Role of adrenergic signaling in fracture healing of non-osteoporotic and osteoporotic bone

Sandra Dieterich[1], Nico Gläser[1], Christoph Kölbl[1], Dorothea Gebauer[1], Miriam E.A. Tschaffon-Müller[1], Oliver Küppers[1], Jana Bleher[1], Verena Fischer[1], Anita Ignatius[1], Melanie Haffner-Luntzer[1]

[1] Institute of Orthopedic Research and Biomechanics, University Medical Center Ulm, Germany
sandra.dieterich@uni-ulm.de

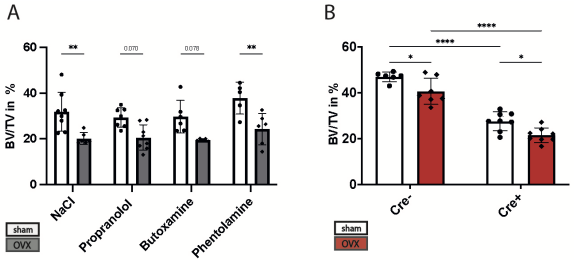
Disclosures: Sandra Dieterich, Nico Gläser, Christoph Kölbl, Dorothea Gebauer, Oliver Küppers, Jana Bleher, Anita Ignatius: None. Miriam E.A. Tschaffon-Müller: Member of ORS ISFR Communications Committee. Melanie Haffner-Luntzer and Verena Fischer: Board of Directors of the German Society of osteological and rheumatological sciences.

INTRODUCTION: Osteoporosis is the most common metabolic bone disease manifested by low bone mass and increased fracture risk. Fracture healing is significantly delayed in osteoporotic patients due to a more challenging fracture fixation in the weak osteoporotic bone and an intrinsic reduction of the healing capacity (Cornell et al., 2003; Nikolaou et al., 2009). Therefore, there is need for new therapeutic strategies to improve osteoporotic bone healing (Burge, 2007). Suggested pathomechanisms of osteoporotic bone healing are for example the estrogen-dependent imbalance of bone formation and resorption (Avioli, 1976), and an overshooting immune response, evidenced by increased recruitment of neutrophils in ovariectomized (OVX), osteoporotic mice after fracture (Haffner-Luntzer et al., 2017). In recent years, it also became evident that the innervation of the bone with sensory and sympathetic nerve fibers plays a crucial role in fracture healing (Niedermair et al., 2020; Shi et al., 2021). In addition, local adrenergic signaling may play a role in the pathomechanisms of osteoporosis (Ma et al., 2013). Male mice treated with the beta adrenoceptor blocker Propranolol immediately before fracture displayed significantly lower numbers of neutrophils in the fracture callus (Haffner-Luntzer et al., 2017), indicating that Propranolol might be useful to block neutrophil recruitment to the fracture site. Therefore, we hypothesized that a short-term treatment of mice with different adrenoceptor blockers during fracture healing modulates the overshooting immune response associated with delayed fracture healing in OVX mice. We also investigated the effects of a genetic deletion of the adrenoceptor beta 2 (*Adrb2*) on neutrophils during fracture healing both in non-osteoporotic and osteoporotic bone.

METHODS: All animal experiments were approved by the local ethical animal welfare committee (Regierungspräsidium Tübingen, Germany) and were performed in compliance with international regulations for laboratory animal welfare and handling. We performed three animal experiments: 1. RNASeq analysis of fracture calli from sham and OVX wildtype mice to determine potential pathomechanisms of delayed healing. 2. Short term blockade of different adrenoceptors during the inflammatory phase of fracture healing in sham and OVX wildtype mice. 3. Long-term effects of a neutrophil-specific deletion of the *Adrb2* on fracture healing in sham and OVX mice. All experiments were conducted in female mice due to postmenopausal osteoporosis; a disease associated in women. First, RNA Sequencing analysis of fracture callus tissue from sham and OVX mice 14 days after fracture was performed to elucidate potential target genes via KEGG pathway analysis as described previously (Haffner-Luntzer et al., 2023). Furthermore, female C57BL/6J and mice with a specific deletion of the *Adrb2* on neutrophils (Adrb2-flox Ly6G-Cre) received either a sham-surgery or bilateral OVX at the age of 12 weeks to induce osteoporosis. After four weeks, a standardized osteotomy of the femur stabilized with an external fixator was performed. C57BL/6J mice received either an adrenoceptor blocker (Propranolol = unspecific β-blocker; Butoxamine = specific β_2-blocker; Phentolamine = unspecific α-blocker) or sodium chloride injection as control subcutaneously on the day of surgery and once a day for three days after the surgery. C57BL/6J mice were analyzed after 1-, 3- and 21-days. Adrb2-Ly6G KO mice were investigated 21 days post-fracture surgery. At days 1 and 3, flow cytometry was performed to analyze the different immune cell populations in the fracture hematoma upon adrenoceptor blocker treatment. The evaluation was done with the AI-based unbiased clustering software Cytolution. On day 21 post fracture, the healing outcome was examined using a three-point-bending test, µCT analysis (voxel resolution of 8 µm, 50 kV, 200 mA) and histology. Statistical analyses were performed by Two-Way-ANOVA with Šidák's post-hoc test. The significance level was set at $p<0.05$. Group size was 3-8.

