Skeletal effects of a gastrin receptor antagonist in H⁺/K⁺ATPase beta subunit KO mice

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Abstract

Epidemiological studies suggest an increased fracture risk in patients taking proton pump inhibitors (PPIs) for long term. The underlying mechanism, however, has been disputed. By binding to the gastric proton pump, PPIs inhibit gastric acid secretion. We have previously shown that proton pump (H⁺/K⁺ATPase beta subunit) KO mice exhibit reduced bone mineral density (BMD) and inferior bone strength compared with WT mice. Patients using PPIs as well as these KO mice exhibit gastric hypoacidity, and subsequently increased serum concentrations of the hormone gastrin. In this study, we wanted to examine whether inhibition of the gastrin/CCK2 receptor influences bone quality in these mice. KO and WT mice were given either the gastrin/CCK2 receptor antagonist netazepide dissolved in polyethylene glycol (PEG) or only PEG for 1 year. We found significantly lower bone mineral content and BMD, as well as inferior bone microarchitecture in KO mice compared with WT mice. Biomechanical properties by three-point bending test also proved inferior in KO mice. KO mice receiving netazepide exhibited significantly higher cortical thickness, cortical area fraction, trabecular thickness and trabecular BMD by micro-CT compared with the control group. Three-point bending test also showed higher Young's modulus of elasticity in the netazepide KO group compared with control mice. In conclusion, we observed that the gastrin receptor antagonist netazepide slightly improved bone quality in this mouse model, suggesting that hypergastrinemia may contribute to deteriorated bone quality during acid inhibition.

Key Words
- gastrin
- netazepide
- bone
- acid inhibition
- osteoporosis
Introduction

Proton pump inhibitors (PPIs) are widely used in the management of acid-related gastrointestinal diseases such as peptic ulcers and gastro-esophageal reflux. The number of prescriptions of PPIs in the USA increased from 134 to 150 million per year from 2007 to 2011, with the PPI omeprazole alone being the sixth most prescribed drug in the US in 2011 (IMS 2012). The consequences of long-term acid inhibition, however, are not fully known.

Several large case-control studies suggest that patients using PPIs have an increased risk of hip fracture (Yang et al. 2006, Targownik et al. 2008, Corley et al. 2010, Khalili et al. 2012). More recently, others have found an association between long-term PPI use and osteoporosis-related fractures overall (Targownik et al. 2008, Gray et al. 2010, Fraser et al. 2013, Moberg et al. 2014), and spine fractures in particular (Roux et al. 2009). Vestergaard and coworkers reported that use of PPIs was associated with increased fracture risk, whereas use of histamine 2 receptor antagonists was associated with reduced risk.

Both animal and clinical studies have revealed an association between PPI use and a reduction in bone mineral density (BMD). Our group previously reported that rats given a PPI for 3 months had reduced BMD (Cui et al. 2001), and two recent prospective studies in humans observed a reduction in BMD in PPI users (Ozdil et al. 2013, Bahtiri et al. 2015). Others, however, have failed to find a decline in BMD (Targownik et al. 2010). The mechanisms underlying the increased fracture risk in PPI users are not fully understood.

PPIs block the gastric H+/K+ATPase, which is responsible for the acidic environment in the stomach. This leads to gastric hypochloridria, which subsequently triggers gastrin release from the G-cells of the gastric antrum (Kleveland et al. 1987, Sandvik et al. 1987). Similarly, patients with chronic atrophic gastritis have impaired gastric acid production due to atrophy of the parietal cells. These patients have decreased BMD (Eastell et al. 1992) as well as an increased risk of osteoporosis-related fractures (Goerss et al. 1992, Merriman et al. 2010). This suggests that the effect of PPIs on bone could be related to gastric hypochloridria and its consequences, rather than a direct drug effect on bone.

While the role of hypochloridria has been examined in both animal and human studies, the effect of hypergastrinemia on bone metabolism has not been addressed. H+/K+ATPase beta subunit knockout (KO) mice display gastric anacidity and hypergastrinemia (Scarff et al. 1999). We have previously shown that these mice exhibit lower bone mineral content (BMC) and BMD, impaired microarchitecture, as well as reduced mechanical bone strength, compared with wild-type mice (Fossmark et al. 2012). In this study, we wanted to explore whether long-term administration of a gastrin/CCK2 receptor antagonist could prevent the development of an osteoporotic phenotype in H+/K+ATPase beta subunit KO mice. We aimed to do so by assessing both BMD and micro-CT-derived structural properties of bones from the test animals, in addition to performing biomechanical tests and dynamic histomorphometric evaluation.

