origin of health and disease (DOHaD) hypothesis suggests that early-life exposure can influence disease outcomes throughout the entire lifespan of an organism and the risk of developing diabetes, cardiovascular disorders, or cancer, increases with age (Barker, 2004). In line with this, it is becoming clearer that low-dose developmental exposure to BPA can contribute to effects observed later in life (Nesan et al., 2018; Pouzaud et al., 2018). Yet, traditional toxicity studies reaches very different conclusions about BPA's safety, compared to low-dose developmental exposure studies (Vandenberg et al., 2019). Along this line, we reported that developmental exposure to BPA at doses between 25 and 50,000 μg/kg BW/day had a sexually dimorphic effect on offspring bone tissue on all doses tested except the highest (Lejonklou et al., 2016). In an effort to clarify differences between traditional regulatory studies and findings from independent investigators a collaborative project called Consortium Linking Academic and Regulatory Insights on Toxicity of BPA (CLARITY–BPA) was launched five years ago (https://ntp.niehs.nih.gov/results/areas/bpa/). However, analysis of bone and marrow was not included as a study focus area of CLARITY-BPA.

Bone marrow is a vital organ and its hematopoietic stem cell niche have the unique ability to give rise to all of the different mature blood cell types and tissues (Morrison and Scadden, 2014). Bone development and later growth is controlled by growth hormone, parathyroid hormone and thyroid hormone, among others, as well as the classical sex hormones estrogen and testosterone (Kovacs, 2014). Estrogen is the primary female sex hormone and reduced estrogen production during menopause induces osteopenia and osteoporosis. Notably, estrogen is also a dominant regulator of bone metabolism in men (Khosla, 2008). Increased blood levels of estradiol are associated with greater risk of developing breast cancer (Breast Cancer, 1997). BPA is a xenoestrogen and has structural resemblance to diethylstilbestrol (DES), another synthetic xenoestrogenic compound which has been linked to vaginal cancer in DES-exposed human offspring (Vandenberg et al., 2012; Gore et al., 2015). In rats, studies of developmental exposure to ethinyl estradiol (a synthetic estrogen used in birth control pills) via food showed that estrogen had a more pronounced effect on the female offspring bone (shorter and thinner) (Hotchkiss et al., 2008). In contrast, we showed in a recent publication, that developmental exposure to BPA, at a dose considerably lower than the current preliminary human tolerable daily intake (TDI) of 4 μg/kg body weight/day set by the European Food Safety Authority (2015)) had a more pronounced effect on male offspring bone (shorter and thinner) (Lind et al., 2017). Thus, developmental low concentration of oral BPA or DES both show negative effects on bone size in young rodent male offspring, while higher concentrations tend to show a positive effect on bone size in female offspring (Rowas et al., 2012). In addition, we showed that BPA exposure increased plasma levels of procollagen type I N-terminal propeptide (P1NP), a peptide formed during type 1 collagen synthesis and a bone anabolic marker, only in female offspring and without increasing bone growth or strength (Lind et al., 2017). Elevated circulating levels of P1NP have been linked to liver fibrosis in humans and in rats (Veidal et al., 2010; Farmer et al., 2015 Luger et al., 2016) and are also associated with a higher risk of developing or having bone metastasis together with a predicted reduced survival in human patients with breast or prostate cancer (de la Piedra et al., 2003; Jung et al., 2011; Dean-Colomb et al., 2013). In rodents, developmental exposure to BPA has been shown to increase the risk of developing tumors in prostate (Ho et al., 2006), breast (Durando et al., 2007; Acevedo et al., 2013), ovary (Newbold et al., 2009) and liver tissue (Weinhouse et al., 2014), as well as induction of fibrosis in liver (Wei et al., 2014) and heart tissue (Belcher et al., 2015). Along this line, *in vitro* experiments have demonstrated that BPA can increase collagen production in cardiac fibroblasts (Hu et al., 2016), adipocytes (Boucher et al., 2016) and osteoblasts (Miki et al., 2016). Interestingly, fibro-osseous lesions (FOLs), characterized by accelerated osteoblastic turnover with concurrent fibroplasia, have been observed in mice treated with high doses of DES (McAnulty and Skydsgaard, 2005), and osteosarcomas arise from these areas after long-term administration. Therefore, in the present study we aimed to examine to what extent developmental BPA exposure affects bones and bone marrow tissues in a long-term investigation to clarify whether increased P1NP levels at a young age are an early sign of fibrosis and FOLs.

2. Materials and methods

2.1. Animals and housing

This study (C26/13) was approved by the Uppsala Ethical Committee on Animal Research, following the Swedish Legislation on Animal Experimentation (Animal Welfare Act SFS1998:56) and the European Union Legislation (Convention ETS123 and Directive 2010/63/EU). The experimental set-up has been described previously (Lejonklou et al., 2017; Lind et al., 2017; Alavian-Ghavanini et al., 2018; Dunder et al., 2018; Spörndly-Nees et al., 2018; Manukyan et al., 2019). Time-mated female Fischer 344/DuCrl (F344) rats, n = 45 (8–9 wks old), were purchased from Charles River, Germany, for housing in an Uppsala University animal facility. Upon arrival at the laboratory on gestational day (GD) 3.5 the dams were weighed and chip-marked, and the dosing started immediately. The study was performed using seven blocks (separated by one week), and all dose groups were equally distributed among blocks. Dams within each block were randomly distributed into three dosing groups (0 (CTRL; n = 18), 0.5 (BPA0.5; n = 12) or 50 (BPA50; n = 15) μg BPA/kg BW/day and housed one dam per cage. Experimental conditions were identical across study groups. Dams were weighed twice per week and housed one by one to be able to measure their individual water consumption. To minimize background BPA exposure, animals were housed in polysulfone cages (Eurostandard IV) with glass water bottles. Cages were housed in a temperature- and humidity-controlled room (22 ± 1 °C and 55 ± 5%, respectively) with a 12-h darkness and light cycle, and an air change rate of ten times per hour. Litters were adjusted to six pups per dam (3 males and 3 females) on postnatal day (PND) 4. At weaning, on PND 22 the dams were sacrificed and two male offspring and two female offspring were selected at random and moved to cages with three offspring in each, divided by sex and dose. Pups of the same sex and dosing group all had different mothers to avoid litter effects. In total, there were 24 control offspring (12 males, 12 females), 16 (BPA0.5) offspring (dams exposed to 0.5 μg/kg BW/day: 8 males, 8 females), and 16 (BPA50) offspring (dams exposed to 50 μg/kg BW/day: 8 males, 8

females). Food and water were available *ad libitum*. Water intake was registered until PND22, twice a week, when fresh solutions were prepared and bottles changed. Dams were fed a standard-type breeding chow pelleted diet, RM3 (NOVA, SCB, Sollentuna, Sweden), and the offspring were fed a maintenance diet after weaning, RM1 (NOVA, SCB, Sollentuna, Sweden). The manufacturer specified the nutrient and phytoestrogen content of the different batches of feed provided to the dams and newborn pups [RME3, batch 9987: 11.2 and < 10 mg/kg of genistein and daidzein, respectively, and 11.3 μg/g total genistein equivalents [TGE = genistein + (daidzein*0.1)], and to offspring after PND22 (RME1, batch 1028: < 10 mg/kg of both genistein and daidzein, and < 10.1 μg/g TGE). All values were well below the upper limit (325–350 μg/g) suggested by the Organization for Economic Co-operation and Development (OECD) (Owens et al., 2003). The offspring were weighed once a week for ten weeks and then once every fourth week until sacrifice at 52 weeks of age (± 2 weeks). The female offspring were sacrificed when in the diestrus cycle stage, with determination and classification conducted as described (Byers et al., 2012). Animals were weighed and subsequently anesthetized using a cocktail of ketamine (90 mg/kg) and xylazine (10 mg/kg) (intraperitoneal injection), according to the Institutional Animal Care and Use Committee anesthesia guidelines for rats (IACUC, 2014). All animals were sacrificed through aortic exsanguinations. Experiments were carried out during daytime in a dedicated laboratory neighboring on the animal facility. All personnel involved in the experiment were blinded, except the project coordinator and the staff member taking notes of weights et cetera, during collection of the different tissues.

