Early Life Stress Does Not Affect Bone Mass In Male Mice But Induces An Osteopenic Phenotype In Female Mice

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INTRODUCTION: Early life stress caused for example by child maltreatment or social neglect is a major risk factor for the development of mental diseases including depression (Gershon et al., 2013), with women being two to three times more affected than men (Vafaei et al., 2016). The underlying mechanisms are not yet fully understood, but evidence from both clinical and preclinical studies support the hypothesis that hyper-responsiveness of the hypothalamus-pituitary-adrenal (HPA) axis as well as activation of innate immunity resulting in chronic low-grade inflammation play an important role. In detail, child maltreatment is associated with increased levels of pro-inflammatory cytokines and white blood cells (Surtees, Wainwright et al. 2003, Danese, Pariante et al. 2007, Baumeister, Akhtar et al. 2016). Three other studies found exaggerated Interleukin 6 (IL-6) responses to *ex vivo* stimulation of toll-like receptors in adolescents raised in harsh family environments (Miller and Chen 2010), in adults raised in low socioeconomic status (Miller, Chen et al. 2009) and in adolescent girls with early life adversity (Ehrlich, Ross et al. 2016, Elwenspoek, Kuehn et al. 2017). In line with this data, depression is highly comorbid with inflammatory somatic diseases, but also with disorders characterized by dysregulation of bone metabolism including osteoporosis (Gebara et al., 2014). In support, we recently demonstrated that chronic psychosocial stress in adolescent mice led to a disturbed endochondral ossification in the growth plate of long bones, negatively affecting bone growth (Foertsch et al., 2017). Less is known about the influence of early life stress on bone metabolism. Therefore, the aim of this study was to investigate how maternal separation, as a model for early life stress in rodents (Veenema et al. 2008), influences bone growth and homeostasis in male and female mice.

METHODS: Animal experiments were approved by the local ethical committee (Regierungspräsidium Tübingen, Germany). Female and male C57BL/6 N mice were subjected to maternal separation (MS) for 3h per day on postnatal days (PNDs) 1-14. Weaning took place on PND 21. 5-6 weeks later, mice were subjected to the open field/novel object test (OF/NO) and social preference/avoidance test (SPAT) for assessment of general and social anxiety-related behavior, respectively, and euthanized one day later. Control animals did not undergo maternal separation, but were sham-handled respectively (no MS animals). Plasma was collected for multiplex cytokine analysis and ELISA for the stress hormone corticosterone. Femurs were collected for µCT analysis and decalcified afterwards before embedded in paraffin. Osteoclasts were counted in tartrate resistant alkaline phosphatase-stained sections. Osteoblasts were counted in sections stained for osteocalcin. Furthermore, immunohistochemical staining for IL-6 and receptor activator of Nf-kB signaling ligand (RANKL) was performed on femur sections. n=10-12 per group. Statistical differences between MS and no MS animals of the same sex were tested by Studen't t-test. p-values of less than 0.05 were considered as statistically significant differences. Data are displayed as single data points with mean and standard deviation.

RESULTS: MS did not affect anxiety-related behavior in both males and females, but caused a hyperactive phenotype in males. In male mice, MS did not have any significant effect on bone parameters. Femoral length, as well as trabecular bone volume to tissue volume ratio, trabecular and cortical mineralization, trabecular and cortical thickness and trabecular number and separation did not differ between MS and no MS males. Furthermore, there were no differences in osteoclast and osteoblast number and activity. In female mice, MS animals displayed significantly reduced femur length (Fig. 1A), trabecular number (Fig. 1B), trabecular thickness (Fig. 1C) and mineralization (Fig. 1D). Cortical parameters did not differ between the groups. On the cellular level, female MS mice displayed significantly increased numbers and activity of osteoclasts in the trabecular metaphyseal area of the femur (NOc/BPm: no MS 8.74 ± 1.68 vs. MS 10.59 ± 1.41 1/mm; OcS/BS: no MS 14.83 ± 2.82 vs. 19.56 ± 2.47 %), whereas osteoblast parameters did not differ. Systemically, no differences were found in plasma cytokine and corticosterone levels in male mice. In female mice, most parameters including plasma corticosterone also did not differ between MS and no MS mice. However, female MS mice displayed significantly increased plasma levels of IL-6, as well as increased IL-6 and RANKL expression locally in the bone.

DISCUSSION: We reported here the effects of early life stress induced by maternal separation on bone metabolism in young adult mice. We found that male mice did not display alterations in bone parameters, nor in inflammatory cytokine levels. In contrast, female mice displayed shorter long bones and an osteopenic bone phenotype 6-7 weeks after maternal separation. This was most likely due to an increased number and activity of osteoclasts in the femoral trabecular bone. On the molecular level, female mice subjected to early life stress showed increased systemic and local expression of the pro-inflammatory cytokine IL-6. Although IL-6 is known to directly suppress osteoclast formation *in vitro* (Wang et al., 2013), IL-6 -stimulated RANKL expression by osteoblasts is known to indirectly support osteoclast formation (McGregor et al., 2019). In conclusion, we hypothesize that increased IL-6 signaling induced by maternal separation in female mice leads to increased RANKL expression by osteoblasts and, therefore, supports osteoclast differentiation and subsequent bone loss. The sex-related differences found in the response to early life stress might be explained by estrogen and androgen-mediated effects on immune system activation under stress conditions which have been shown previously (Mackey et al. 2020).

SIGNIFICANCE: Child maltreatment and social neglect have a high prevalence of over 10% in the general population in western countries (Plener et al., 2017) and are well known risk factors for many psychosomatic disorders. Our results extend this knowledge by showing that early life stress in mice, probably via psychoneuroimmunological pathways, in a sex-dependent manner, compromises bone metabolism resulting in an osteopenic phenotype specifically in females. Investigating underlying molecular mechanisms might help to develop future treatment strategies to counteract the negative and sex-dependent effects of early life stress on bone health.

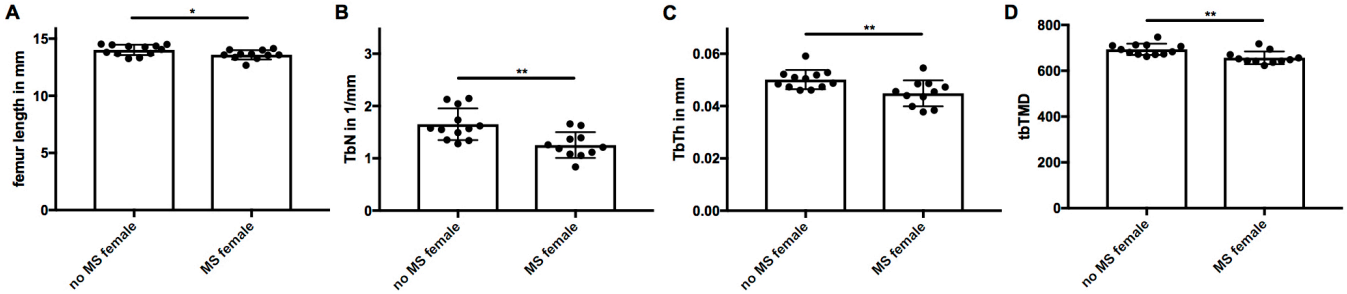

Fig. 1: µCT analysis of bone parameters in female mice subjected to maternal separation (MS) or sham-handling (no MS). A) Femoral length. B) Trabecular number, C) trabecular thickness and D) trabecular mineralization in the metaphyseal part of the femur. n=10-12. *0.05>p≥0.01; **0.01>p>0.001.