Materials and methods

Animals and genotyping

H+/K+ATPase beta subunit KO mice, originally with a BalbC/black6 (Scarff et al. 1999) background, were back-crossed eight times onto BalbC mice (Møllegaard, Skensved, Denmark) before study start. DNA was isolated from tail samples using the high pure PCR template preparation kit, according to the manufacturer’s instruction (Roche Diagnostics). A PCR assay was set up to genotype mice and was used to distinguish between homozygous KO mice (−/−), heterozygous mice (+/−) and homozygous wild-type (WT) mice (+/+). For detection of H+/K+ATPase WT alleles, specific primers (forward primer: GGACCAACTGACTTCTGGGA and reverse primer: ACCTGCATGGCAGTCTCTCT, product length: 472 bp) were based on H+/K+ATPase beta subunit (Atp4b) sequence (Ensembl: ENSMUSG00000031449) and located approximately 100 bp upstream and downstream of exon 1 (the target for the homologous recombination). A PCR product with size of 472 bp would appear only if the WT allele was present. Inserted construct (PKG-neo) alleles were detected using specific primers selected from the PKG-neo sequence (Scarff et al. 1999) (forward primer: AGACAATCGAGTCTGCTGGGA and reverse primer: ATACTTTCTCCGAGCAGGAGCA, product length: 261 bp), and a PCR product with the size 261 bp would only appear if the KO allele was present. All variants were genotyped by combining these two PCR reactions using the FastStart PCR Master kit according to the manufacturer’s instruction (Roche Diagnostics), and visually inspected after gel electrophoresis separation and ethidium bromide staining.
For the experiments, we used female H+/K+ATPase beta subunit KO mice (n=21) and WT controls (n=21), 6 weeks of age. They were housed in cages with aspen woodchip bedding from B&K Universal Ltd (Hull, United Kingdom). The room temperature was 24±1°C with a relative humidity of 40–50% and a 12 h light:12 h darkness cycle. RM1 (E) diet from SDS (Essex, UK) and tap water were provided ad libitum. The study was approved by the Norwegian National Animal Research Authority.

**Study design**

Netazepide (NTZ), previously named YF476 (Hammersmith Medicines Research, London, UK), has been shown to be a potent and highly selective competitive antagonist of the gastrin/CCK2 receptor (Takinami et al. 1997). It was dispersed in polyethylene glycol 300 (PEG) to a concentration of 12 mg/mL, and given as subcutaneous injections at a dose of 40 mg/kg (80 µmol/kg) to 11 KO mice (KO/NTZ) and 10 WT mice (WT/NTZ) from 6 weeks of age (baseline) once every 2 weeks for 12 months. The remaining animals received an equivalent volume of PEG (KO/PEG, 10 mice, and WT/PEG, 11 mice). The mice were included in the study consecutively after birth, and siblings were allocated to either PEG or NTZ groups. All animals were subcutaneously injected with 20 mg/kg body weight of calcein (Sigma-Aldrich) 7 and 2 days before necropsy.

**DXA measurements**

DXA measurements were performed using a Hologic QDR 4500A and small animal software (Hologic, Bedford, MA, USA). Body weight, fat mass and lean mass, BMC and BMD were measured at 6 weeks of age and again at termination. The animals were anesthetized with 5% isoflurane at −20°C as described (Erben 1997). Three-micrometer-thick sections of the proximal tibiae were prepared using a Microm HM 355S microtome (Thermo Scientific). Sections were stained for tartrate-resistant acid phosphatase (TRACP) enzyme activity according to standard protocols (Erben 1997). For fluorochrome analysis, undeplasticized and unstained sections were mounted with Fluoromount (Serva, Heidelberg, Germany). Cancellous bone histomorphometry was performed as described (Schneider et al. 2009) using OsteoMeasure 3.0 (OsteoMetrics, Decatur, GA, USA) software. The area within 0.25 mm from the growth plate was excluded from the measurements.

**Micro-computed tomography (µCT) measurements**

The right femurs were dissected and the lengths measured, before the bones were snap-frozen in liquid nitrogen and stored at −80°C until µCT scan by SkyScan 1172 (Bruker, Kontich, Belgium). For analysis of trabecular bone, a volume of interest (VOI) of 250 slices was selected from the distal femur, starting 50 slices proximal to the growth plate. Cortical values were calculated from a VOI of 100 slices, starting 500 slices from the growth plate. The analyses were performed with the Skyscan program CTAn (version 1.13.4.0).

**Cancellous bone dynamic histomorphometry**

The left tibiae were dissected, defleshed and fixed in 70% ethanol before embedding in methyl methacrylate at −20°C as described (Erben 1997). Three-micrometer-thick sections of the proximal tibiae were prepared using a Microm HM 355S microtome (Thermo Scientific). Sections were stained for tartrate-resistant acid phosphatase (TRACP) enzyme activity according to standard protocols (Erben 1997). For fluorochrome analysis, undeplasticized and unstained sections were mounted with Fluoromount (Serva, Heidelberg, Germany). Cancellous bone histomorphometry was performed as described (Schneider et al. 2009) using OsteoMeasure 3.0 (OsteoMetrics, Decatur, GA, USA) software. The area within 0.25 mm from the growth plate was excluded from the measurements.

