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The effect of oral dabigatran etexilate on bone density, strength, and microstructure in healthy mice

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ABSTRACT

Thrombin is a key component in the coagulation cascade where it converts factor V, VIII, XI, and fibrinogen. In addition to the abundant production of thrombin in the liver, osteoclasts synthesize and secrete thrombin as well. Osteoblasts express thrombin receptors, and it has been reported that thrombin stimulates the expression of RANKL relatively to OPG, resulting in greater osteoclast activation and bone degradation. Pradaxa (dabigatran etexilate, DE) is a new anticoagulant, which has recently been approved for clinical use. DE is a direct thrombin inhibitor with potential to modulate the RANKL/OPG ratio and thereby limit osteoclast activation and bone degradation. The purpose of the study was to investigate whether DE can increase bone density, bone strength, and bone microstructure in healthy male and female mice and to investigate whether the effect of DE is sex-dependent. Twenty-eight 14-week-old male C57BL/6 mice were stratified by weight into 4 groups: 1. Control 3 weeks; 2. DE 3 weeks; 3. Control 6 weeks; 4. DE 6 weeks. An identical study design was applied to twenty-four 14-week-old female C57BL/6 mice. Chow mixed with DE was offered ad libitum, resulting in a dose of 1.70 mg DE/g body weight and 1.52 mg DE/g body weight, to female and male mice, respectively. The animals were euthanized after 3 or 6 weeks. Bone mineral density (aBMD) and bone mineral content (BMC) were evaluated with DEXA, 3D microstructural properties were determined with μCT, bone strength was determined with mechanical testing, and bone formation and resorption was evaluated with bone histomorphometry. In female mice, DE resulted in significant higher tibial aBMD values after 6 weeks of intervention. Furthermore, DE significantly increased tibial diaphyseal cortical bone area and tissue area, which was accompanied by significantly increased strength of the tibial shaft. DE had no effect on femoral cortical bone or on femoral and vertebral trabecular 3D microstructure. Finally, bone histomorphometry showed that DE had no effect on MS/BS or Oc.S/BS. In male mice, no bone positive effects of DE were found in any of the parameters investigated. In conclusion, intervention with DE may result in a weak positive site specific effect at tibial cortical bone in female mice, and importantly, no major deleterious effects of DE on bone tissue were seen in either female or male mice despite the relatively high dose of DE used.

1. Introduction

Dabigatran is a non-vitamin K antagonist oral anticoagulant that competitively and reversibly inhibits thrombin in a concentration-dependent manner and thereby reduces platelet aggregation (Wienen et al., 2007). Dabigatran is administered as dabigatran etexilate (DE), the prodrug of dabigatran, which facilitates the gastrointestinal absorption, as dabigatran is a highly polar substance and therefore not available orally (Stangier and Clemens, 2009). The facilitation of gastrointestinal absorption is achieved by adding an ethyl group at the carboxylic acid group and a hexyloxycarbonyl side chain at the amidine group of dabigatran resulting in an absolute bioavailability of 6.5% after oral administration (Stangier et al., 2007; Stangier and Clemens, 2009). After oral administration, DE is rapidly hydrolysed to its active form, dabigatran, by non-specific ubiquitous esterases (Stangier and Clemens, 2009).

Thrombin is a trypsin-like serine protease that plays a key role in the coagulation cascade. However, it also affects other organ systems due to the presence of thrombin receptors (TRs) outside the vascular domains (Abraham et al., 1998; Sokolova and Reiser, 2008). The TR is a seven transmembrane G-protein-coupled receptor that has been reported to be expressed, among others, in osteoblasts, macrophages, endothelial cells, and muscle cells (Abraham et al., 1998; Abraham and Mackie, 1999; Tudpor et al., 2015). Thrombin has been reported to be

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synthesized and secreted by osteoclasts, in addition to the more abundant production in the liver (Karlström et al., 2011). However, the physiological role of TR and thrombin on bone metabolism and bone morphology has not yet been fully elucidated.

It has been reported, that thrombin stimulates the expression of interleukin 6 (IL-6), prostaglandin E_2 (PGE_2) and cyclooxygenase 2 (COX-2) mRNA in osteoblasts (Kozawa et al., 1997; Pagel et al., 2009). IL-6 and PGE_2 have been shown to increase the expression of RANKL relative to OPG (Braun and Zwerina, 2011; Okada et al., 2000), resulting in a greater activation of osteoclasts and thereby a more rapid degradation of the mineralized bone matrix. This is consistent with the findings of Tudpor et al. (2015) who recently reported that TR deficiency leads to a high bone mass phenotype due to a reduction in RANKL and an upregulation of OPG secretion from osteoblasts leading to a decreased RANKL/OPG ratio in male mice. However, they also reported preliminary findings in female TR deficient mice showing no influence on either trabecular or cortical bone. Moreover, Aronovich et al. (2013) reported that TR knock-out female mice had decreased bone mass and compromised bone architecture. Taken together these finding indicate that thrombin deficiency may influence the skeleton in female and male mice differently.

Thrombin also plays a role in direct cleavage of the bone protein osteopontin (Senger et al., 1994), which is primarily produced by osteoblasts (Haylock and Nilsson, 2006), and is important for anchoring osteoclasts to the mineral matrix of bones (Reinholt et al., 1990; Senger et al., 1994; Sivagurunathan et al., 2013).

A recent study published in abstract form only has indicated that DE-induced inhibition of thrombin in C57BL/6 mice, resulted in significantly increased femoral aBMD as well as trabecular BV/TV (Kalinowski et al., 2014).

Therefore, the aim of the present study was to investigate whether DE can increase aBMD, bone strength, bone formation, and bone microstructure in healthy female and male mice, and to investigate whether the effect of DE on bone is sex-dependent.

2. Materials and methods

2.1. Animals and study design

The study comprised twenty-four female and twenty-eight male 14-week-old C57BL/6JBomTac mice (Taconic, Denmark). Female and male mice were stratified according to their body weight (BW), which were 22.48 g ± 0.90 g and 27.61 g ± 2.68 g, respectively. The mice were divided into the following four subgroups: 1. Control (Ctrl) 3 weeks; 2. DE 3 weeks; 3. Ctrl 6 weeks; 4. DE 6 weeks and subsequently allowed three full days of acclimatization to adapt to the new surroundings before the study begun. The animals were housed in cages equipped with laboratory animal bedding material, nesting material (SizzleNest, Scanbur, Denmark), and environmental enrichment devices. The female mice were housed with 3 mice per cage, and the male mice were housed with 4, 5, or 7 mice per cage. The cages were stored at room temperature (21 °C and approximately 30% humidity) with 12 h light/12 h dark cycles.

All mice were given either placebo-chow or DE-chow (BIBR1048MS 15 mg DE/g (Sparkenbaugh et al., 2014)) and water ad libitum. A recent study by Kalinowski et al. (2014) used a relatively high dosage of DE, and we therefore chose a DE dose that resulted in a plasma dabigatran concentration in the same range as Kalinowski et al. in order to detect any positive or negative effects of DE on bone tissue. The DE-chow was the same as the placebo-chow with DE added. Both chows were provided by Boehringer Ingelheim, Germany. New chow was offered every Monday, Wednesday, and Friday and any leftover chow were removed from the cages and weighed using a digital scale (Mettler AT250, Mettler Instrumente AG, Greifensee, Switzerland) in order to monitor daily food intake. During replacement of chow, the mice were assessed for visible spontaneous bleedings. Finally, all mice were weighed once a week and at the end of the study using the digital scale.

All animals were injected s.c. with calcein (20 mg/kg) and tetracycline (20 mg/kg) 8 and 4 days prior euthanization, respectively.

Before euthanization, the animals were anaesthetized by inhalation of 4% isoflurane (IsoFlo Vet, Orion Pharma, Abbott). Blood was drawn from the inferior vena cava, and the heart removed to ensure euthanization. Tibia, femur, and a segment of the columna consisting of vertebras Th13-L5 were removed for later investigations. Tibial and femoral lengths were measured using a digital caliper. The right tibiae and femora and the column vertebra segments were stored at −29 °C in Ringer's solution, while the left tibiae and femora, were fixed in 4% formaldehyde.