2.2. Exposure

Results on the effects on the same animals (or their siblings) have been reported in previous studies (Lejonklou et al., 2017; Lind et al., 2017; Alavian-Ghavanini et al., 2018; Dunder et al., 2018; Spörndly-Nees et al., 2018; Manukyan et al., 2019). To mimic the most likely route of human exposure, dams were exposed to BPA via their drinking water directly from their arrival at GD 3.5 till sacrifice at weaning on PND 22. Bisphenol A (80-05-7, $(CH_3)_2C(C_6H_4OH)_2$, ≥99% purity) (Sigma Aldrich) was dissolved in ethanol and subsequently diluted with well-flushed tap water to defined final concentrations (1% ethanol in the final solutions). We aimed for the doses 0.5 μg BPA/kg BW/day (BPA0.5), a dose well below the current European Food Safety Authority (EFSA) TDI of 4 μg BPA/kg BW/day, and a dose corresponding to the former EFSA and current U.S FDA TDI of 50 μg BPA/kg BW/day (BPA50). BPA solutions were freshly made, changed, and consumption measured twice every week. Control dams were given well-flushed tap water containing 1 vol% ethanol (vehicle). The pups were exposed mainly through the placenta *in utero* and via milk during lactation but may also have been exposed directly through drinking the water during the last days of lactation. The concentration of BPA in the solution was verified at the Division of Occupational and Environmental Medicine in Lund, Sweden, applying the modified method described in (Bornehag et al., 2015). The limit of detection (LOD) for the analysis of BPA was 0.2 ng/ml. The division in Lund is a reference laboratory chosen for the European biomonitoring project (Consortium to Perform Human Biomonitoring on a European Scale (COPHES); www.eu-hbm.info/democophes).

2.3. Histology and immunohistochemistry

The bones were removed and cut in half length and immersion-fixed for 48 h in 4% phosphate-buffered paraformaldehyde at room temperature, followed by decalcified for 48 h in 20% formic acid. The head region part was then cut in half to expose the bone marrow center, dehydrated, and then embedded in paraffin wax. Longitudinal sections (5 μm thick, starting from the marrow center) were cut, transferred to slides (SuperFrost, Menzel-Gläser, Germany) and then deparaffinized and rehydrated. Bones (randomly selected) were sectioned in the same orientation in order to produce comparable sections, and initial examination of hematoxylin and eosin stained sections (1/animal, 3 random animals/group of females) was performed blinded by a pathologist. Bone marrow lesions were defined as pale stained areas devoid of fat cells or large blood vessels and were quantified from 2 consecutive sections per animal (from 6 random animals/group of females) using a low power magnification (4x). Trichrome Stain (Masson) kit and reticulin silver plating kit according Gordon & Sweets were used according to the manufacturer's instructions (Sigma-Aldrich). The reticulin stain was used to score for fibrosis severity, number of animals for each group = 4–6 (2 sections/animal) (Kuter et al., 2007). Immunostaining was achieved by antibodies for cleaved caspase 3 (ab2301, Abcam); osteopontin as described before (Lind et al., 2011); Fabp4 (BAF1443, R&D Systems); Tnf (ab6671, Abcam) and Timp1 (AF580, R&D Systems) in blocking solution (Background sniper, Biocare Medical). Bone sections were pretreated sequentially with 3% H_2O_2 and trypsin (Carezyme I: Trypsin Kit, Biocare Medical), at room temperature for 10 min. Between treatments sections were washed in PBS with 0.05% Tween 20 (3 times à 3 min). Visualization of the antibodies where achieved by incubation with a secondary biotinylated antibody at a dilution of 1:200 followed by an avidin–biotin–peroxidase complex incubation using the Vectastain ABC-kit (Vector Laboratories) and the substrate diaminobenzidine tetrahydrochloride (DAB, DAKO).

2.4. Determination of plasma markers for bone turnover and inflammation

Blood from exsanguination was collected in ethylenediaminetetraacetic acid (EDTA)/protease inhibitor-treated tubes, and centrifuged (2500 × *g*; 10 min, 4 °C) to prepare plasma, as previously described (Lejonklou et al., 2017; Lind et al., 2017). Aliquots were stored at −70 °C. Commercially available ELISA kits were utilized for measurement of plasma levels as follows: C-terminal telopeptides of type I collagen (CTX-1), RatLaps TM (Nordic Bioscience Diagnostics), procollagen type I N-terminal propeptide (P1NP) enzyme immunoassay (Immunodiagnostic Systems, Boldon, UK) and transforming growth factor, beta 1 (Tgfb1, R&D Systems). A Meso Scale Discovery multiplex cytokine immunoassay panel was used to quantitate tumor necrosis factor (Tnf), C-C motif chemokine ligand 2 (Ccl2 or MCP-1), interleukin 10 (Il10), chemokine (C-X-C motif) ligand 1 (Cxcl1 or KC/GRO) and TIMP metallopeptidase inhibitor 1 (Timp1), according to the manufacturer's instructions (Meso Scale Discovery, https://www.mesoscale.com).

2.5. Hormone analysis

The plasma hormone analysis results from the male offspring in the present study have recently been published (Spörndly-Nees et al., 2018). Briefly, testosterone (Te), estrone (E1), estradiol (E2), hydroxylamine, formic acid, trifluoroacetic acid, dansyl chloride and sodium carbonate were purchased (Sigma Chemical Company, St Louis, MO). The internal standards were deuterium-labeled analogs of the steroids d3-Te (Cambridge Isotope Laboratories, Andover, MA), d5-DHEA (Ceriliant, Round Rock, TX), d7- A4 (Steraloids Inc.), d4-E1 and d3-E2, (CDN Isotopes, Toronto, ON). All other chemicals were of the highest purity commercially available.

Plasma samples were analyzed for DHEA (dehydroepiandrosterone), A4 (androstenedione), Te, E1 and E2 using liquid chromatography tandem mass spectrometry methods (LC–MS/MS) as previously described (Kushnir et al., 2008, 2010a; 2010b). Briefly, steroids were extracted from samples; DHEA, A4, Te, were derivatized with hydroxylamine to form oxime derivatives; estrone and estradiol were derivatized with dansyl chloride to form dansyl derivatives (Kushnir et al., 2008). Limit of quantification (LOQ) was 10 pg/mL for Te and A4, 50 pg/mL for DHEA, 1 pg/ml for E1 and E2 (Kushnir et al., 2008, 2010a). The intra-assay and inter-assay imprecision (coefficient of

Table 1
Effects of developmental exposure to bisphenol A (0.5 μg/kg BW/day or 50 μg/kg BW/day) on body weight and femur length in 52-weeks-old offspring.

	Control (n = 12)	0.5 μg/kg/daym (n = 8)	% diff.	50 μg/kg/day (n = 8)	% diff.	*p*-value
Females						
Body weight (gram)	218 ± 10	215 ± 10	−1.6	227 ± 20	+4.0	*0.37*
Femur length (mm)	33.5 ± 0.33	33.6 ± 0.47	+0.2	33.7 ± 0.28	+0.5	*0.63*
Males						
Body weight (gram)	455 ± 20	449 ± 18	−1.4	448 ± 20	−1.6	*0.66*
Femur length (mm)	40.9 ± 0.64	40.7 ± 0.78	−0.5	40.5 ± 0.87	−0.8	*0.59*

Results are presented as means ± SD, %-diff. is the % change of mean in BPA exposed rat offspring compared to controls. *p*-values are from ANOVA analysis.

variation) for all measured analyses was < 8% and 11%, respectively (Kushnir et al., 2008, 2010a). All steroids were analyzed in positive ion mode using electrospray ion source on a triple quadruple mass spectrometer (API5500; AB Sciex, Foster City, CA). The HPLC system consisted of series 1260 and series 1290 HPLC pumps (Agilent Technologies) and an HTC PAL autosampler (LEAP Technologies, NC) equipped with a fast-wash station. The quadrupoles Q1 and Q3 were tuned to unit resolution, and the mass spectrometer conditions were optimized for maximum signal intensity of each steroid. Two mass transitions were monitored for each steroid and its internal standard. Quantitative data analysis was performed using Analyst™ 1.6.1 software. Calibration curves were generated with every set of samples using six calibration standards; three quality-control samples were analyzed along with every set of samples. Specificity of the analysis for each steroid in every sample was evaluated by comparing concentrations determined using the primary and the secondary mass transitions of each steroid and its internal standard (Kushnir et al., 2005, 2010b).