**Mechanical testing**

A three-point bending (3pb) test was used to evaluate global mechanical bone strength. After µCT, the right femurs were refrozen at −80°C in 4% phosphate-buffered saline until the bending test was performed using Instron 5944 2kN single column (ITW Ltd, Buckinghamshire, UK). Breaking force and stiffness were derived directly from the load–displacement curves obtained by 3pb. The µCT data for the middle part of the beam were used to calculate actual cross-section area and cross-section second area moment. Due to the high ratio of cross-section depth to beam length, shear deformations contribute to the overall deflection. Therefore, Timoshenko beam theory was employed to derive a global Young's modulus (Spatz et al. 1996). The breaking stress was determined from beam theory, based on actual cross-section shapes and ultimate global loads.

**Intragastric pH and stomach weight**

Intragastric pH was measured immediately after euthanasia using a pediatric pH catheter, and the lowest
measured pH was recorded. The stomachs were removed and rinsed in saline, and thereafter weighed.

**Plasma gastrin, receptor activator of nuclear factor κB ligand (RANKL), osteoprotegerin (OPG), osteocalcin, leptin and sclerostin**

At termination of the study, blood was drawn from the inferior caval vein during isoflurane anesthesia, and plasma frozen at −20°C until analyses. Gastrin was measured by radioimmunoassay as described previously (Kleveland et al. 1985). OPG, osteocalcin, leptin and sclerostin in plasma were analyzed using multianalyte profiling Milliplex MAP assay, and RANKL using a single-analyte assay (Millipore). Analyses were performed by a Luminex-100 analyzer (Luminex, Austin, TX, USA).

**Bone marrow cell preparation, primary osteoblasts and osteoclasts for gene expression studies**

Bone marrow cells were obtained from femurs and tibiae from four young WT mice (12 weeks of age) by a modified version of a protocol described by Dobson and coworkers (1999). Briefly, tibiae and femurs were collected and all soft tissue was removed. The proximal ends were cut off, and the bones centrifuged (1000 g, 10 s). The bone marrow pellets from all animals were pooled and resuspended in culture medium (10% FCS. Eagle’s Medium (DMEM, Gibco) supplemented with 2% bovine serum albumin, 20 ng/mL RANKL and 100 ng/mL M-CSF (R&D Systems) for osteoclast differentiation in Dulbecco’s Modified Eagle’s Medium (DMEM, Gibco) supplemented with 10% fetal calf serum (FCS) (EuroClone, Pero, Italy) and seeded in 6-well plates. After 24 h, unattached cells were removed and reseeded in new 6-well plates for osteoclast differentiation in Dulbecco’s Modified Eagle’s Medium (DMEM, Gibco) supplemented with 10% FCS.

For osteoblast differentiation, the attached cells were grown until 70% confluence, and further differentiation up to 21 days with addition of 5.0 × 10⁻⁵ M ascorbic acid (Sigma–Aldrich), 2.0 × 10⁻⁶ M β-glycerophosphate (Sigma–Aldrich) and 1.0 × 10⁻⁹ M dexamethasone (Sigma–Aldrich). Cells were harvested for RNA isolation at day 1, 7, 14 and 21. To verify osteoblast differentiation from precursors, cells from parallel wells were stained for mineralization with alizarin red (Sigma–Aldrich).

For osteoclast differentiation, cells were grown until confluence and growth medium were enriched with 50 ng/mL RANKL and 100 ng/mL M-CSF (R&D Systems) for up to 6 days. Cells were harvested for RNA isolation at day 1, 3 and 6. To verify osteoclast differentiation from precursors, cells from parallel wells were stained for tartrate-resistant acid phosphatase (TRAP) activity at day 6, using Naphthol AS-BI phosphate and Fast Garnet in the presence of sodium tartrate, as described by the manufacturer (Sigma–Aldrich). TRAP-positive, multinuclear (3 or more nuclei) cells were regarded as osteoclasts. All experiments were performed in triplicates.