The study complied with the guiding principles in the European Communities Council Directive of 24 November 1986 (86/609/EEC), and was approved by the Danish Animal Experiments Inspectorate (2012-15-2934-00769).

2.2. Peripheral dual energy X-ray absorptiometry (pDEXA)

Tibiae, femora, and the vertebral segments were scanned in a pDEXA scanner (Sabre XL, Norland Stratec, Pforzheim, Germany) at a pixel size of 0.1 mm × 0.1 mm and a scan speed of 3.0 mm/s as previously described in detail (Lodberg et al., 2015).

The coefficient of variation of femoral aBMD determined with pDEXA is 2.8% in our laboratory (same sample measured 10 times).

2.3. Micro-computed tomography (μCT)

Prior to μCT scanning, L4 was carefully separated from the vertebral segment, soft tissue removed, and corpus vertebra isolated. The scanning was performed using a desktop μCT scanner (μCT 35, Scanco Medical AG, Switzerland). The distal femoral epiphyses and metaphyses were both scanned, as site specific trabecular changes has previously been reported (Vegger et al., 2017). Tibiae, femora, and L4s were scanned at different sessions with different settings. The distal femoral epiphysis and metaphysis, proximal tibial epiphysis, and L4 were scanned with 1000 projections/180° at an isotropic voxel size of 3.5 μm, an X-ray tube voltage of 55 kVp, a current of 145 μA, and an integration time of 800 ms. The tibial and femoral mid-diaphyses were scanned with 500 projections/180°, at an isotropic voxel size of 7.0 μm, an X-ray tube voltage of 55 kVp, a current of 145 μA, and an integration time of 300 ms.

Subsequently, volumes of interest (VOIs) were semi interactively drawn using the software provided with the μCT scanner for data evaluation (μCT Evaluation V6.5-1, Scanco Medical AG, Switzerland). The femoral epiphyseal VOI started at the point where the lateral and medial epicondyle fused to one coherent structure and ended where the growth plate first appeared, and contained trabecular bone only. The distal femoral metaphyseal VOI and proximal tibial metaphyseal VOI both started 200 μm below the point where the mineralized cartilage from the growth plate fused and ended 1000 μm further below, and contained trabecular bone only (Fig. 1). The L4 vertebral VOI was defined as the whole trabecular network from upper to lower growth plate. Finally, the VOIs at the tibial and femoral mid-diaphysis were selected as a 0.82-mm-high region centred on the mid-point (50%) of the bone, thus containing cortical bone only (Fig. 1). Dimensions of the femoral mid-diaphysis are reported as average cross-sectional areas determined as volumes divided by the height of the VOI.

The data were low-pass filtered using a Gaussian filter ($\sigma = 0.8$ and support = 1) and segmented with a fixed threshold filter. The threshold was determined automatically with IPL (v. 5.11, Scanco Medical AG, Switzerland) as the minimum point between marrow and bone peak in

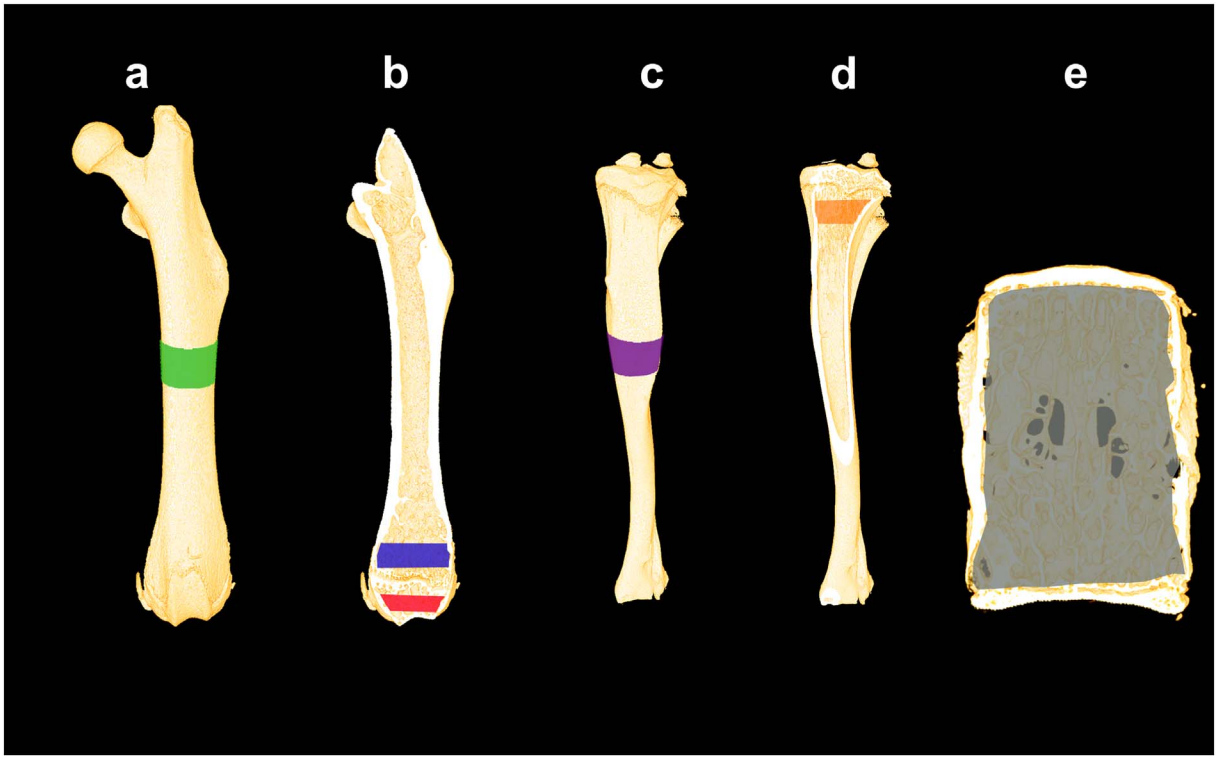

Fig. 1. Micro-computed tomography (μCT) scans of a mouse femur, tibia, and vertebra L4. a: Green area represents analyzed volume of interest (VOI) of the femoral mid-diaphysis consisting of cortical bone only. b: Blue area represents analyzed VOI of the trabecular bone at the distal femoral metaphysis; red area represents analyzed VOI of the trabecular bone at the distal femoral epiphysis. c: Purple area represents analyzed VOI of the tibial mid-diaphysis consisting of cortical bone only. d: Orange area represents analyzed VOI of the trabecular bone at the proximal tibial metaphysis. e: Grey area represents analyzed VOI of vertebra L4 consisting of trabecular bone only.

the attenuation histogram, for at least three samples from each group and the median value was used as threshold for evaluation of all samples. The thresholds for evaluation of female mice data were: Femoral epiphysis and metaphysis and tibial metaphysis: 525.5 mg HA/cm^3; femoral diaphysis: 550.2 mg HA/cm^3; tibial diaphysis: 556.4 mg HA/cm^3; and L4: 519.4 mg HA/cm^3. The thresholds for evaluation of male mice data were: Femoral epiphysis and metaphysis and tibial metaphysis: 519.4 mg HA/cm^3; femoral diaphysis: 556.4 mg HA/cm^3; tibial diaphysis: 562.5 mg HA/cm^3: and L4: 519.4 mg HA/cm^3. The assessment of the bone microstructure using μCT was performed in accordance with the current guidelines (Bouxsein et al., 2010).

The coefficient of variation of distal femoral metaphyseal BV/TV determined with μCT is 1.8% in our laboratory (same sample measured 10 times).

2.4. Mechanical testing

Mechanical properties of femora, tibiae, and L4 were determined with femoral neck test, femoral and tibial 3-point bending test, and vertebral compression test, respectively. Different site specific bone tests were used, as treatment with bone acting agents have been shown to exhibit site specificity of the mechanical properties (Mosekilde et al., 1999).