RESULTS: RNA sequencing of fracture calli of sham and OVX mice revealed a significant regulation of the KEGG term "adrenergic signaling" in OVX mice, with a 0.8976-log2fold and 1.2145-log2fold overexpression of the genes *Adrb2* and *Adra1d*, indicating locally enhanced adrenergic signaling in osteoporotic bone. C57BL/6J mice + blocker treatment: Flow cytometry performed 1- and 3-days post fracture revealed distinct effects of the blockers on immune cell populations during fracture healing. Significant differences were observed between sham and OVX mice after Phentolamine treatment. Here, a significantly higher proportion of CD19+, CD3+ and CD3+/CD8+ cells was observed in sham mice upon Phentolamine treatment on day 1. Neutrophil numbers were significantly increased in sham mice upon Propranolol treatment after 1 day ($p = 0.002$). On day 21 after fracture, µCT analysis revealed that OVX resulted in impaired fracture healing in all treatment groups as indicated by reduced bone volume/tissue volume (BV/TV) (Fig.1, A) and reduced bone volume (BV) in the fracture callus (BV fracture callus sham vs. OVX: NaCl = 2.86 ± 0.48 vs. 1.71 ± 0.65; Propranolol = 2.55 ± 0.37 vs. 1.72 ± 0.24; Butoxamine = 2.77 ± 0.19 vs. 1.72 ± 0.23; Phentolamine = 2.87 ± 0.27 vs. 1.57 ± 0.30). Histological analysis confirmed these results. Adrb2-Ly6G-KO mice: µCT analysis indicated impaired fracture healing in OVX mice compared to the respective sham group (Fig. 1, B). This effect was independent of *Adrb2* deletion. Furthermore, both sham and OVX knockout mice displayed significantly delayed healing compared to their respective controls (BV fracture callus sham vs. OVX: Cre- = 3.89 ± 0.56 vs. 2.56 ± 0.53; Cre+ = 2.29 ± 0.29 vs. 1.56 ± 0.21).

DISCUSSION: Although our initial data from the RNASeq screening indicated increased local adrenergic signaling during fracture healing in OVX mice, we showed that short-term administration of Propranolol, Butoxamine and Phentolamine in the early inflammatory phase did not improve fracture healing in OVX mice. In contrast to previous results in male mice, Propranolol treatment could not reduce neutrophil recruitment to the fracture hematoma but rather increased the number of these inflammatory cells in female mice. This indicates a sex-specific effect of adrenoceptor blockers during fracture healing in mice and might be an explanation why blocker treatment was not effective to enhance fracture healing in the current experimental setting. Furthermore, a specific deletion of the *Adrb2* on neutrophils did not improve fracture healing but rather showed negative effects both in non-osteoporotic and osteoporotic mice. In conclusion, in contrast to our initial hypothesis, adrenergic signaling specifically in immune cells of the early inflammatory phase seems to be critical for proper fracture healing in female mice no matter of their estrogen status.



SIGNIFICANCE: Beta blocker drugs such as Propranolol are widely used clinically for the treatment of cardiovascular diseases. It is therefore of high clinical relevance for the patient to investigate potential effects of these drugs on fracture healing. Our study showed that specifically *Adrb2* signaling in neutrophils might be critical for proper fracture healing and short-term blocker treatment does not have a beneficial effect.

Fig. 1. BV/TV (%) at day 21 after fracture from C57BL/6J mice (A) and Adrb2-Ly6G-KO mice (B). A: Significant reduction of the BV/TV for control and Phentolamine-treated OVX mice. Only tendency for reduction in Propranolol- and Butoxamine-treated OVX mice compared to sham mice. **B:** Significant reduction of the BV/TV between sham and OVX mice for Cre- and Cre+ mice. Cre+ mice with a significant BV/TV reduction compared to Cre- mice of the respective group. (N=5-8) (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$)