### Table 1 Bone mineral content and density, body composition and body length in KO and WT mice.

<table>
<thead>
<tr>
<th></th>
<th>WT/PEG</th>
<th>WT/NTZ</th>
<th>KO/PEG</th>
<th>KO/NTZ</th>
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<tbody>
<tr>
<td><strong>Baseline measures</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total mass (g)</td>
<td>19.05±1.17</td>
<td>19.76±1.60</td>
<td>19.92±1.58</td>
<td>18.09±1.37a</td>
</tr>
<tr>
<td>Fat (%)</td>
<td>12.0±6.3</td>
<td>12.2±3.1</td>
<td>11.4±2.8</td>
<td>11.4±2.8</td>
</tr>
<tr>
<td>Lean mass (%)</td>
<td>86.1±6.2</td>
<td>85.8±3.1</td>
<td>86.8±3.0</td>
<td>86.8±3.0</td>
</tr>
<tr>
<td>BMC (g)</td>
<td>0.38±0.05</td>
<td>0.42±0.07</td>
<td>0.34±0.03c</td>
<td>0.34±0.06</td>
</tr>
<tr>
<td>BMD (g/cm²)</td>
<td>0.063±0.005</td>
<td>0.063±0.004</td>
<td>0.056±0.004d</td>
<td>0.058±0.006</td>
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<tr>
<td><strong>Terminal measures</strong></td>
<td></td>
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</tr>
<tr>
<td>Total mass (g)</td>
<td>29.63±5.67</td>
<td>30.19±2.10</td>
<td>28.33±2.80</td>
<td>24.51±1.43a</td>
</tr>
<tr>
<td>Fat (%)</td>
<td>19.0±14.3</td>
<td>12.2±5.7</td>
<td>14.4±5.7</td>
<td>12.5±3.1</td>
</tr>
<tr>
<td>Lean mass (%)</td>
<td>78.3±14.4</td>
<td>85.1±5.7</td>
<td>83.5±5.7</td>
<td>85.0±3.0</td>
</tr>
<tr>
<td>Femur length (mm)</td>
<td>16.7±0.5</td>
<td>16.6±0.5</td>
<td>16.9±0.4</td>
<td>16.5±0.5</td>
</tr>
<tr>
<td>Body length (cm)</td>
<td>10.1±0.6</td>
<td>10.1±0.5</td>
<td>10.2±0.9</td>
<td>9.9±0.3</td>
</tr>
<tr>
<td>BMC (g)</td>
<td>0.83±0.18</td>
<td>0.80±0.09</td>
<td>0.61±0.09b</td>
<td>0.60±0.05</td>
</tr>
<tr>
<td>BMD (g/cm²)</td>
<td>0.080±0.005</td>
<td>0.082±0.002</td>
<td>0.070±0.004g</td>
<td>0.070±0.003</td>
</tr>
</tbody>
</table>

All variables are presented as mean ± s.d.

BMC, bone mineral content; BMD, bone mineral density; Body length, measured without tail; KO/NTZ, KO mice receiving netazepide; KO/PEG, KO mice receiving only vehicle; WT/NTZ, WT mice receiving netazepide; WT/PEG, wild-type mice receiving only vehicle.

*Significantly lower than KO/PEG, P = 0.012; †Significantly lower than KO/PEG, P = 0.009; ‡Significantly lower than WT/PEG, P = 0.037; §Significantly lower than WT/PEG, P = 0.003; ¶Significantly lower than KO/PEG, P = 0.033; ‡‡Significantly lower than WT/PEG, P = 0.004; ‡‡‡Significantly lower than WT/PEG, P < 0.001.
RNA isolation, cDNA synthesis and real-time PCR analyses

Gastric corpus from three WT mice were snap-frozen on liquid nitrogen and lysed in lysis buffer using a knife homogenizer, and primary osteoblasts and osteoclasts were lysed using a syringe and a needle in lysis buffer. Total RNA was isolated using the RNeasy Plus mini kit (Qiagen). The amount of total RNA in each sample was measured using a NanoDrop spectrophotometer, and equal amounts of total RNA from each sample were applied directly to obtain first-strand cDNA using the iScript cDNA synthesis kit with oligo-dT synthesis primer (Bio-Rad). The real-time PCR analyses were performed with TaqMan Gene Universal Master mix II and the TaqMan Gene Expression Assays (primers and probes) for gastrin/CCK2 receptor (assay ID MM00432329_m1) and Rn18s (assay ID: Mm0392899_gl) (Life Technologies, Thermo Fisher Scientific) and carried out on a StepOne Real-Time PCR system (Applied Biosystems) according to the manufacturer’s protocol. Data were calculated using the relative standard curve method applying Rn18s as the housekeeping gene.

Statistics

Values are presented as mean ± S.D. The WT/PEG and KO/PEG groups were first compared with confirm differences between the two genetic variants. Gastric weight and pH of all groups were compared to verify the effect of netazepide. For all other parameters, a comparison of the two WT groups and the KO groups separately was performed to evaluate the effects of netazepide. Where baseline data were available (BMC, BMD and body composition), we used a univariate ANOVA with the baseline values as covariate. For all other data, a two-tailed Student’s t-test or Mann–Whitney U test was applied, depending on data distribution. P<0.05 was considered significant. D’Agostino & Pearson omnibus normality test and Brown–Forsythe test were used to assess distribution and variances of the data. Analyses were performed by IBM SPSS statistics 20 (ANOVA analysis) and GraphPad Prism 6.