The mechanical integrity of the cortical bone at the tibial and femoral mid-diaphyses was determined using a 3-point bending test as previously described in detail (Henriksen et al., 2011). The tibiae or femora were placed on two supporting bars separated by either 10.33 mm (tibia) or 7.14 mm (femur) in a material testing machine (Instron model 5566, United Kingdom). Vertical load was applied using a third rounded bar at a constant deflection rate of 2 mm/min at the mid-diaphysis until fracture was achieved.

Femoral neck test was performed by placing the remaining proximal femur in a custom-made fixation device supporting the bone shaft while exposing the neck and applying vertical load to the top of the femoral head at a constant deflection rate of 2 mm/min until fracture (Henriksen et al., 2011).

Finally, the L4 was compression tested by placing it in the materials testing machine, stripped from any cartilage remains and intervertebral discs, and vertical load was applied with a steel cylinder at a deflection rate of 2 mm/min until fracture (Wendelboe et al., 2016).

During the mechanical testing data was recorded and stored with Merlin v. 3.21 (Instron, United Kingdom) and subsequently analyzed with a custom-made computer program.

2.5. Dynamic bone histomorphometry

After the mechanical test of the tibial mid-diaphysis, the region just above the fracture site of the proximal part of the tibial mid-diaphysis was used for dynamic bone histomorphometry. Two approximately 200-μm-thick cross-sectional slices were sawed with a diamond parallel precision saw (EXAKT Apparatebau, Germany) and mounted with Pertex on glass slides.

The fluorescent labels were detected using a light microscope equipped for fluorescence light (Nikon Eclipse i80, Japan). Live images of the slides were shown on a computer screen and analyzed with NewCAST (Visiopharm NewCAST v. 4.6.3.857, Denmark). A randomly rotated star-shaped grid with 24 radiating arms was used to count intersections with bone labels at the periosteal and endosteal bone surfaces (Lodberg et al., 2015). The centre of the grid was placed in the centre of the medullar cavity, so that the arms of the grid randomly intersected the endosteal and periosteal bone surfaces. The distances between calcein and tetracycline labels were measured using the

Table 1
Final number of animals, chow consumption, and start and final body weight (BW) of female and male mice treated with either placebo or DE for 3 or 6 weeks, respectively. Mean (SD). *$p < 0.05$ vs. start BW.

	Female				Male			
	Final no. of animals	Chow consumption (g/day)	Start BW (g)	Final BW (g)	Final no. of animals	Chow consumption (g/day)	Start BW (g)	Final BW (g)
Ctrl 3 weeks	6	2.30 (1.25)	22.50 (0.79)	22.60 (1.46)	7	2.30 (0.95)	27.44 (3.11)	25.79* (2.02)
DE 3 weeks	6	2.60 (0.94)	22.4 (1.17)	23.07 (1.38)	7	2.20 (0.81)	28.00 (1.94)	24.76* (0.73)
Ctrl 6 weeks	6	2.80 (0.17)	22.47 (1.03)	23.03 (1.19)	7	3.30 (1.28)	27.19 (3.40)	25.07* (2.76)
DE 6 weeks	6	2.70 (0.16)	22.55 (0.80)	23.25* (0.03)	7	3.10 (0.62)	27.80 (2.30)	25.16* (1.75)

Table 2
Tibial and femoral length and tibial, femoral and vertebral (Th13-L5) BMC (bone mineral content) in female and male mice treated with either placebo or DE for 3 or 6 weeks, respectively. Mean (SD).

	Female					Male				
	Tibial length (mm)	Femoral length (mm)	Tibial BMC (mg)	Femoral BMC (mg)	Vertebral BMC (mg)	Tibial length (mm)	Femoral length (mm)	Tibial BMC (mg)	Femoral BMC (mg)	Vertebral BMC (mg)
Ctrl 3 weeks	17.79 (0.18)	15.25 (0.24)	152 (23)	189 (15)	315 (13)	17.69 (0.30)	15.42 (0.35)	174 (28)	204 (24)	313 (26)
DE 3 weeks	17.86 (0.18)	15.32 (0.29)	168 (24)	197 (11)	314 (24)	17.61 (0.27)	15.19 (0.28)	166 (21)	183 (20)	290 (20)
Ctrl 6 weeks	17.73 (0.32)	15.02 (0.37)	155 (25)	185 (15)	290 (19)	17.69 (0.39)	15.44 (0.48)	167 (18)	197 (15)	304 (30)
DE 6 weeks	17.76 (0.33)	15.30 (0.17)	170 (16)	197 (12)	314 (23)	17.80 (0.22)	15.48 (0.16)	175 (19)	191 (16)	282 (69)

integrated measuring tool in the stereology software. All dynamic bone histomorphometry measurements were performed in a blinded fashion.

MS/BS were calculated as the number of intersections with calcein and tetracycline labels (double labels) plus half the number of intersections with either calcein or tetracycline (single labels) divided by the total number of intersections with an intact bone surface. Mineral apposition rate (MAR) was calculated as the average distance between calcein and tetracycline labels divided by the inter-labelling period of 4 days. Bone formation rate per bone surface (BFR/BS) was calculated as MS/BS × MAR. MS/BS, MAR, and BFR/BS of the cortical bone were determined at a magnification of ×1190. In addition, osteoclast covered surfaces (Oc.S/BS) were estimated in a similar way using proximal tibial sections stained for Cathepsin K as described in details by Vegger et al. (2016). Osteoclasts were defined as Cathepsin K-positive cells with at least one nucleus situated at the bone surface at a final magnification of ×1190.

The coefficient of variation of tibial fluorochrome labels determined with dynamic bone histomorphometry is 10.6% in our laboratory (same sample measured 10 times).

2.6. Blood serum analysis

Blood samples were kept in 5 ml heparinized Eppendorf tubes on ice for 15 min, centrifuged for 10 min at 4000*g*, and stored at −80 °C for the subsequent analyses.

In order to determine the plasma concentration of dabigatran, the diluted thrombin time (dTT) was compared with a standard curve generated by measuring TT of normal mouse plasma containing known concentrations of dabigatran. Before determination of dTT, heparin in the samples was neutralized by heparinase, and replaced by sodium citrate. Furthermore, it was controlled that this replacement did not influence dTT.

2.7. Statistical analysis

Data are given as mean ± SD, and differences were considered significant at $p < 0.05$. Statistical analysis was performed with SigmaPlot v. 12.5 (Systat Software, USA). An asterisk denotes a significant difference ($p < 0.05$) for the effect of DE using two-way ANOVA followed by a Holm-Sidak post hoc test.

A priori power calculations (power = 0.8) on healthy C57BL/6JBomTac mice showed that a 7% difference in aBMD (CV: 3%) can be demonstrated between groups with $n = 6$ animals.

3. Results

3.1. Food consumption, body weight, and length of tibia and femur

On average female and male mice consumed the same amount of chow per day regardless of whether they were served placebo or DE enriched chow. In addition, the food consumption did not differ between 3- and 6-weeks groups or between the first and second half of the study (Table 1). The daily mean dose of DE was 1.70 mg DE/g BW and 1.52 mg DE/g BW for female and male mice, respectively.

The start BW did not differ between the DE-groups and the control groups for either female or male mice. Female DE mice increased their BW significantly ($p = 0.018$) after 6 weeks, whereas the initial BW did not differ from the final BW in any of the other female mice. In contrast, all male mice lost approximately 10% ($p = 0.014$) of their BW during the first week, after which the BW stabilized. In male mice, the final BW was significantly lower in all groups compared to their initial BW. DE had no effect on BW in either female or male mice compared to controls (Table 1).

In female mice, the plasma levels of dabigatran were 933 ± 121 ng/ml and 1143 ± 650 ng/ml after 3 and 6 weeks, respectively. In male mice, the plasma levels of dabigatran were 1305 ± 389 ng/ml and 2365 ± 787 ng/ml after 3 and 6 weeks, respectively. Despite the relatively high plasma levels of dabigatran, no bleeding episodes were detected, and no animals died prematurely during the study.

The femoral or tibial length did not differ between DE and control groups, indicating that DE had no effect on longitudinal bone growth at these two skeletal sites (Table 2).