2.6. Micro-computed tomography measurements

This method (Bouxsein et al., 2010) is used to follow the general bone development *in vivo* during the course of the entire study. Due to the time consuming nature of this procedure, the right tibia of a subset of randomly chosen offspring (Cont, n = 14; BPA0.5, n = 14; BPA50 n = 16) was analyzed *in vivo* by micro-computed tomography (μCT, SkyScan 1176, Kontich Belgium) at 5, 20, 42 weeks of age and *ex vivo* at 52 weeks, at a voltage of 80 kVp and a current of 313 mA with an Al-Cu filter. For the *ex vivo* analysis, bone was removed and covered with a piece of sterile non-woven compress and submerged in a centrifuge tube filled with Ringer's solution (pH 7.4, Tris 0.3 g/l, NaCl 9 g/l, $CaCl_2$:$2H_2O$ 0.24 g/l, KCl 0.4 g/l, 2.05×10^{-3} M HCl) and frozen down at −18 °C until used for μCT analysis in air. The exposure time was set to 85 ms; frame averaging to 1; and the rotation step to 0.70°: Images were acquired using an isotropic pixel size of 36 μm. The settings were optimized to minimize the radiation dose to the animals without compromising image quality. The anesthetized animal (isoflurane 1.0%–2.5% in 50%/50% medical oxygen: air at 450 ml/min) was placed on a gantry bed heated through hot air to prevent hypothermia. Reconstruction of cross-sections was done using the software package NRecon (Bruker microCT, Kontich, Belgium). The software package DataViewer (Bruker microCT, Kontich, Belgium) was used to align the tibia and to provide high-resolution cross-sectional images. Two volumes of interest (VOI) were selected to analyze the trabecular and cortical bone, respectively (figs1(S1 Fig.). At a distance of 1.5 mm below the tibial growth plate, a 1 mm-high VOI was set to analyze the trabecular bone. A second VOI of the same height was placed 10 mm below the growth plate to analyze the cortical bone. Bone tissue morphometric analysis was done with the software CTAn (Bruker microCT, Kontich, Belgium).

2.7. Peripheral quantitative computed tomography measurements

This method was used to get a detailed analysis of the bone structure, which requires high energy radiation and is not compatible with living tissue. The peripheral quantitative computed tomography (pQCT) measurements were performed as previously described (Lind et al., 2011; Lejonklou et al., 2016). Briefly, the right femurs were dissected out and cleaned after sacrifice. Numbers of samples were for females: Cont, n = 12; BPA0.5, n = 7; BPA50 n = 7; and for males: Cont, n = 12; BPA0.5, n = 8; BPA50 n = 8. Due to dissection error (severing of bone end), the reference line could not be accurately placed for two female bones (1 from BPA0.5 and 1 from BPA50) and therefore these bones could not be analyzed by pQCT in air. Femur lengths were measured using a slide caliper with an accuracy of 0.1 mm from the proximal to the distal end of the bone, with the ruler parallel to the bone. Subsequently the bone was covered with a piece of sterile non-woven compress and submerged in a centrifuge tube filled with Ringer's solution (pH 7.4, Tris 0.3 g/l, NaCl 9 g/l, $CaCl_2$:$2H_2O$ 0.24 g/l, KCl 0.4 g/l, 2.05×10^{-3} M HCl) and frozen down at −18 °C until used for pQCT analysis. A pQCT machine (Stratec XCT Research SA+, Stratec Medizintechnik, Pforzheim, Germany, software version 5.50 R) was used to measure femur dimension and density. The manufacturer-specified reference object (phantom) was used for calibration of the machine before start of measurements every day. pQCT scans were performed at the metaphysis (20% of bone length from the condyle) and at (mid-)diaphysis (at 50% of bone length) figs1).

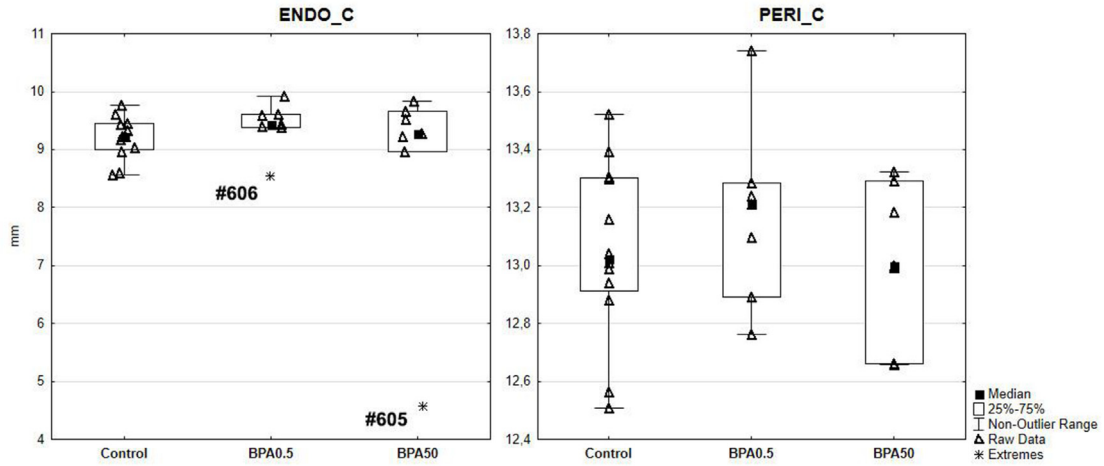
2.8. Mechanical three-point bending test of femur diaphyses

The three-point bending test evaluate the material properties and strength of the bone and was performed as previously described (Lind et al., 2011; Lejonklou et al., 2016) on the right femurs (after pQCT analysis) using an electromechanical material testing machine (Avalon Technology Inc., Rochester, MN, USA) with a span length of 13 mm and a crosshead speed of 0.48 mm/s. Numbers of samples were for females and males: Control, n = 12; BPA0.5, n = 8; BPA50 n = 8. Bone strength was measured at (mid-)diaphysis (i.e. at 50% of bone length). Load and displacement were sampled with a frequency of 50 Hz and stored digitally (Jepsen et al., 2015). Displacement at failure (mm) and the maximum load applied (N) before the bone fractured were recorded. The area under the curve was defined as the amount of energy absorbed until failure (work-to-fracture, N x mm). The slope of the linear part of the load–displacement curve was defined as the bone's stiffness (N/mm).

2.9. Statistics

Levene's test was used to verify homogeneity of variance and Shapiro-Wilk normality test (SW-W) to determine whether data was normally distributed. The body weight, pQCT and biomechanical results were evaluated using analysis of variance (One-way ANOVA), followed by Dunnett's post-hoc test. Plasma markers of bone turnover were analyzed using the Mann-Whitney *U* test. Due to outliers/extremes in more than one group, the median test was used for estradiol and testosterone level analysis. Student's t-test was used for marrow lesion numbers and fibrosis scoring. In every case, $p < 0.05$ was considered statistically significant. Software used for data analysis was Statistica, version 13.4.0.14 (TIBCO Software Inc.).