Results

During the study period, two animals died, one in the WT/NTZ group and one in the KO/PEG group, leaving 11 mice in the WT/PEG and KO/PEG groups and 9 in

Table 2  Right femur micro-CT and left tibia histomorphometry results in KO and WT mice.

<table>
<thead>
<tr>
<th></th>
<th>WT/PEG</th>
<th>WT/NTZ</th>
<th>KO/PEG</th>
<th>KO/NTZ</th>
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<tbody>
<tr>
<td><strong>Cortical bone morphology</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TL.Ar (mm²)</td>
<td>1.980 ±0.171</td>
<td>2.088 ±0.100</td>
<td>1.810 ±0.044</td>
<td>1.669 ±0.123</td>
</tr>
<tr>
<td>CT.Ar (mm²)</td>
<td>1.043 ±0.075</td>
<td>1.064 ±0.047</td>
<td>0.822 ±0.069</td>
<td>0.830 ±0.050</td>
</tr>
<tr>
<td>CT.Th (mm)</td>
<td>0.131 ±0.008</td>
<td>0.131 ±0.002</td>
<td>0.114 ±0.009</td>
<td>0.121 ±0.004</td>
</tr>
<tr>
<td>CT.Ar/TL.Ar (%)</td>
<td>0.528 ±0.024</td>
<td>0.510 ±0.016</td>
<td>0.454 ±0.044</td>
<td>0.498 ±0.019</td>
</tr>
<tr>
<td>Cort.BMD (g/cm³)</td>
<td>1.938 ±0.087</td>
<td>1.896 ±0.083</td>
<td>1.948 ±0.045</td>
<td>1.961 ±0.053</td>
</tr>
<tr>
<td><strong>Trabecular bone morphology</strong></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>BV/TV (%)</td>
<td>1.730 ±0.618</td>
<td>1.663 ±1.019</td>
<td>0.629 ±0.210</td>
<td>0.768 ±0.620</td>
</tr>
<tr>
<td>Tb.N (1/mm)</td>
<td>0.482 ±0.156</td>
<td>0.448 ±0.249</td>
<td>0.230 ±0.071</td>
<td>0.234 ±0.150</td>
</tr>
<tr>
<td>Tb.Th (mm)</td>
<td>0.035 ±0.004</td>
<td>0.036 ±0.003</td>
<td>0.027 ±0.002</td>
<td>0.032 ±0.005</td>
</tr>
<tr>
<td>Tb.Sp (mm)</td>
<td>0.425 ±0.045</td>
<td>0.482 ±0.120</td>
<td>0.524 ±0.033</td>
<td>0.610 ±0.143</td>
</tr>
<tr>
<td>Trab.BMD (g/cm³)</td>
<td>1.338 ±0.098</td>
<td>1.310 ±1.040</td>
<td>1.227 ±0.070</td>
<td>1.321 ±0.108</td>
</tr>
<tr>
<td><strong>Histomorphometry</strong></td>
<td></td>
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<td></td>
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<tr>
<td>MAR (µm/day)</td>
<td>0.22 (0.15, 0.29)</td>
<td>0.19 (0.10, 0.39)</td>
<td>0.28 (0.20, 0.37)</td>
<td>0.23 (0.22, 0.29)</td>
</tr>
<tr>
<td>MS/BS (%)</td>
<td>22.4 (15.5, 26.0)</td>
<td>23.4 (13.6, 40.2)</td>
<td>32.8 (26.2, 39.5)</td>
<td>38.6 (18.5, 51.3)</td>
</tr>
<tr>
<td>BFR/BS (µm²/µm²/day)</td>
<td>0.92 (0.78, 1.15)</td>
<td>0.81 (0.67, 0.97)</td>
<td>0.85 (0.77, 0.93)</td>
<td>0.71 (0.47, 1.35)</td>
</tr>
<tr>
<td>N.Oc/B.Pm (1/mm)</td>
<td>1.81 (0.63, 2.48)</td>
<td>0.73 (0.48, 1.07)</td>
<td>0.60 (0.59, 1.82)</td>
<td>0.84 (0.56, 0.96)</td>
</tr>
</tbody>
</table>

Micro-CT variables are presented as mean ± S.D. Histomorphometry is presented as median (interquartile range).

BFR/BS, bone formation rate per bone surface; BV/TV, bone volume fraction; Cort.BMD, cortical bone mineral density; CT.Ar, cortical bone area; CT.Ar/TL.Ar, cortical area fraction; CT.Th, cortical thickness; KO/NTZ, KO mice receiving netazepide; KO/PEG, KO mice receiving only vehicle; MAR, mineral apposition rate; MS/BS, mineralizing surface per bone surface; N.Oc/B.Pm, number of osteoclast bone per bone perimeter (osteoclast surface density); Tb.N, trabecular number; Tb.Sp, trabecular separation; Tb.Th, trabecular thickness; Trab.BMD, trabecular bone mineral density; Tt.Ar, total cross sectional area inside the periostal envelope; WT/NTZ, WT mice receiving netazepide; WT/PEG, wild-type mice receiving only vehicle.