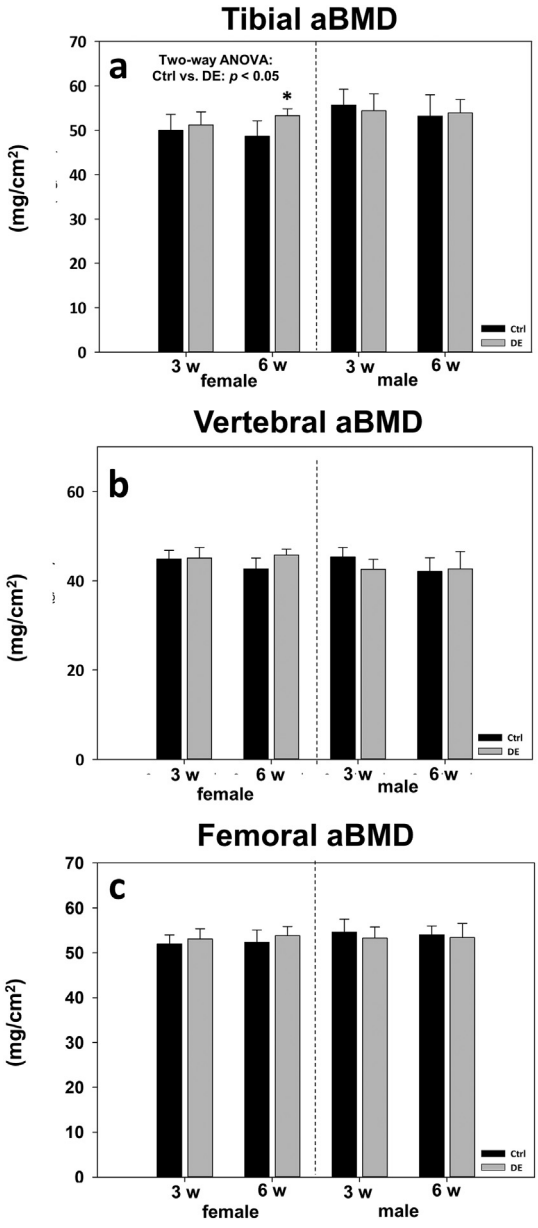

Fig. 2. Areal bone mineral density (aBMD) in female and male mice treated with either placebo or DE for 3 or 6 weeks (w), respectively. Comparison between groups was made using a two-way ANOVA with a post hoc Holm Sidak test. An * denotes significant differences ($p < 0.05$) between DE and controls.

3.2. Peripheral dual energy X-ray absorptiometry (pDEXA)

Female mice treated with DE had significantly higher tibial aBMD (two-way ANOVA: $p < 0.05$), and resulted in an 8.6% increase after 6 weeks of intervention ($p < 0.05$) compared to controls. Intervention with DE also tended to increase tibial BMC, however, this did not reach the level of significance (two-way ANOVA: $p = 0.099$). No differences in aBMD or BMC were observed in either the segment of column vertebrae or femur in female mice.

DE did not affect aBMD or BMC in male mice at any of the investigated skeletal sites (Fig. 2 and Table 2).

3.3. Micro-computed tomography (μCT)

3.3.1. Cortical bone

Intervention with DE resulted in a larger bone area (two-way ANOVA: $p < 0.05$) and tissue area (two-way ANOVA: $p < 0.05$) of the tibial mid-diaphysis in female mice. After 6 weeks of DE intervention diaphyseal bone area increased by 6.72% ($p < 0.05$) and tissue area increased by 5.91% ($p < 0.05$) compared to controls, whereas DE did not influence either tibial marrow area or cortical thickness in female mice (Fig. 3) (Table 3).

Finally, DE had no effect on any of the femoral or tibial cortical bone parameters in male mice, or any effect on femoral cortical bone parameters in female mice.

3.3.2. Trabecular bone

DE had no effect on the trabecular bone parameters at the femoral epiphysis or metaphysis, the tibial metaphysis, or the L4 in either female or male mice (Table 3).

3.4. Mechanical testing

The tibial 3-point bending test showed that DE significantly increased fracture strength for female mice (two-way ANOVA: $p < 0.05$) compared to Ctrl (Fig. 4). DE did not affect bone strength at the femoral mid-diaphysis, femoral neck, or L4 in female mice.

DE did not influence either tibial or femoral bone fracture strength in male mice.

3.5. Dynamic bone histomorphometry and osteoclast covered bone surfaces

DE did not influence MS/BS, MAR, BFR/BS, and Oc.S/BS in either female or male mice (Table 4).

4. Discussion

The present study demonstrated that DE administrated orally had a weak positive and site specific effect on bone in female mice and no positive effect on bone in male mice.

The effect of thrombin and the thrombin receptor in relation to bone have been investigated in both in vitro and in vivo experiments (Pagel et al., 2006; Sivagurunathan et al., 2013; Wienen et al., 2007). In vitro studies of cell cultures have shown that thrombin can increase the expression of RANKL relatively to OPG mediated by IL6 and PGE_2, thereby enhancing osteoclast activation and bone degeneration (Kozawa et al., 1997; Pagel et al., 2009). Therefore, it is reasonable to assume that inhibition of thrombin will result in a higher bone mass. This assumption has been investigated in thrombin receptor knockout (KO) C57BL/6 mice, which exhibited a decreased RANKL/OPG ratio and higher bone mass phenotype (Tudpor et al., 2015).

The outcome of previous in vivo studies of the effect of DE on rodent bone has been contradicting. In a study comparing the impact of DE and warfarin on bone structure, Fusaro et al. (2015) investigated three different bone sites (tibia, femur, and vertebra) using bone histomorphometry. They studied female Sprague-Dawley rats given 1.0 mg DE/g chow for 6 weeks and found that DE did not influence trabecular bone microstructural parameters such as BV/TV, Tb.Th, or Tb.Sp, and had no effect on either MS/BS or BFR/BS. In the present study, we found that DE weakly increased tibial aBMD as well as tibial cortical bone area and tissue area in female mice, and that this was accompanied by increased tibial bone strength. In contrast, no effect of DE was seen in femur or L4 for any bone parameter investigated, and no effect of DE was detected

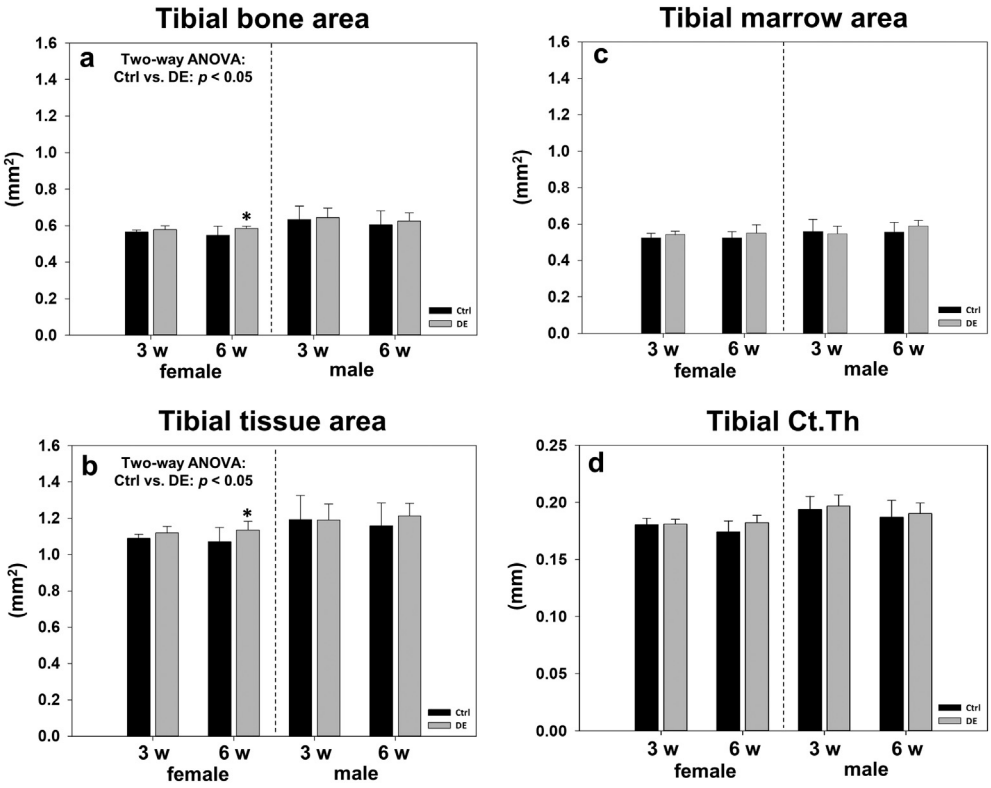

Fig. 3. Cortical bone parameters in female and male mice treated with either placebo or DE for 3 or 6 weeks (w), respectively. (a) Tibial bone area. (b) Tibial tissue area. (c) Tibial marrow area. (d) Tibial cortical thickness (Ct.Th). An * denotes significant differences ($p < 0.05$) between DE and controls.

