The effects of prenatal alcohol exposure on the skeleton is sexually dimorphic in skeletally mature mice

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INTRODUCTION:
Prenatal alcohol exposure (PAE) can result in lifelong disabilities known as fetal alcohol spectrum disorder (FASD). FASD is characterised by a variety of cognitive and physical impairments, notably its effects on brain development and facial dysmorphology. In the skeletal system, PAE is associated with an increased risk of long bone fractures in children and FASD adolescents are typically shorter in stature; however, sexual dimorphisms in these cohorts have not been investigated[1,2]. Whilst the role of PAE on fetal growth has been examined in murine models, its effect on skeletally mature mice is unknown. Therefore, the purpose of this study was to fully characterise the skeletal phenotype, including mechanical testing, of both an adult and juvenile murine model of PAE, in male and female mice.

METHODS:
Pregnant C57Bl/6J females received 5% ethanol in their drinking water during gestation. Male and female offspring from control and alcohol-exposed dams were sacrificed at 4- and 12-weeks of age. Fluid intake between control (water-only) and alcohol-exposed females was unchanged. Skeletal phenotyping was conducted at the tibia by volumetric and 2D microcomputed tomography (microCT) scans at 5µm, growth plating bridging, porosity, and histological analysis. Mechanical testing was performed by femoral 3-point bending tests. Primary calvarial osteoblasts were isolated from 3–5-day old offspring from control and alcohol-exposed dams and cultured for ≤21 days in Alpha Minimum Essential Medium supplemented with 10% FBS, 100 U/ml penicillin, 100µg/ml streptomycin, 2mM β-glycerophosphate and 50µg/ml L-ascorbic acid. The effect of PAE on mineralised bone nodule formation was investigated and gene expression after 7 days of culture was assessed using a PCR array. All animals utilised in these studies were kept in controlled conditions at the University of Brighton and conducted in line with the UK Animals (Scientific Procedures) Act 1986 set by the UK Home Office with local ethical approval. Statistical analysis was performed using a two-way ANOVA with post-hoc comparisons in Graphpad Prism or R.

RESULTS:
Despite no changes in total bone length, histological analysis revealed a decrease in total growth plate width in PAE male mice compared to controls at 12-weeks of age ($p<0.05$; n=4/group), whilst growth plate bridging showed a trend towards an increase in the number of bridges, suggestive of accelerated growth plate fusion. PAE female mice however were unaffected (n=4/group). MicroCT analysis of 12-week-old male mice (n=8 control vs n=11 PAE) revealed that PAE reduced trabecular bone parameters including tissue volume, bone volume, and intersection surface ($p\leq0.001$). The structure of the trabeculae was also altered in PAE male mice (trabecular number, trabecular pattern factor, and fractal dimension; $p\leq0.05$), indicating a more disconnected and less complex architecture. However, female mice did not display these negative effects in the trabecular compartment (n= 9 control vs n=5 PAE). Conversely, microCT analysis showed that both male and female mice had significant reductions in several cortical parameters (cortical bone area, tissue area, tissue perimeter; $p\leq0.05$). Whilst male mice were negatively affected along the entire length of the tibia, female mice were only affected at the distal end. Similarly, PAE altered parameters associated with cortical bone shape (predicted resistance to torsion [*J*], maximum [I_{max}] and minimum [I_{min}] moments of inertia; $p\leq0.05$) along the length of the male tibia, but only at the distal end of the female tibia. Interestingly, PAE female mice had higher % total cortical porosity within the posterior region of the tibiofibular junction ($p=0.014$) compared to control, whilst male mice were unaffected. To assess bone strength, mechanical testing revealed that in 12-week-old mice, work to failure ($p=0.049$) and stiffness ($p=0.0162$) were elevated in female PAE mice versus control; load at maximum stiffness was reduced in both PAE males and females ($p=0.0109$ and $p=0.0135$, respectively). To assess whether the dimorphic effects of PAE on the skeleton are observed throughout skeletal development, we next examined juvenile mice. However, in 4-week-old mice, the altered trabecular and cortical parameters observed by microCT at 12-weeks of age were not present in either males (n=5 control vs n= 9 PAE) or females (n=10 control vs n=7 PAE). In the 4-week-old mice, fracture load ($p=0.006$) and load at maximum stiffness ($p=0.021$) were reduced in PAE females. Stiffness was increased in the PAE females ($p=0.0346$), whilst the males were unaffected. Given the observed differences in bone micro- and macro-architecture, we next examined primary osteoblast function *in vitro*. PAE osteoblasts from male and female mice displayed reduced bone nodule formation compared to control (≤30%; $p<0.001$). A PCR microarray showed that compared to control, PAE differentially affects gene expression in both males and females, including upregulation of genes for *Bglap* (Osteocalcin) and *Ahsg* (Fetuin A). Whilst *Vegfa* was also downregulated in both sexes, *Bmp6*, *Tgfbr1*, and *Flt1* (VEGFR1) were downregulated in male mice only.

DISCUSSION:
Evidence herein suggests that PAE has detrimental and sexually dimorphic effects on the skeleton of mature male and female mice. In 12-week-old animals, differences are observed in spatially distinct regions; male mice are negatively affected from the growth plate and adjacent trabecular bone region, along almost the entire length of the tibia, whilst female mice are predominantly affected only at the distal end. Despite alterations in femoral mechanical properties, differences in tibial architecture and shape are not observed in 4-week-old mice, suggesting that the effects of PAE on the skeleton may occur over time in response to mechanoadaptation. Inherent differences in osteoblast function from PAE mice, indicated by changes to nodule formation and gene expression, could account for these differences, however, work to determine the mechanistic basis for these observations is ongoing.

SIGNIFICANCE/CLINICAL RELEVANCE:
Data here demonstrate that PAE has negative effects on the mature skeleton in male and female mice, however, this is in spatially different regions of the bone. This adds further evidence to indicate that the *in utero* environment has lasting impacts on skeletal health and whilst sex-specific changes to bone composition have not yet been reported in the human FASD population, data here suggests the need for further investigation into this potential sexual dimorphism.

REFERENCES:
[1]Parviainen R, Auvinen J, Serlo W, Järvelin MR, Sinikumpu JJ. Maternal alcohol consumption during pregnancy associates with bone fractures in early childhood. A birth-cohort study of 6718 participants. Bone. 2020 Aug;137:115462.
[2]Young SL, Gallo LA, Brookes DSK, Hayes N, Maloney M, Liddle K, James A, Moritz KM, Reid N. Altered bone and body composition in children and adolescents with confirmed prenatal alcohol exposure. Bone. 2022 Nov;164:116510.