*Significantly lower values than WT/PEG, P<0.010; **Significantly lower values than WT/PEG, P<0.001; "Significantly lower values than KO/PEG, P<0.004; 5Significantly higher values than KO/PEG, P=0.039; 6Significantly higher values than KO/PEG, P=0.019; 7Significantly higher values than KO/PEG, P=0.017; 8Significantly higher than WT/PEG, P<0.001; 9Significantly lower values than WT/PEG, P=0.006; "Significantly higher than KO/PEG, P=0.042.

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the two other groups. No differences were observed in animal welfare between the different groups.

**DXA measurements and femur lengths**

There was no significant difference in body composition between KO and WT groups at baseline. However, the KO group receiving netazepide had a significantly lower fat percentage ($P = 0.038$), and total mass ($P = 0.012$) at baseline than the KO group receiving only PEG. At termination, fat percentage, lean mass percentage, femur length and body length did not differ significantly between the groups. Total mass in the KO/NTZ group was significantly lower than in KO/PEG ($P = 0.033$), but no differences were seen between the other groups. Subtraction of stomach weight did not influence the results. KO mice had lower whole body BMC ($P = 0.035$) and BMD ($P = 0.003$) than WT mice at baseline. At termination, KO/PEG mice displayed a lower BMC ($P = 0.015$) and BMD ($P = 0.001$) than WT/PEG, whereas no differences were found between the netazepide and PEG groups (Table 1).

**Bone microarchitecture of the femur evaluated by µCT**

Trabecular BMD, trabecular thickness (Tb.Th), cortical area fraction (Ct.Ar/Tt.Ar) and cortical thickness (Ct.Th) were significantly lower in KO/PEG than in WT/PEG ($P < 0.01$), and were also significantly higher in the KO/NTZ group compared with KO/PEG ($P < 0.05$). Mean total cortical area (Tt.Ar) was lower in KO/PEG than in WT/PEG, and significantly lower in KO/NTZ compared with KO/PEG. Cortical area (Ct.Ar), trabecular number (Tb.N) and trabecular bone volume fraction (BV/TV) were significantly lower ($P < 0.001$), and trabecular separation (Tb.Sp) higher ($P < 0.001$) in KO/PEG compared with WT/PEG. These parameters (Ct.Ar, Tb.N, BV/TV and Tb.Sp) were not affected by netazepide administration (Table 2).

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**Figure 1**

Three-point bending tests of right femurs. KO/NTZ, KO mice receiving netazepide; KO/PEG, KO mice receiving only vehicle; WT/NTZ, WT mice receiving netazepide; WT/PEG, wild-type mice receiving only vehicle. H-K-ATPase KO have lower stiffness and higher failure displacement than WT. KO mice receiving the gastrin receptor antagonist netazepide develop higher Young's modulus than KO mice receiving only polyethylene glycol (PEG). *$P < 0.05$; **$P < 0.001$. A two-tailed Student's $t$-test was used for all statistical analyses.
Dynamic histomorphometry

As a result of very fragile bones at the age of 13 months, several tibiae were lost to analysis due to fracturing. The number of tibiae finally available for histomorphometric analysis was 10 WT/PEG, 9 WT/NTZ, 4 KO/PEG and 5 KO/NTZ. Of these bones, 4 tibiae did not display any trabecular bone (2 KO/PEG and 2 WT/NTZ), and in 5 tibiae (2 WT/PEG, 2 WT/NTZ and 1 KO/NTZ), osteoclasts could not be identified. The remaining bones, which were also scarce in trabecular bone, did not differ significantly in mineral apposition rate, bone formation rate or osteoclast numbers between the groups (data presented in Table 2).

Mechanical testing

Three-point bending tests revealed higher stiffness (146.0 ± 27.3 N/mm vs 101.7 ± 14.5 N/mm, \( P < 0.001 \)) and lower failure displacement (0.113 ± 0.029 mm vs 0.153 ± 0.125 mm \( P = 0.013 \)) in WT/PEG compared with KO/PEG and a trend toward higher values in KO/NTZ compared with KO/PEG (119.3 ± 24.4 N/mm vs 101.7 ± 14.5 N/mm, \( P = 0.063 \)). Breaking force tended to be higher in WT/PEG than in KO/PEG (16.22 ± 2.92 N vs 13.98 ± 2.38 N, \( P = 0.081 \)), whereas no differences were observed between the KO groups. For Young’s modulus, we did not find significant differences between WT and KO, but higher values in the KO/NTZ group compared...
with KO/PEG (15.07 ± 5.32 GPa vs 11.10 ± 1.93 GPa, \( P = 0.048 \)). There was no significant difference in breaking stress and work to failure between the groups (Fig. 1). Figure 2 shows individual load versus displacement curves from the 3pb tests.