A



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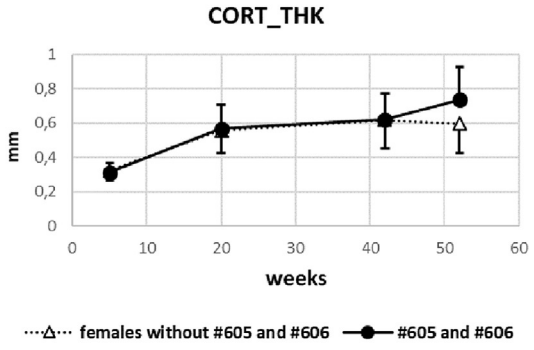

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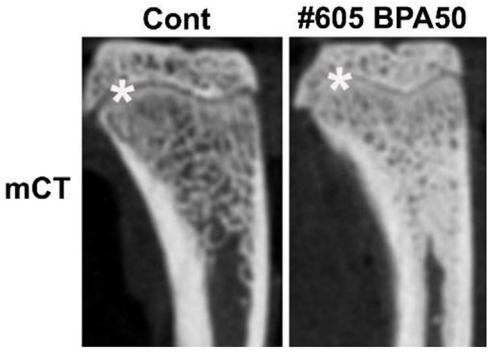

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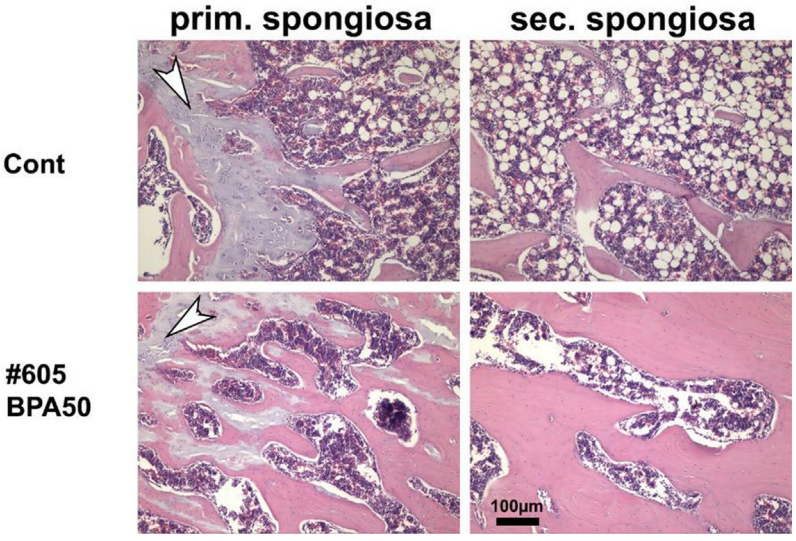

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Fig. 1. The bone phenotype in female F344 offspring developmentally exposed to 0.5 or 50 μg BPA/kg BW/day. (A) Box plot of female offspring endocortical circumference (ENDO_C) and periostal circumference (PERI_C) as determined by peripheral quantitative computed tomography (pQCT). Dots outside the boxes are extreme values. #605 and #606 are the rat individuals with the extreme readings. Numbers of samples for pQCT were: Cont, n = 12; (BPA0.5) (0.5 μg BPA/kg BW/day), n = 7; (BPA50) (50 μg BPA/kg BW/day) n = 7. **(B)** Cortical thickness (CORT_THK) of female tibia as determined by *in vivo* micro-computed tomography (μCT) plotted against rat offspring age. Numbers of samples in "females without #605 and #606" were: Cont, n = 7; (BPA0.5) (0.5 μg BPA/kg BW/day), n = 6; (BPA50) (50 μg BPA/kg BW/day) n = 7. Numbers of samples in "extremes" are two: rat #606 (BPA0.5) and #605 (BPA50). **(C)** High resolution μCT picture of the tibia from the female rat with the most extreme pQCT value, #605 from (BPA50) and a control. Asterisk denotes growth plate. **(D)** Hematoxylin staining of trabecular bone in humerus (primary and secondary spongiosa) close to the growth plate (white arrowhead), from rat #605 and a control. Bar = 100 μm.

3. Results

3.1. Body weight, femur length and bone geometry

Body weight and bone length are independent factors influencing bone size and strength. The mean body weight and femur length of BPA-exposed offspring did not differ significantly from controls in either sex (Table 1). pQCT analysis of the femoral metaphysis (for trabecular bone) and diaphysis (for cortical/compact bone) did not show any significant differences between BPA-exposed offspring compared to unexposed controls, in either sex (Supplemental S1 table). However, of the total of 58 femurs examined, extreme values were observed in two of the BPA-exposed female offspring (#605 and #606). These animals had reduced endocortical (but not periosteal) circumference (ENDO_C, smaller bone marrow volume), and the reduction was more pronounced after BPA50 exposure than after BPA0.5 exposure (Fig. 1A). The excessive cortical bone growth in female offspring #605 and #606 began to deviate from normal growth after week 42, as assessed by *in vivo* microCT cortical thickness (CORT_THK) measurements of tibia at week 5, 20, 42 and *ex vivo* analysis at week 52 (Fig. 1B). μCT scan of tibia and histological analysis of the humerus from #605 showed excessive, but apparently normal, bone formation in the marrow compartment (Fig. 1C and D).

3.2. Plasma levels of bone remodeling markers and sex hormones

Developmental BPA exposure to BPA50 increased the circulating bone anabolic marker P1NP (+59%, $p < 0.05$) in 52-week-old female offspring compared to control, whereas no differences were seen in 52-week-old male offspring (Fig. 2A). Blood levels of the bone resorption marker CTX-1 were not affected by developmental BPA exposure in either sex (Fig. 2B). Sex-hormone analysis revealed a trend towards increased levels of estradiol (+84%, $p = 0.05$) in the female offspring exposed to BPA50, albeit not statistically significant. The values for testosterone (+150%), androstenedione (+83%) and estrone (+83%) levels were not significantly different altered (Fig. 2C, Supplemental S2 table). Dehydroepiandrosterone (DHEA) concentrations in all samples were below LOQ of the method (50 pg/mL).

3.3. Biomechanical properties: the three-point bending test

Femur stiffness was significantly reduced in female offspring: −12% ($p < 0.05$) in the BPA0.5-dose group and −11% ($p < 0.05$) in the BPA50-dose group (Table 2). No other parameter was significantly changed in BPA-exposed females compared to controls. Bones from male offspring did not differ from controls in the three-point bending test (Table 2). As the three-point bending test results are highly dependent on cortical thickness and as 2 of 14 (14%) BPA-exposed females had extremely thick cortical bone, we also analyzed the biomechanical data after exclusion of these two animals. As expected, stiffness was then slightly more reduced, by 15% ($p < 0.01$) in BPA0.5 and by 14% ($p < 0.01$) in BPA50. After exclusion of females #605 and #606 the results did not change for load, displacement, or work-to-fracture.

3.4. Histology on bone and marrow

Initial examination of hematoxylin and eosin stained decalcified bone-marrow sections from female offspring, developmentally exposed to 0.5 or 50 μg BPA/kg BW/day ([BPA0.5]- and [BPA50]), identified 2 out of the 9 sections (1 section/animal) including 3 controls, 3 BPA0.5 and 3 BPA50 with areas of pathological changes such as blebbing and intracellular vacuoles. Both sections showing blebbing and intracellular vacuoles were from BPA-exposed offspring. This prompted us to conduct a more in depth bone marrow examination in female offspring, which revealed marrow lesions, visible as pale areas, in bones from female offspring developmentally exposed to BPA50 (Fig. 3A). Staining with reticulin showed that these lesions contained collagen type 3 fibers, indicating fibrotic lesions (Fig. 3B). Fibrosis scoring, using the modified Bauermeister scale, showed that the bone marrow lesions in BPA-exposed female offspring had more and thicker reticulin fibers. More importantly, the fibrotic lesions in the BPA-exposed females were also positive for Masson's trichrome stain, indicating presence of collagen type 1 fibers, which is linked to a severe type of fibrosis and disease. Male bone marrow did not show fibrotic lesions (data not shown). Immunostaining of these lesions for osteopontin (expressed in FOLs and osteosarcomas), Fabp4 (expressed in breast cancer) and activated caspase 3 (apoptosis and breast cancer) are shown in Fig. 3C. The lesions appear positive for Fabp4 and caspase 3, whereas osteopontin staining is weak (Fig. 3C).