on dynamic histomorphometry and the 3D trabecular microstructure at any of three bone sites investigated. However, it should be noted that the concentration of DE in the chow used in the present study was 15 mg/g and resulted in a mean dabigatran plasma concentration of 1143 ng/ml, whereas Fusaro et al. (2015) used a diet with a DE concentration of 1 mg/g and reported a mean dabigatran plasma concentration of 150 ng/ml. Therefore, a possible reason for the different outcome of the two studies could be the 15 times higher DE dosage used in the present study, which resulted in an almost 8 times higher dabigatran plasma concentration.

Although a week positive effect of DE was seen in female mice in tibial bone, no significant effect of DE was seen in other parameters in female mice or in any parameter in male mice. However, as the only significant effect of DE was observed in tibial cortical bone in female mice, we cannot exclude that the minor, but significant, effect of DE on tibial cortical bone was a result of a statistical type-1 error. On the other hand the minor, but significant, effect of DE was observed for three different properties i.e. aBMD, outer cortical bone dimensions, and cortical bone fracture strength, which make the probability of a type-1 error less likely.

The findings of the present study are in contrast to findings reported in an abstract by Kalinowski et al. (2014). They investigated 8-week-old C57BL/6 male mice treated with 8.5 mg/kg DE twice daily, given as i.p. injections for 6 weeks, and found a significant increase of 71% in femoral trabecular BV/TV, but no change in cortical bone mass (Kalinowski et al., 2014). These findings were accompanied by an increase in serum P1NP and a decrease in serum CTX. No data on dabigatran plasma levels was given. However, according to Zhou et al. (2011), a dosage of 9 mg/kg BW of DE administered i.p. to C57BL/6 male mice results in a maximum dabigatran plasma level of approximately 1200 ng/ml during the first hours after injection, and a dabigatran plasma level below 200 ng/ml 4 h after injection. In the present study, the dabigatran plasma levels of male mice fed DE chow was 1305 ng/ml and 2365 ng/ml after 3 and 6 weeks, respectively. Although, it cannot be precluded, a difference in DE concentration is probably not the most likely explanation for the discrepancies between the study by Kalinowski et al. (2014) and the present study. When DE is administered i.p., peak plasma dabigatran concentration will appear in spikes after each successive injection as shown by Zhou et al. (2011), in contrast to when DE is administered in the chow, which will result in a more stable dabigatran concentration. It is possible that spikes in dabigatran plasma concentration is necessary in order to generate the positive bone effect – similar to what is seen with PTH (Aslan et al., 2012). In order to detect any positive or negative effects of DE on bone tissue the present study used same intervention time of 6 weeks as the study by Kalinowski et al. (2014) and Fusaro et al. (2015). However, no studies have investigated the effect of DE on bone tissue in a dose-dependent manner, and therefore no optimal duration of intervention or dosage reference exists.

Previous in vivo studies of KO mice have suggested both sex- and bone dependent effects of DE. Tudpor et al. (2015) reported that thrombin receptor deficiency leads to a high femoral bone mass phenotype in male C57BL/6 thrombin receptor KO mice, while no effect was seen in female mice. In contrast, Aronovich et al. (2013) reported lowered tibial BV/TV and BMD in female C57BL/6 thrombin receptor KO mice. However, no previous study has compared the skeletal response in both sexes in the same study. In addition, Tudpor et al. (2015) and Aronovich et al. (2013) used KO mice, while the present study used drug induced inhibition of the thrombin receptor. The differences in study design make it difficult to compare the present study to the studies performed by Tudpor et al. (2015) and Aronovich et al. (2013) on KO mice. However, both the present study and the KO mice studies suggest that sex- and bone dependency might be present.

Oral anti-coagulants are widely used in the prevention and treatment of arterial and venous thrombo-embolic diseases, cardiovascular diseases such as deep vein thrombosis, and atrial fibrillation (chest

Table 3
Femoral and vertebral cortical and trabecular bone parameters in female and male mice treated with either placebo or DE for 3 or 6 weeks, respectively. B.Ar: bone area, Ma.Ar: marrow area, T.Ar: tissue area, Ct.Th: cortical thickness, pMOI: polar moment of inertia, BV/TV: bone volume/tissue volume, Tb.Th: trabecular thickness, Tb.Sp: trabecular spacing and SMI: structure model index, CD: connectivity density. Mean (SD).