**Intragastric pH and stomach weight**

Both KO groups had significantly higher intragastric pH than WT/PEG (\( P = 0.001 \)) and WT/NTZ (\( P = 0.003 \)). Also, the WT/NTZ mice had higher intragastric pH than WT/PEG (\( P = 0.044 \)). In the KO groups, lower pH levels were observed in the group receiving netazepide (\( P = 0.001 \)). Stomach weight was higher in the KO/PEG group than in all other groups (\( P = 0.001 \)), and the KO/NTZ group had higher stomach weight than both WT groups (\( P = 0.001 \)) (Fig. 3).

**Plasma gastrin, OPG, RANKL, osteocalcin, leptin and sclerostin**

Gastrin levels were higher in the KO groups compared with WT (\( P < 0.001 \)), and higher in WT/NTZ mice compared with WT/PEG (\( P = 0.005 \)). No significant differences between the two KO groups were observed (Fig. 3). We found a higher OPG level (\( P = 0.006 \)), as well as OPG/RANKL ratio (\( P = 0.043 \)), in KO/PEG mice compared with WT/PEG. The other parameters did not differ significantly between the groups (Table 3).

**Primary cells and gene expression**

Differentiation of primary mouse osteoblasts and osteoclasts resulted in osteoblasts as assessed by mineralization at day 21 and osteoclasts assessed by TRAP-positive multinucleated cells at day 6 (data not shown).

Neither primary osteoblasts nor osteoclasts expressed detectable gene products for the gastrin/CCK2 receptor, while control samples (corpus from WT mice) showed abundant gene expression of the gastrin/CCK2 receptor (data not shown).

**Discussion**

We have previously shown that H+/K+ATPase beta subunit KO mice exhibit an osteoporotic phenotype. In the current study, we gave these mice a potent and highly selective gastrin/CCK2 receptor antagonist, netazepide (Takinami et al. 1997), to examine whether circulating gastrin may influence their bone phenotype. Plasma analysis confirmed the hypergastrinemic state of the KO mice and gastric weight was significantly higher in KO compared with WT mice, probably reflecting a trophic effect of gastrin on the gastric mucosa (Bakkelund et al. 2010). Intragastric pH in WT mice receiving netazepide was elevated, and the gastric weight markedly reduced in the KO group receiving netazepide. This suggests that the netazepide dose has been sufficient to block the gastrin receptors of the ECL cells in the gastric corpus. We observed a slight improvement in bone structure assessed by µCT. Concerning mechanical properties, we find an increased Young’s modulus in KO/NTZ compared with KO/PEG, advocating improved elastic properties. Although not significant, a similar trend was observed for three-point bending test stiffness. We found no significant differences in histomorphometric parameters of cancellous bone turnover between the groups; however, the scarce amount of trabecular bone made it difficult to draw firm conclusions. A number of large studies have reported increased risk of fractures in long-term users of PPIs (Vestergaard et al. 2006, Yang et al. 2006, Targownik et al. 2008, Roux 2008, Vestergaard et al. 2006).
et al. 2009, Corley et al. 2010, Gray et al. 2010, Khalili et al. 2012) and patients with chronic atrophic gastritis (Goerres et al. 1992, Merriman et al. 2010). Histing and coworkers demonstrated delayed fracture healing in mice given the PPI pantoprazole (Histig et al. 2012), and our group has previously found reduced BMC, BMD and mechanical bone strength in $H^+\text{/}K^+$ATPase beta subunit KO mice (Fossmark et al. 2012). Gastric hypoacidity and secondary hypergastrinemia are common features of PPI users, patients with chronic atrophic gastritis and the $H^+\text{/}K^+$ATPase beta subunit KO mice. This suggests that low gastric acid or elevated serum gastrin could be of importance for the bone deterioration seen in the presence of a nonfunctioning gastric proton pump.

In the current study, we observed significantly lower BMC and BMD in KO mice compared with WT both at baseline and termination, which indicates that skeletal changes caused by $H^+\text{/}K^+$ATPase beta subunit deficiency start early in life. BMC and BMD at termination were significantly lower in KO mice also when corrected for baseline values, which shows that bone loss proceeds throughout life in KO mice. This was reflected in inferior bone quality, as well as impaired mechanical strength in KO mice compared with WT.