3.5. Plasma markers of inflammation

Screening for circulating markers of inflammation showed that tumor necrosis factor (Tnf) levels were significantly higher (+41%, $p < 0.01$) in female offspring developmentally exposed to BPA0.5 (Fig. 4A). In addition, TIMP metallopeptidase inhibitor 1 (Timp1) levels were significantly higher (+33%, $p < 0.05$), but only in female offspring developmentally exposed to BPA50 (Fig. 4B). Circulating levels of C-C motif chemokine ligand 2 (Ccl2 or MCP-1), interleukin 10 (IL-10), and chemokine (C-X-C motif) ligand 1 (Cxcl1 or KC/GRO) were not altered by BPA exposure in either sex (data not shown). Although, female offspring developmentally exposed to BPA showed excess bone marrow fibrosis, the plasma transforming growth factor beta 1 (Tgfb1) levels were generally not elevated in these animals (Fig. 4C). However, the one female rat (#605, from the BPA50 group) with the most extremely thick cortical bone, showed a 4-fold higher level of Tgfb1 in the circulation in comparison to all other females (Fig. 4C). This female had a high, but not extreme P1NP plasma level (0.44 ng/ml), normal estradiol plasma level (5.4 pg/mL) and less than average body weight (212 g). Immunostaining of the BPA-induced bone lesions for Tnf and Timp1 show that lesion cells are positive for these inflammation markers.

4. Discussion

In this study, we found sex-specific, late-life pathologies in female F344 rat offspring after developmental exposure to BPA at a dose eight times lower than the current preliminary human TDI of 4 μg/kg BW/day set by EFSA , 2015. A BPA exposure of 0.5 μg/kg BW/day between GD 3.5 and postnatal day 22, via maternal water intake, induced

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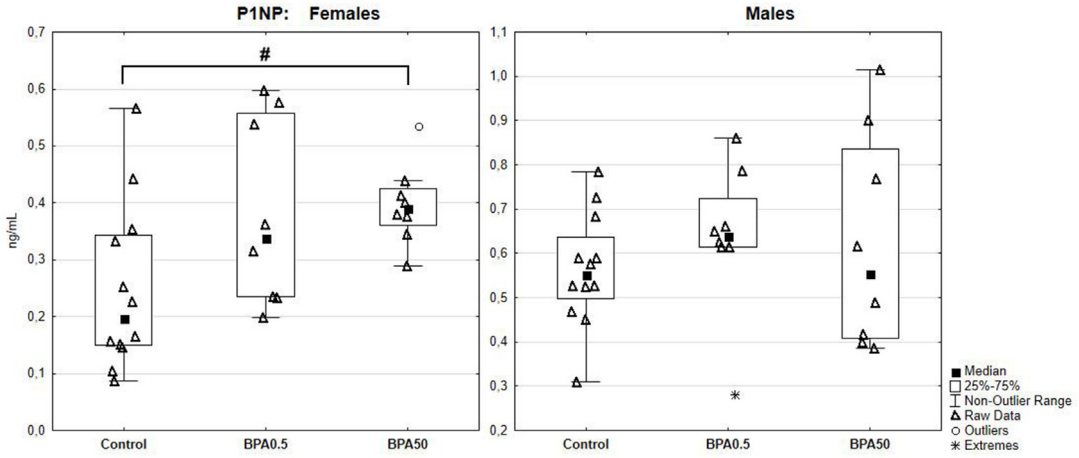

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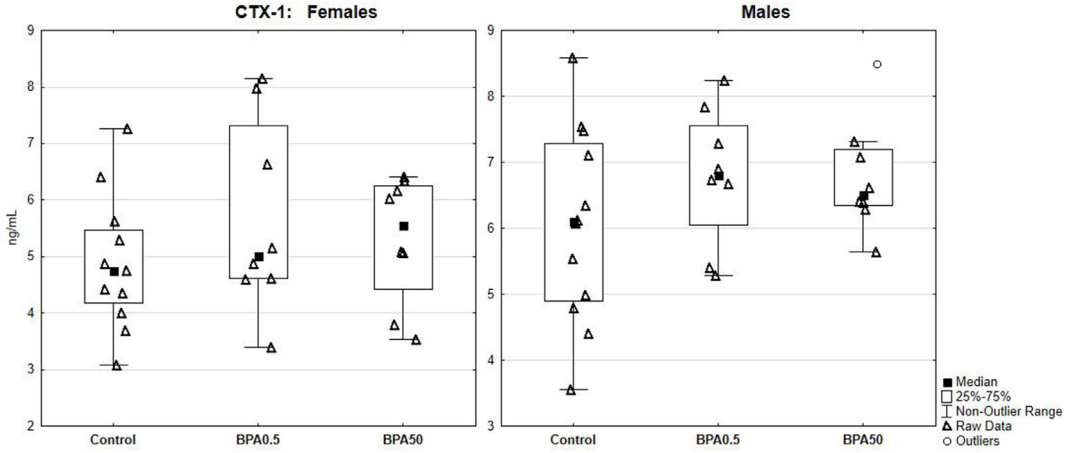

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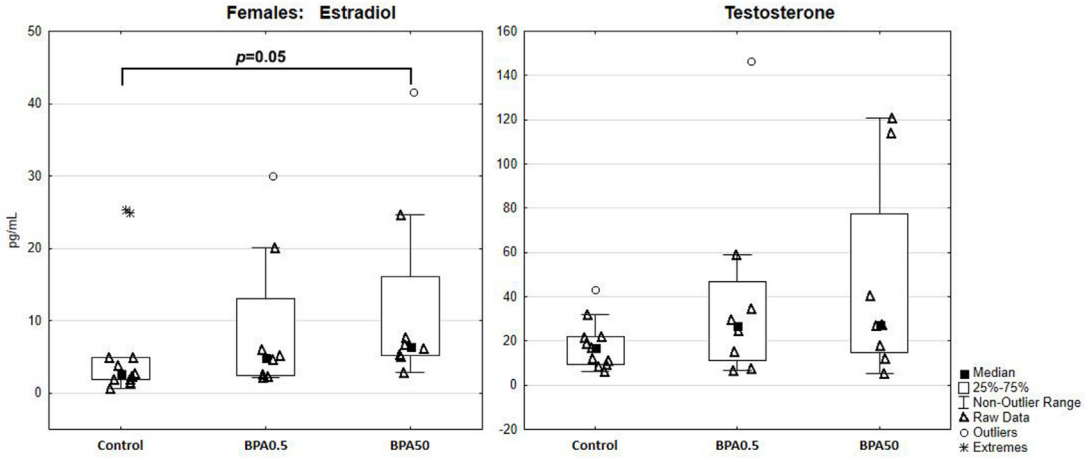

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Fig. 2. Plasma levels of markers for bone turnover and sex hormones in F344 offspring developmentally exposed to 0.5 or 50 μg BPA/kg BW/day. (A) Collagen type 1 synthesis marker: P1NP (procollagen type I N-terminal propeptide), **(B)** collagen type 1 breakdown marker: CTX-1 (C-terminal telopeptides of type I collagen) and **(C)** sex hormone levels in female offspring. Numbers of samples in (A) and (B) were for females and males: Control, n = 12; (BPA0.5) (0.5 μg BPA/kg BW/day), n = 8; (BPA50) (50 μg BPA/kg BW/day) n = 8. Numbers of samples in (C) were for females: Control, n = 11; BPA0.5, n = 8; BPA50 n = 8. Results are given as means +SD or +SEM (estradiol and testosterone). *$p < 0.05$ *vs* Cont.

Table 2
Effects of developmental exposure to bisphenol A (0.5 μg/kg BW/day or 50 μg/kg BW/day) on 3-point bending test of femur in 52-week-old offspring.