	Femoral diaphyseal cortical bone parameters									
	Female					Male				
	B.Ar (mm^2)	T.Ar (mm^2)	Ma.Ar (mm^2)	Ct.Th (mm)	pMOI (mm^4)	B.Ar (mm^2)	T.Ar (mm^2)	Ma.Ar (mm^2)	Ct.Th (mm)	pMOI (mm^4)
Ctrl 3 weeks	0.729 (0.031)	1.835 (0.059)	1.106 (0.055)	0.165 (0.007)	0.357 (0.028)	0.810 (0.099)	1.997 (0.205)	1.187 (0.108)	0.175 (0.013)	0.438 (0.090)
DE 3 weeks	0.751 (0.026)	1.905 (0.069)	1.154 (0.055)	0.164 (0.006)	0.387 (0.030)	0.780 (0.061)	1.936 (0.170)	1.157 (0.119)	0.173 (0.008)	0.407 (0.070)
Ctrl 6 weeks	0.699 (0.050)	1.827 (0.110)	1.129 (0.076)	0.158 (0.009)	0.346 (0.043)	0.730 (0.079)	1.898 (0.214)	1.168 (0.147)	0.162 (0.011)	0.379 (0.077)
DE 6 weeks	0.737 (0.023)	1.848 (0.052)	1.112 (0.039)	0.166 (0.004)	0.366 (0.065)	0.741 (0.047)	1.938 (0.120)	1.197 (0.083)	0.161 (0.008)	0.397 (0.047)
	Femoral metaphyseal trabecular bone parameters									
	Female					Male				
	BV/TV	Tb.Th (mm)	Tb.Sp (mm)	SMI	CD (mm^{-3})	BV/TV	Tb.Th (mm)	Tb.Sp (mm)	SMI	CD (mm^{-3})
Ctrl 3 weeks	0.070 (0.014)	0.032 (0.002)	0.270 (0.022)	1.881 (0.155)	188 (44.3)	0.145 (0.032)	0.039 (0.004)	0.202 (0.015)	1.246 (0.368)	306 (59.4)
DE 3 weeks	0.056 (0.017)	0.031 (0.002)	0.291 (0.024)	0.291 (0.024)	150 (48.5)	0.139 (0.020)	0.039 (0.003)	0.197 (0.013)	0.291 (0.024)	320 (23.5)
Ctrl 6 weeks	0.047 (0.013)	0.032 (0.003)	0.306 (0.016)	2.313 (0.259)	102 (11.6)	0.117 (0.016)	0.038 (0.004)	0.218 (0.008)	1.527 (0.147)	227 (22.2)
DE 6 weeks	0.056 (0.012)	0.035 (0.003)	0.300 (0.019)	2.189 (0.224)	108 (17.2)	0.118 (0.019)	0.038 (0.003)	0.217 (0.006)	1.521 (0.219)	223 (14.2)
	Femoral epiphyseal trabecular bone parameters									
	Female					Male				
	BV/TV	Tb.Th (mm)	Tb.Sp (mm)	SMI	CD (mm^{-3})	BV/TV	Tb.Th (mm)	Tb.Sp (mm)	SMI	CD (mm^{-3})
Ctrl 3 weeks	0.223 (0.019)	0.053 (0.004)	0.246 (0.022)	0.112 (0.151)	194 (29.9)	0.287 (0.085)	0.052 (0.008)	0.180 (0.008)	− 0.068 (0.806)	319 (48.3)
DE 3 weeks	0.213 (0.016)	0.053 (0.004)	0.253 (0.017)	0.221 (0.109)	187 (20.1)	0.306 (0.011)	0.052 (0.002)	0.172 (0.002)	− 0.249 (0.072)	359 (19.2)
Ctrl 6 weeks	0.216 (0.012)	0.053 (0.003)	0.250 (0.021)	0.172 (0.035)	178 (14.6)	0.278 (0.023)	0.050 (0.004)	0.178 (0.004)	− 0.000 (0.164)	323 (28.7)
DE 6 weeks	0.22 (0.013)	0.055 (0.003)	0.242 (0.021)	0.153 (0.021)	167 (21.7)	0.283 (0.026)	0.050 (0.002)	0.176 (0.002)	− 0.084 (0.183)	336 (28.1)
	Tibial metaphyseal trabecular bone parameters									
	Female					Male				
	BV/TV	Tb.Th (mm)	Tb.Sp (mm)	SMI	CD (mm^{-3})	BV/TV	Tb.Th (mm)	Tb.Sp (mm)	SMI	CD (mm^{-3})
Ctrl 3 weeks	0.064 (0.019)	0.037 (0.004)	0.298 (0.027)	2.056 (0.281)	80.7 (26.9)	0.139 (0.026)	0.043 (0.004)	0.193 (0.020)	1.718 (0.237)	198 (63.6)
DE 3 weeks	0.052 (0.008)	0.037 (0.002)	0.322 (0.021)	2.154 (0.154)	62.1 (19.7)	0.125 (0.017)	0.040 (0.003)	0.189 (0.022)	1.861 (0.150)	197 (35.4)
Ctrl 6 weeks	0.050 (0.012)	0.039 (0.003)	0.333 (0.037)	2.301 (0.302)	49.3 (22.1)	0.123 (0.022)	0.040 (0.004)	0.199 (0.015)	1.806 (0.235)	160 (26.7)
DE 6 weeks	0.054 (0.011)	0.042 (0.003)	0.350 (0.040)	2.180 (0.115)	46.9 (6.55)	0.125 (0.036)	0.040 (0.004)	0.198 (0.023)	1.827 (0.332)	183 (75.6)
	Vertebral trabecular bone parameters									
	Female					Male				
	BV/TV	Tb.Th (mm)	Tb.Sp (mm)	SMI	CD (mm^{-3})	BV/TV	Tb.Th (mm)	Tb.Sp (mm)	SMI	CD (mm^{-3})
Ctrl 3 weeks	0.155 (0.009)	0.034 (0.002)	0.240 (0.009)	0.404 (0.070)	485 (143)	0.194 (0.026)	0.035 (0.003)	0.183 (0.008)	0.335 (0.200)	891 (174)
DE 3 weeks	0.154 (0.018)	0.034 (0.002)	0.242 (0.014)	0.496 (0.159)	428 (83.4)	0.180 (0.013)	0.033 (0.002)	0.187 (0.008)	0.477 (0.123)	798 (181)
Ctrl 6 weeks	0.147 (0.020)	0.034 (0.002)	0.251 (0.014)	0.607 (0.201)	426 (42.9)	0.176 (0.014)	0.033 (0.003)	0.187 (0.008)	0.505 (0.081)	672 (131)
DE 6 weeks	0.157 (0.014)	0.036 (0.002)	0.247 (0.016)	0.525 (0.082)	360 (70.9)	0.179 (0.022)	0.033 (0.003)	0.186 (0.011)	0.448 (0.129)	588 (125)

guidelines (Guyatt et al., 2012)), and for many cases treatment should be continued for many years (Guyatt et al., 2012). Some of the most frequently used anti-coagulants are vitamin K antagonist such as warfarin and phenprocoumon (Guyatt et al., 2012), which inhibits the γ-carboxylation of glutamatic acid residues of factor II, VII, IX, and X in the coagulation cascade. In clinical studies, warfarin has been associated with increased risk of rib and vertebral fractures (Sugiyama et al., 2007), whereas data have been conflicting when it comes to whether warfarin treatment lead to decreased BMD (Simon et al., 2002). In rodent studies, warfarin treatment has been shown to have negative effects on bone i.e. decreased BV/TV, decreased bone strength and ultimate stress (Fusaro et al., 2015; Simon et al., 2002; Sugiyama et al., 2007). In this context, it is should be emphasized that even with a fairly high dosage of DE no deleterious effects on bone structure or bone strength were found in the present study.

The doses used in the present study resulted in dabigatran plasma levels, which were 10–20 times higher than the dabigatran plasma concentrations used for prevention of non-valvular atrial fibrillation. In the RE-LY trial, the efficacy of DE to prevent stroke and systemic embolism was compared to warfarin in patients with atrial fibrillation (Beckett et al., 2009). High dose DE, 150 mg twice daily, resulted in dabigatran mean plasma levels of 175 ng/ml and 91 ng/ml 1–3 h and 10–16 h after oral administration, respectively (Reilly et al., 2014). Therefore, even though no reference guide exists for dabigatran plasma concentrations in relation to its effect on bone tissue, it is fair to say that a high plasma concentration was assured in the present study. When the weak bone effect of the relatively high DE plasma concentrations obtained in the present study is taken into consideration, it seems unlikely that a more pronounced bone effect will occur at lower plasma concentrations.

A limitation of the study was the unexpected loss of BW observed in male mice during the first week of the study, after which the loss of BW ceased. In contrast, no loss of BW was seen in female mice. The reason for this loss of BW around week 1 could be explained by a lower consumption of chow during the first half of the study compared to the last half. Furthermore, greater variations in aBMD standard derivations were found in the present study than what was expected a priori, suggesting a larger number of animals in each group should be considered for future studies. However, given the results from the present study it seems unlikely that DE exerts a general positive effect on bone tissue and that the overall results would be different if more animals were included in each group, even though it cannot completely be precluded.

In conclusion, DE may induce a site specific weak increase of tibial aBMD, tibial cortical bone area, tibial tissue area, and tibial bone strength in female mice, whereas no effect was found in femoral or vertebral bone, moreover, no positive bone effects were found in male mice. Importantly, no major deleterious effects of DE on bone tissue were seen despite the relatively high dose of DE used.

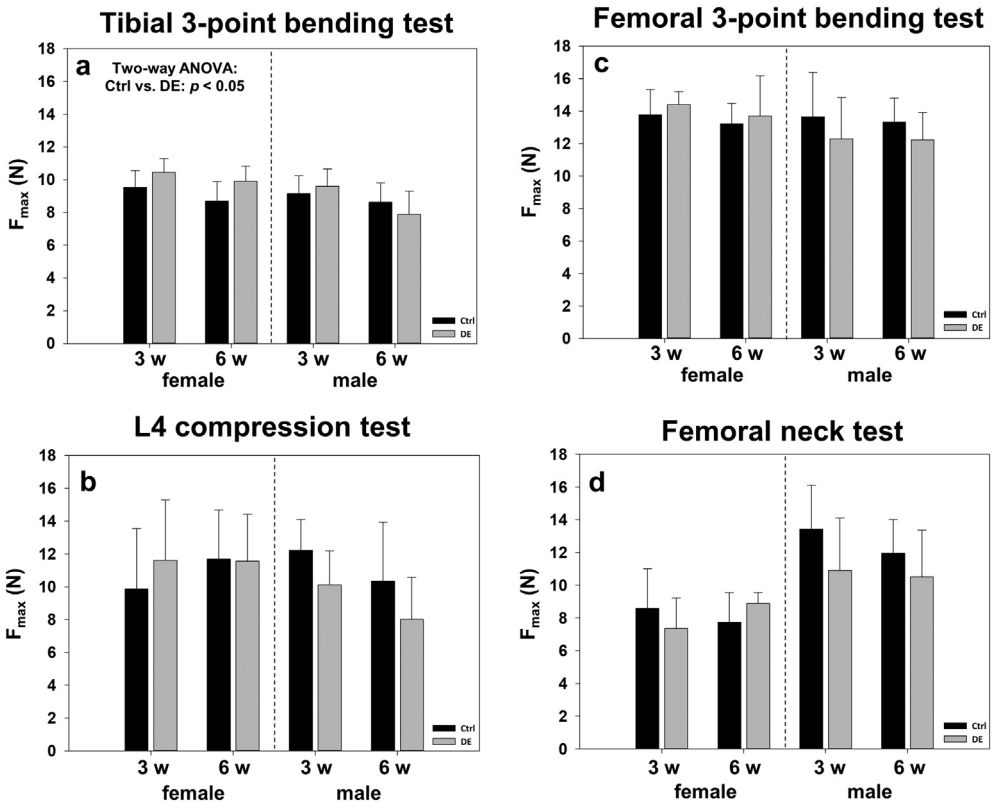

Fig. 4. Mechanical test of female and male mice treated with either placebo or DE for 3 or 6 weeks (w), respectively. (a) Tibial 3-point bending test. (b) L4 compression test. (c) Femoral 3-point bending test. (d) Femoral neck test.

Table 4
Tibial dynamic bone histomorphometry in female and male mice treated with either placebo or DE for 3 or 6 weeks, respectively. MS/BS: mineralizing surface/bone surface, MAR: mineral apposition rate and BFR/BS: bone formation rate/bone surface, Oc.S/BS: osteoclast covered surfaces. Mean (SD).

	Endosteal surface							
	Female				Male			
	MS/BS (%)	MAR (μm/day)	BFR/BS (μm/day)	Oc.S/BS (%)	MS/BS (%)	MAR (μm/day)	BFR/BS (μm/day)	Oc.S/BS (%)
Ctrl 3 weeks	5.1 (4.7)	0.013 (0.012)	0.001 (0.002)	0.58 (0.70)	9.9 (10.6)	0 (0)	0 (0)	1.55 (1.07)
DE 3 weeks	8.3 (5.5)	0.021 (0.014)	0.002 (0.002)	2.50 (1.91)	5.6 (9.6)	0 (0)	0 (0)	1.11 (1.11)
Ctrl 6 weeks	17.5 (21.0)	0.044 (0.052)	0.017 (0.034)	3.94 (3.02)	8.3 (5.1)	0 (0)	0 (0)	2.92 (1.92)
DE 6 weeks	22.2 (22.5)	0.055 (0.056)	0.023 (0.042)	2.78 (1.16)	13.4 (11.6)	0.066 (0.17)	0.012 (0.032)	2.46 (1.99)

	Periosteal surface					
	Female			Male		
	MS/BS (%)	MAR (μm/day)	BFR/BS (μm/day)	MS/BS (%)	MAR (μm/day)	BFR/BS (μm/day)
Ctrl 3 weeks	1.3 (1.3)	0.003 (0.003)	1×10^{-4} (9×10^{-5})	5.6 (9.6)	0 (0)	0 (0)
DE 3 weeks	0.5 (0.6)	0.001 (0.002)	0.000 (1.61×10^{-5})	3.1 (7.1)	0 (0)	0 (0)
Ctrl 6 weeks	2.0 (1.9)	0.005 (0.005)	2×10^{-4} (2.04×10^{-4})	1.18 (1.8)	0 (0)	0 (0)
DE 6 weeks	1.4 (2.4)	0.004 (0.006)	2×10^{-4} (4.31×10^{-4})	5.4 (2.6)	0.076 (0.20)	0.006 (0.016)

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References

Abraham, L.A., Mackie, E.J., 1999. Modulation of osteoblast-like cell behavior by activation of protease-activated receptor-1. J. Bone Miner. Res. 14, 1320–1329. http://dx.doi.org/10.1359/jbmr.1999.14.8.1320.

Abraham, L., Jenkins, A.L., Stone, S.R., Mackie, E.J., 1998. Expression of the thrombin receptor in developing bone and associated tissues. J. Bone Miner. Res. 13, 818–827. http://dx.doi.org/10.1359/jbmr.1998.13.5.818.

Aronovich, A., Nur, Y., Shezen, E., Rosen, C., Zlotnikov Klionsky, Y., Milman, I., Yarimi, L., Hagin, D., Rechavi, G., Martinowitz, U., Nagasawa, T., Frenette, P.S., Tchorsh-Yutsis, D., Reisner, Y., 2013. A novel role for factor VIII and thrombin/PAR1 in regulating hematopoiesis and its interplay with the bone structure. Blood 122, 2562–2571. http://dx.doi.org/10.1182/blood-2012-08-447458.

Aslan, D., Andersen, M.D., Gede, L.B., de Franca, T.K., Jørgensen, S.R., Schwarz, P., Jørgensen, N.R., 2012. Mechanisms for the bone anabolic effect of parathyroid hormone treatment in humans. Scand. J. Clin. Lab. Invest. 72, 14–22. http://dx.doi.org/10.3109/00365513.2011.624631.

Beckett, N.S., Peters, R., Fletcher, A.E., Staessen, J.A., Liu, L., Dumitrascu, D., Stoyanovsky, V., Antikainen, R.L., Nikitin, Y., Anderson, C., Belhani, A., Rajkumar, C., Thijs, L., Banya, W., Bulpitt, C.J., 2009. Dabigatran versus warfarin in patients with atrial fibrillation. N. Engl. J. Med. 361, 1139–1151.

Bouxsein, M.L., Boyd, S.K., Christiansen, B.A., Guldberg, R.E., Jepsen, K.J., Müller, R., 2010. Guidelines for assessment of bone microstructure in rodents using micro-computed tomography. J. Bone Miner. Res. http://dx.doi.org/10.1002/jbmr.141.

Braun, T., Zwerina, J., 2011. Positive regulators of osteoclastogenesis and bone resorption in rheumatoid arthritis. Arthritis Res. Ther. 13, 235. http://dx.doi.org/10.1186/ar3380.

Fusaro, M., Carbonare, L.D., Dusso, A., Arcidiacono, M.V., Valenti, M.T., Aghi, A., Pasho, S., Gallieni, M., 2015. Differential effects of dabigatran and warfarin on bone volume and structure in rats with normal renal function. PLoS One 10, 1–15. http://dx.doi.org/10.1371/journal.pone.0133847.

Guyatt, G.H., Akl, E.A., Crowther, M., Gutterman, D.D., Schünemann, H.J., 2012. Executive summary: antithrombotic therapy and prevention of thrombosis. In: American College of Chest Physicians Evidence-based Clinical Practice Guidelines, 9th ed. 141. pp. 7–47. Chest. https://doi.org/10.1378/chest.1412S3.

Haylock, D.N., Nilsson, S.K., 2006. Osteopontin: a bridge between bone and blood. Br. J. Haematol. http://dx.doi.org/10.1111/j.1365-2141.2006.06218.x.

Henriksen, K., Flores, C., Thomsen, J.S., Brüel, A.M., Thudium, C.S., Neutzsky-Wulff, A.V., Langenbach, G.E.J., Sims, N., Askmyr, M., Martin, T.J., Everts, V., Karsdal, M.A., Richter, J., 2011. Dissociation of bone resorption and bone formation in adult mice with a non-functional V-AtPase in osteoclasts leads to increased bone strength. PLoS One 6. http://dx.doi.org/10.1371/journal.pone.0027482.

Kalinowski, J., Jastrzebski, S., Won, H.Y., Mirza, F., Sun-Kyeong, L., Lorenzo, J., 2014. Dabigatran etexilate, a new direct thrombin inhibitor, enhances bone mass, inhibits bone resorption and stimulates bone formation in mice. In: ASBMR 2014 Annu. Meet, http://dx.doi.org/10.1613/jair.301.