	Control (n = 12)	0.5 μg/kg BW/day (n = 8)	% diff.	50 μg/kg BW/day (n = 8)	% diff.	*p*-value
Females						
Load (N)	134 ± 13	129 ± 12	−3.7	131 ± 11	−2.7	*0.64*
Stiffness (N/mm)	**241 ± 19**	**213 ± 33**	**−12**	**214 ± 22**	**−11**	***0.025***[a,b]
Displacement (mm)	0.80 ± 0.11	0.88 ± 0.22	+10	0.88 ± 0.09	+10	*0.34*
Work-to-fracture (N x mm)	64 ± 12	70 ± 24	+9.7	73 ± 30	+13	*0.62*
Males						
Load (N)	217 ± 19	216 ± 14	−0.3	211 ± 21	−2.6	*0.78*
Stiffness (N/mm)	333 ± 33	344 ± 23	+3.4	348 ± 44	+4.5	*0.60*
Displacement (mm)	1.26 ± 0.11	1.29 ± 0.11	+1.8	1.24 ± 0.09	−1.5	*0.73*
Work-to-fracture (N x mm)	182 ± 23	193 ± 23	+5.8	181 ± 37	−1.0	*0.62*

Results are presented as means ± SD, %-diff. is the % change of mean in BPA exposed rat offspring compared to controls. Statistically significant values are highlighted in bold. *p*-values are from ANOVA followed by Dunnett's post-hoc analysis. [a][Control] *vs* [0.5 μg/kg BW/day]: $p = 0.036$, [b][Control] *vs* [50 μg/kg BW/day]: $p = 0.045$.

elevated plasma levels of the proinflammatory marker Tnf and reduced bone stiffness. In line with this, exposure to a higher BPA dose (50 μg/kg BW/day) induced a similar, but more severe, phenotype with not only increased plasma levels of the inflammation marker Timp1 and reduced bone stiffness, but also increased bone marrow fibrotic lesions and persistently increased plasma P1NP levels. No comparable phenotype was observed in male offspring.

It is well established that exposure of the xenoestrogen, DES, during pregnancy affects daughters more severely than sons (Al Jishi and Sergi, 2017). Along this line, our study show that developmental BPA exposure affected female but not male offspring. The mechanism behind this sex-specific effect is still unclear, but it might involve regulation via brain estrogen receptor 1, which has been found to be specifically downregulated in female rats by BPA (and possibly other xenoestrogens) (Cao et al., 2012; Rebuli et al., 2014). This receptor was recently shown to be important in controlling bone growth and body weight exclusively in female mice (Herber et al., 2019) and found to interact with BPA differently from estradiol (Gould et al., 1998). We show here that low-dose developmental BPA exposure in rats increased the circulating levels of inflammation markers only in female offspring. A similar observation was made during intestinal barrier function experiments in Wistar rats, where only female rat offspring, developmentally exposed to low doses of BPA, showed severe inflammation in the gut (Braniste et al., 2010). In humans, a correlation was observed between increased BPA exposure and higher circulating levels of TNF exclusively in women (Yang et al., 2016). It appears that postmenopausal women are more susceptible to BPA-induced inflammation and correlated detrimental health effects than younger women (Yang et al., 2009). The origin of the increased circulating levels of Tnf observed in our study is unknown, although we show that the BPA-induced bone marrow lesions are positive for Tnf and Timp1 protein. Previously, it has been shown that bone-marrow derived mast cells from adult mice produce more Tnf if they have been developmentally exposed to BPA (O'Brien et al., 2014). Chronic inflammation constitutes an important link between obesity and its metabolic consequences (Saltiel and Olefsky, 2017). Along this line, we recently showed that the female offspring in the present study, exposed to the BPA50 dose, had a significantly higher body weight increase when taking into account (adjusting for) food consumption over time (Dunder et al., 2018). Combining it with the fact that the female offspring siblings of the present study, analyzed at the age of 5 weeks, showed no indication of increase in body weight after developmental BPA exposure (Lejonklou et al., 2017) suggests that increasing age contributes to the effects of BPA exposure on body weight gain. As with BPA, developmental exposure to DES is capable of causing adult weight gain in female mice offspring which is not apparent at birth or in infancy (Newbold et al., 2005).

Fibrosis typically occurs as a result of tissue inflammation. In agreement with this, we found fibrous lesions in the bone marrow and increased levels of plasma markers of inflammation in female offspring in the present study. This bone pathology is not unlike the description of FOLs, which is characterized by accelerated osteoblastic turnover with concurrent fibroplasia, that have been found in mice treated with high doses of DES (McAnulty and Skydsgaard, 2005). Notably, FOLs have mainly been described to occur in female mice (Sass and Montali, 1980). Further studies are needed to determine if the fibrotic lesions observed in the present study, caused by developmental BPA exposure, will develop into osteosarcomas. The PBA-induced fibrotic lesions in the present study showed only weak osteopontin staining, which has been used to characterize FOLs and osteosarcoma (Sakamoto et al., 1999; Dalla-Torre et al., 2006). However, two cases of female offspring with extremely thick cortical bone were observed after developmental exposure to BPA. Both these cases appeared to have normal cortical thickness up to 42 weeks of age when systematically analyzed with *in vivo* microCT scanning during the study, suggesting that a late event started the extreme cortical bone growth after the age of 42 weeks. Notably, the BPA-exposed female offspring with the most extreme cortical thickness had a 4-fold higher Tgfb1 plasma levels but normal plasma levels of estradiol, which may implicate Tgfb1 rather than estradiol as a driver of this excess bone growth.

Circulating P1NP levels in the BPA50-exposed female group was increased by 59%, compared to a smaller non-significant increase of 13% in the corresponding male group. P1NP is a peptide formed during type 1 collagen synthesis and is mainly used as a bone anabolic marker but can also be used as a marker for fibrosis and overall survival in prostate cancer patients with bone metastasis (de la Piedra et al., 2003; Veidal et al., 2010; Jung et al., 2011; Dean-Colomb et al., 2013; Farmer et al., 2015; Luger et al., 2016). Timp1, an inhibitor of collagen breakdown and a marker for inflammation, was also increased in plasma in female offspring exposed to the BPA50 dose. Interestingly, and in line with the higher body weight increase in aged BPA50 female offspring, Timp1 has been found to be associated with high-fat diet-induced glucose intolerance and hepatic steatosis in mice (Fjaere et al., 2015) and with obesity and metabolic disorders in humans (Cardoso et al., 2018). Put together, the fact that BPA-exposed female offspring

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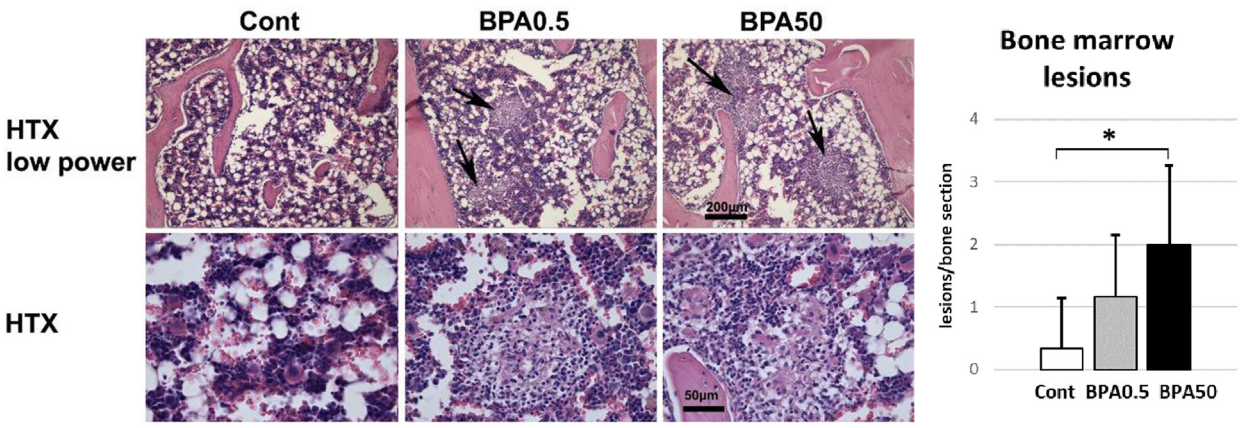

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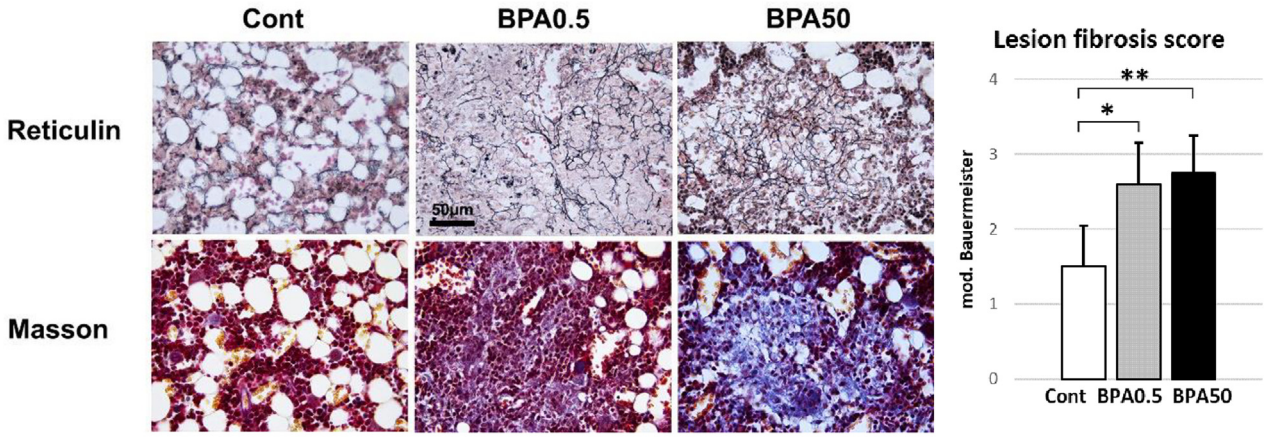

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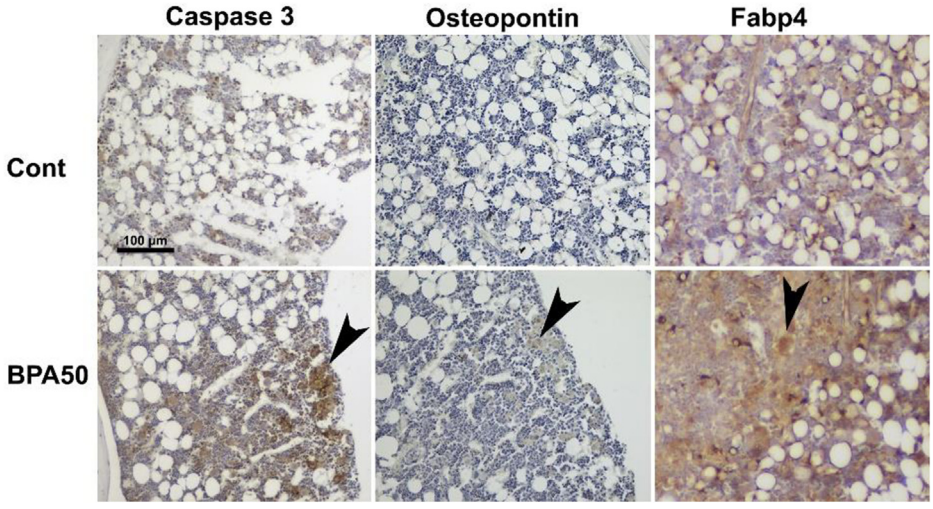

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Fig. 3. Histological analysis of bone marrow in female F344 offspring developmentally exposed to 0.5 or 50 μg BPA/kg BW/day. (A) Representative images of decalcified bone sections from 52-week-old female offspring developmentally exposed to BPA. Arrows on hematoxylin and eosin stained (HTX) sections indicate pale areas (lesions) found more frequently in BPA-exposed female bone marrow (HTX low power) and (HTX). Right panel shows result from manual counting of these lesions, as described in the Methods section. Number of animals for each group was n = 6 (2 sections/animal). **(B)** Representative images of reticulin and Masson's trichrome staining of the pale lesions. Right panel shows reticulin fibrosis scoring of bone marrow in controls (Cont) versus data from both female groups exposed to BPA. Bar = 50 μm. Number of animals for each group were n = 4–6 (2 sections/animal). Results are given as means +SD. $^{*}p < 0.05$ *vs* Cont and $^{**}p < 0.01$ *vs* Cont. **(C)** Representative images of bone marrow immunostainings (brown) for caspase 3, osteopontin and Fabp4. Arrowheads indicate stained cells in BPA-induced lesions. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

show areas of collagen type 1 fibrosis (positive for Masson trichrome) in bone marrow but unchanged bone growth suggests that the elevated plasma P1NP levels in these rats are signs of fibrosis. Thus, the most probable explanation for the elevated circulating P1NP levels in 5-week-old female offspring developmentally exposed to BPA is early signs of fibrosis (Lind et al., 2017).

We observed a trend towards increased levels of estradiol in female offspring developmentally exposed to BPA50 (although not statistically significant, $p = 0.05$), while other hormones where unaffected. Some previous studies show increased estradiol levels after developmental BPA exposure (Xi et al., 2011; Gámez et al., 2015; Lite et al., 2019) but others not (Patel et al., 2017), the reason for this is not known but it might depend on the experimental design, such as rat strain and method of exposure (via drinking water or forced feeding). In the skeleton, estradiol is a key hormone for maintaining bone mass, and estradiol deficiency is known to be associated with age-related bone-mineralization defects caused by high bone turnover and reduced bone stiffness (Riggs et al., 2002; Wehrli et al., 2010). However, in spite of the small increase in estradiol levels observed in the present study, developmental BPA exposure was associated with increased concentrations of a bone anabolic marker (P1NP) and reduced bone stiffness. It appears that developmental exposure to BPA may have a long-lasting antagonizing effect of estradiol in bone. BPA has been shown to have an antagonizing effect on estradiol stimulatory effects on peroxidase activity and progesterone levels but not uterine weight in female Sprague-Dawley rats (Gould et al., 1998). In addition to a positive effect of estradiol on bone mineralization, increased levels of estradiol are also associated with greater risk of developing breast cancer (Breast Cancer, 1997). Elevated concentrations of estradiol in rats exposed to BPA may explain earlier reports of developmental BPA exposure as a risk factor for breast cancer in rats (Durando et al., 2007; Acevedo et al., 2013). Along this line, we observed high expression of activated caspase 3 and Fabp4, two proteins associated with breast cancer (Guaita-Esteruelas et al., 2017; Blázquez et al., 2006), in the BPA-induced bone marrow lesions. Bone metastasis is a common complication of breast cancer. Whether fibrotic lesions in the bone marrow correlate with breast cancer or other non-hematologic tumors is not yet known (Xiao et al., 2009) and should be subject to future studies.

The present study further showed that low-dose developmental BPA exposure had a distinct effect on bone stiffness, which was the only bone quality parameter consistently altered, and it was only seen in aged female offspring. The reason behind this effect on bone quality is unknown. However, as bone geometry and density were not changed, it is tempting to speculate that the extracellular bone matrix composition has been affected. In our previous study of 12-week-old Wistar rats, femur stiffness in BPA-exposed female offspring seemed the most clearly affected parameter in biomechanical testing (−11%), albeit not statistically significant ($p = 0.07$), while apparently less affected in male offspring (−3.4%, $p = 0.30$) in the 250 μg/kg BW/day dose group (Lejonklou et al., 2016). We have also shown that the siblings of the present study, analyzed at 5 weeks of age showed no statistically significant changes of femur stiffness in female (+7,3%, $p = 0.67$) or male (−0.8%, $p = 0.34$) offspring exposed to BPA50 (Lind et al., 2017). In contrast, a recent study in mice showed reduced bone stiffness only in male offspring, developmentally exposed to a low BPA dose (Xin et al., 2018). The main differences from our study was that their exposure started at GD 0 and that they used same-sex siblings (5 males from 3 litters and 4 females from one litter) for the determination of the effect of BPA exposure. Together, these studies indicate that developmental exposure to BPA appears also to affect bone stiffness in an age- and sex-specific way, as with body weight. Along these lines, sex-specific effects of BPA-exposure have been reported previously not only on body weight (Rubin et al., 2017; Dunder et al., 2018), but also on food intake (Mackay et al., 2013), liver weight (van Esterik et al., 2014), adipocyte mass and number (Somm et al., 2009; Mackay et al., 2013; Lejonklou et al., 2017). Additionally, blood levels of key factors in metabolic control, such as of leptin, insulin (Bansal et al., 2017) and adiponectin (van Esterik et al., 2014), as well as glucose tolerance (Mackay et al., 2013), have been observed to be sex-specifically altered after BPA-exposure. Interestingly, behavioral alterations, including increased activity or hyperactivity following BPA-exposure, have mainly been reported in female rodents (Rubin et al., 2017, Anderson et al., 2013).