Karlström, E., Norgård, M., Hultenby, K., Somogyi-Ganss, E., Sugars, R., Andersson, G., Wendel, M., 2011. Localization and expression of prothrombin in rodent osteoclasts and long bones. Calcif. Tissue Int. 88, 179–188. http://dx.doi.org/10.1007/s00223-010-9443-3.

Kozawa, O., Tokuda, H., Kaida, T., Matsuno, H., Uematsu, T., 1997. Thrombin Regulates Interleukin-6 Synthesis Through Phosphatidylcholine Hydrolysis By Phospholipase D in Osteoblasts. 345. pp. 10–15.

Lodberg, A., Vegger, J.B., Jensen, M.V., Larsen, C.M., Thomsen, J.S., Brüel, A., 2015. Immobilization induced osteopenia is strain specific in mice. Bone Rep. 2, 59–67. http://dx.doi.org/10.1016/j.bonr.2015.04.001.

Mosekilde, L., Thomsen, J.S., Orhii, P.B., McCarter, R.J., Mejia, W., Kalu, D.N., 1999. Additive effect of voluntary exercise and growth hormone treatment on bone strength assessed at four different skeletal sites in an aged rat model. Bone 24, 71–80. http://dx.doi.org/10.1016/S8756-3282(98)00169-0.

Okada, Y., Lorenzo, J.A., Freeman, A.M., Tomita, M., Morham, S.G., Raisz, L.G., Pilbeam, C.C., 2000. Prostaglandin G/H synthase-2 is required for maximal formation of osteoclast-like cells in culture. J. Clin. Invest. 105, 823–832. http://dx.doi.org/10.1172/JCI8195.

Pagel, C.N., Sivagurunathan, S., Lay, H.L., Tudor, E.M., Pike, R.N., Mackie, E.J., 2006. Functional responses of bone cells to thrombin. Biol. Chem. 387, 1037–1041. http://dx.doi.org/10.1515/BC.2006.128.

Pagel, C.N., Song, S.J., Loh, L.H., Tudor, E.M., Murray-Rust, T.A., Pike, R.N., Mackie, E.J., 2009. Thrombin-stimulated growth factor and cytokine expression in osteoblasts is mediated by protease-activated receptor-1 and prostanoids. Bone 44, 813–821. http://dx.doi.org/10.1016/j.bone.2008.12.031.

Reilly, P.A., Lehr, T., Haertter, S., Connolly, S.J., Yusuf, S., Eikelboom, J.W., Ezekowitz, M.D., Nehmiz, G., Wang, S., Wallentin, L., 2014. The effect of dabigatran plasma concentrations and patient characteristics on the frequency of ischemic stroke and major bleeding in atrial fibrillation patients: the RE-LY trial (Randomized Evaluation of Long-term Anticoagulation Therapy). J. Am. Coll. Cardiol. 63, 321–328. http://dx.doi.org/10.1016/j.jacc.2013.07.104.

Reinholt, F.P., Hultenby, K., Oldberg, A., Heinegård, D., 1990. Osteopontin - a possible anchor of osteoclasts to bone. Proc. Natl. Acad. Sci. U. S. A. 87, 4473–4475. http://dx.doi.org/10.1073/pnas.87.12.4473.

Senger, D.R., Perruzzi, C.A., Papadopoulos-Sergiou, A., Van de Water, L., 1994. Adhesive properties of osteopontin: regulation by a naturally occurring thrombin-cleavage in close proximity to the GRGDS cell-binding domain. Mol. Biol. Cell 5, 565–574. http://dx.doi.org/10.1091/mbc.5.5.565.

Simon, R.R., Beaudin, S.M., Johnston, M., Walton, K.J., Shaughnessy, S.G., 2002. Long-term Treatment With Sodium Warfarin Results in Decreased Femoral Bone Strength and Cancellous Bone Volume in Rats I. pp. 353–358.

Sivagurunathan, S., Pagel, C.N., Loh, L.H., Wijeyewickrema, L.C., Pike, R.N., Mackie, E.J., 2013. Thrombin inhibits osteoclast differentiation through a non-proteolytic mechanism. J. Mol. Endocrinol. 50, 347–359. http://dx.doi.org/10.1530/JME-12-0177.

Sokolova, E., Reiser, G., 2008. Prothrombin/thrombin and the thrombin receptors PAR-1 and PAR-4 in the brain: localization, expression and participation in neurodegenerative diseases. Thromb. Haemost. 100, 576–581. http://dx.doi.org/10.1160/TH08-03-0131.

Sparkenbaugh, E.M., Chantrathammachart, P., Mickelson, J., Van Ryn, J., Hebbel, R.P., Monroe, D.M., Mackman, N., Key, N.S., Pawlinski, R., 2014. Differential contribution of FXa and thrombin to vascular inflammation in a mouse model of sickle cell disease. Blood 123, 1747–1756. http://dx.doi.org/10.1182/blood-2013-08-523936.

Stangier, J., Clemens, A., 2009. Pharmacology, Pharmacokinetics, and Pharmacodynamics of Dabigatran Etexilate, an Oral Direct Thrombin Inhibitor. 15. pp. 9S–16S.

Stangier, J., Rathgen, K., Stahle, H., Gansser, D., Roth, W., 2007. The pharmacokinetics, pharmacodynamics and tolerability of dabigatran etexilate, a new oral direct thrombin inhibitor, in healthy male subjects. Br. J. Clin. Pharmacol. 64, 292–303. http://dx.doi.org/10.1111/j.1365-2125.2007.02899.x.

Sugiyama, T., Takaki, T., Sakanaka, K., Sadamaru, H., Mori, K., Kato, Y., Taguchi, T., Saito, T., 2007. Warfarin-induced impairment of cortical bone material quality and compensatory adaptation of cortical bone structure to mechanical stimuli. J. Endocrinol. 194, 213–222. http://dx.doi.org/10.1677/JOE-07-0119.

Tudpor, K., van der Eerden, B.C.J., Jongwattanapisan, P., Roelofs, J.J.T.H., van Leeuwen, J.P.T.M., Bindels, R.J.M., Hoenderop, J.G.J., 2015. Thrombin receptor deficiency leads to a high bone mass phenotype by decreasing the RANKL/OPG ratio. Bone 72, 14–22. http://dx.doi.org/10.1016/j.bone.2014.11.004.

Vegger, J.B., Brüel, A., Sørensen, T.G., Thomsen, J.S., 2016. Systemic treatment with strontium ranelate does not influence the healing of femoral mid-shaft defects in rats. Calcif. Tissue Int. 98, 206–214. http://dx.doi.org/10.1007/s00223-015-0077-3.

Vegger, J.B., Brüel, A., Brent, M.B., Thomsen, J.S., 2017. Disuse osteopenia induced by botulinum toxin is similar in skeletally mature young and aged female C57BL/6J mice. J. Bone Miner. Metab. http://dx.doi.org/10.1007/s00774-017-0830-y.

Wendelboe, M.H., Thomsen, J.S., Henriksen, K., Vegger, J.B., Brüel, A., 2016. Zoledronate prevents lactation induced bone loss and results in additional post-lactation bone mass in mice. Bone 87, 27–36. http://dx.doi.org/10.1016/j.bone.2016.03.012.

Wienen, W., Stassen, J.M., Priepke, H., Ries, U.J., Hauel, N., 2007. Effects of the direct thrombin inhibitor dabigatran and its orally active prodrug, dabigatran etexilate, on thrombus formation and bleeding time in rats. Thromb. Haemost. 98, 333–338. http://dx.doi.org/10.1160/TH07-02-0113.

Zhou, W., Schwarting, S., Illanes, S., Liesz, A., Middelhoff, M., Zorn, M., Bendszus, M., Heiland, S., Van Ryn, J., Veltkamp, R., 2011. Hemostatic therapy in experimental intracerebral hemorrhage associated with the direct thrombin inhibitor dabigatran. Stroke 42, 3594–3599. http://dx.doi.org/10.1161/STROKEAHA.111.624650.