The main strength of the present study was the careful design aimed at exploring the effects of developmental exposure to low doses of BPA. We used an inbred rat strain (F344) as it is more stable, uniform, and better defined than outbred strains; which allowed us to use fewer animals. Furthermore, compared to the Sprague-Dawley rat, F344 excretes a larger portion of circulating BPA via the kidneys, similar to human excretion (Snyder et al., 2000). To reduce animal stress, we used a natural (non-forced) administration protocol, i.e. via drinking water, as the oral route of exposure is highly relevant for humans (Vandenberg et al., 2007). Exposure started at GD 3.5 to cover for effects as early as possible. Moreover, we used glass water bottles and the animal cages were made of polysulfone to reduce the risk that BPA would leach from the plastic. The diet was analyzed with regard to phytoestrogen content and found to be low according to the Organization for Economic Co-operation and Development (OECD) standards (Owens et al., 2003). The main limitation was the limited number of animals, particularly in the groups of BPA-exposed offspring. The reason for this is unclear, although there were indications that dams exposed to the higher dose (BPA50) appeared more likely to have fewer pups or to be without litter (Lejonklou et al., 2017). Another limitation was, due to unfortunate technical reasons, lack of information on internal BPA doses in the animals. All in all, the findings of the present study, despite the small size, provide additional evidence of low-dose, sex-specific and later in life detrimental effects of developmental BPA exposure.

In conclusion, we found that developmental exposure to BPA (0.5 and 50 μg/kg BW/day) induces signs of fibrosis and chronic inflammation in adult female but not male rat offspring, even at a dose that is considerably lower than the current preliminary human TDI of 4 μg/kg BW/day set by EFSA. Together with the findings of reduced bone stiffness despite elevated P1NP levels in 52-week-old females, this supports our hypothesis that the P1NP increase described previously at 5 weeks (Lind et al., 2017), is an early sign of fibrosis in offspring developmentally exposed to BPA. Our findings further strengthens the evidence for sex-specific effects of BPA and suggest that bone tissue might be extra sensitive to low doses of BPA during development.

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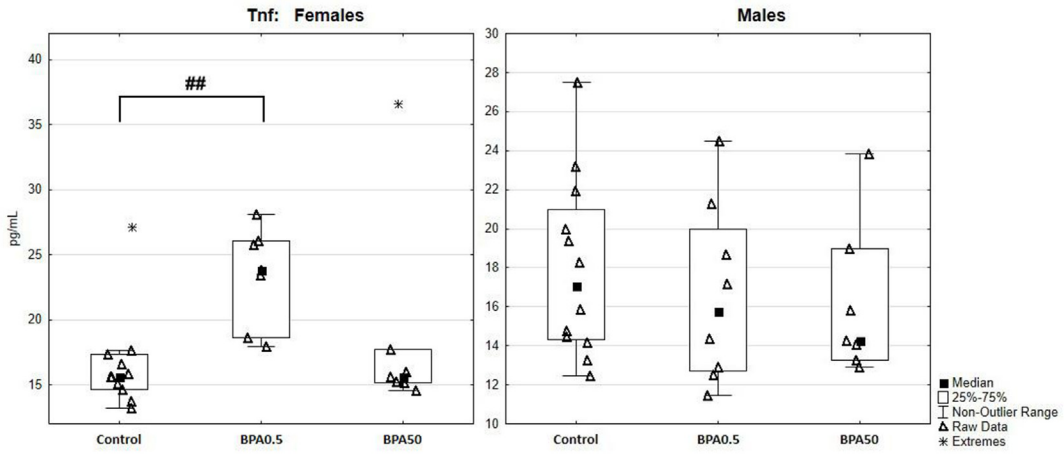

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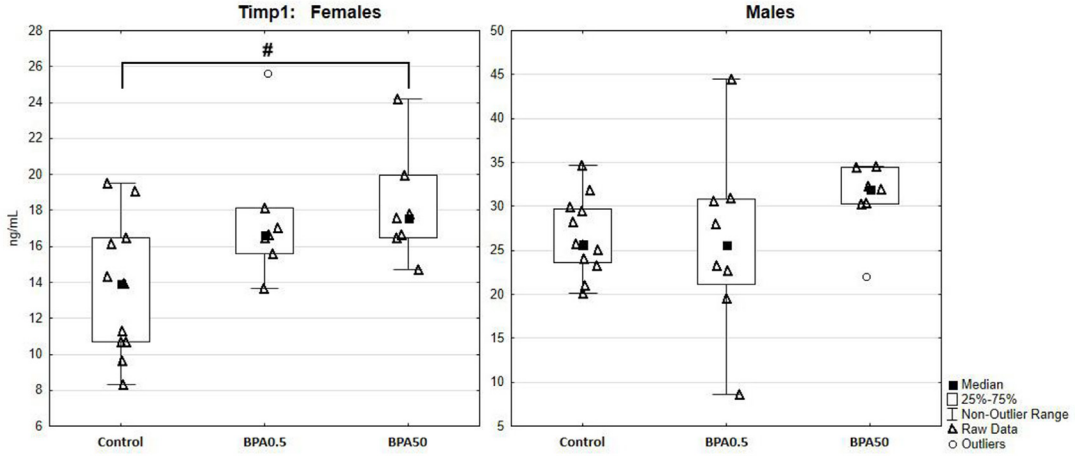

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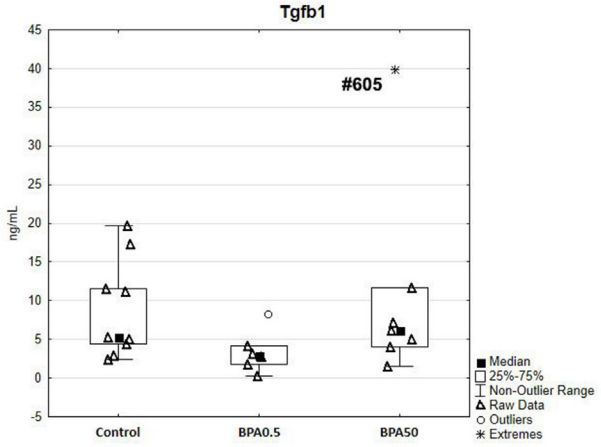

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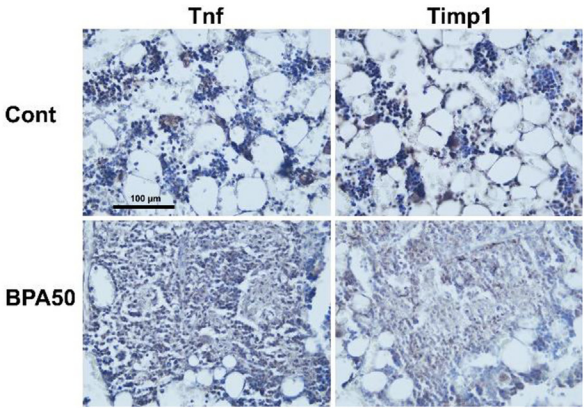

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Fig. 4. Plasma levels of inflammation markers in F344 offspring developmentally exposed to 0.5 or 50 μg BPA/kg BW/day. (A) Tumor necrosis factor (Tnf), **(B)** TIMP metallopeptidase inhibitor 1 (Timp1) and **(C)** Transforming growth factor beta 1 (Tgfb1). Dot in panel C represents extreme value for rat #605. Numbers of samples were for females and males: Cont, n = 12; (BPA0.5) (0.5 μg BPA/kg BW/day), n = 8; (BPA50) (50 μg BPA/kg BW/day) n = 8. Results are given as means +SD. *$p < 0.05$ *vs* Cont and **$p < 0.01$ *vs* Cont. **(D)** Representative images of immunostainings (brown) for Tnf and Timp1 of BPA-induced bone marrow lesions. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Conflicts of interest

The authors declare no competing financial interests.

Animal study approval

The present study (C26/13) was approved by the Uppsala Ethical Committee on Animal Research, following the Swedish Legislation on Animal Experimentation (Animal Welfare Act SFS1998:56) and the European Union Legislation (Convention ETS123 and Directive 2010/63/EU).

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envres.2019.108584.

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