Several hypotheses have been proposed to explain the increased fracture risk in PPI users. Gastric anacidity has been suggested to affect bone metabolism through malabsorption of calcium, giving rise to secondary hyperparathyroidism. This is in accordance with our previous study on KO mice, showing higher plasma PTH in KO mice at 6 months of age (Fossmark et al. 2012). A study by Schinke and coworkers revealed that CCK2-deficient mice also had an osteoporotic phenotype (Schinke et al. 2009). In line with our KO mice, these mice had gastric hypoacidity and secondary hyperparathyroidism. Modest hypocalcemia was also observed, and calcium supplementation was found to rescue the osteoporotic phenotype (Schinke et al. 2009). Joo and coworkers also demonstrated increased osteoclast activity in ovariectomized rats receiving a combination of PPI and a low calcium diet compared with a low calcium diet alone (Joo et al. 2013). This implies that hypocalcemia may play a part in the osteoporotic process in $H^+\text{/}K^+$ATPase beta subunit KO mice.

Whether this also applies to humans with reduced gastric acid remains controversial. Though absorption of calcium from carbonate compounds in fasting, anacidic patients has been reported to be significantly lower than in controls (Recker 1985, O’Connell et al. 2005), three double-blind crossover studies failed to demonstrate altered absorption of isotopic-labeled calcium during PPI use in nonfasting conditions (Recker 1985, Wright et al. 2008, Hansen et al. 2010). This suggests that a possible effect of hypoacidity on calcium absorption in humans occurs only during fasting conditions, and does not affect calcium absorbed from a normal diet. It is also in accordance with other clinical studies, which fail to find elevated PTH levels in PPI users (Maggio et al. 2013, Sharara et al. 2013).

Reduced calcium absorption does not seem to provide a complete explanation of the differences seen between our KO and WT mice, nor the improvement in bone parameters in the KO/NTZ group. Even though the KO/NTZ mice had a significantly lower gastric pH than the KO/PEG group, both KO groups had severe hypoacidity (median pH above 5.8), and this difference is unlikely to influence calcium absorption. Moreover, gastric pH in the WT/NTZ group was significantly higher than in WT/PEG, though no skeletal differences were found between these groups.

The changes in bone parameters in KO mice receiving netazepide suggest that gastrin may play a role in the osteoporotic process observed in these mice. CCK2-deficient mice are referred to as a model for PPI use due to their hypoacidity (Schinke et al. 2009). Though caution should be applied in extrapolating findings in animal models to humans, our KO mice exhibit more similarities with PPI users, by presenting hypergastrinemia with intact gastrin receptors. Gastrin stimulates release of histamine, and other substances from the ECL cell. Hypergastrinemia would also affect gastrin receptors elsewhere in the body, and any influence of gastrin on bone homeostasis would be missed in a model with impaired gastrin receptors.

Research on direct gastrin effects on bone is scarce. Gastrin receptors have been detected in C-cells of the thyroid gland (Blaker et al. 2002) and in the brain (Lee et al. 1993), but have to our knowledge not been identified in bone. In the current study, PCR analyses of gastrin receptor expression in primary mouse osteoblasts and osteoclasts were also negative. A gastrin effect via other mediators, therefore, seems more plausible than a direct effect on bone.

Our previous study on this mouse model revealed elevated plasma PTH, interpreted as secondary hyperparathyroidism. Another possible explanation could be an effect of gastrin on PTH. To our knowledge, however, gastrin receptors have not been detected in the parathyroid, and the few studies performed on gastrin’s effect on PTH have given diverging results. One study on humans showed an increase in calcitonin and PTH after...
pentagastrin administration (Vantini et al. 1981), while others failed to find a relationship between gastrin and PTH (Coetzee et al. 1980). Due to low blood volumes available from mice, this analysis was not repeated in the current study and we are not able to draw firm conclusions in this matter.

Gastrin stimulates histamine release from ECL cells (Sandvik et al. 1987, Bakke et al. 2001), and histamine subsequently stimulates exchange of K+ and H+ by the H+/K+-ATPase of the parietal cell (Kleveland et al. 1987). It is known that systemic mastocytosis, a condition with elevated histamine in the systemic circulation, predisposes to osteoporosis, and expression of histamine receptor-1 and -2 mRNA has been demonstrated in both primary osteoclasts and osteoblasts in vitro (Biosse-Duplan et al. 2009). We previously showed that rats receiving the PPI omeprazole for 6 months exhibited elevated plasma histamine (Cui et al. 2001), and a clinical study revealed significantly elevated plasma histamine levels in patients using acid suppressing histamine-2-blockers (Waldum et al. 1989). Other murine and human studies have demonstrated transient increase in blood histamine after intravenous administration of pentagastrin (Man et al. 1984, Watanabe et al. 1996). Increased systemic histamine secondary to hypergastrinemia could play a role in the negative skeletal effects observed in PPI users and in our KO mice.

While no differences were found in other bone biomarkers, KO mice had elevated OPG and OPG/RANKL ratio in plasma compared with WT. These findings probably reflect compensatory mechanisms. It should, however, be kept in mind that OPG in plasma may be derived from other sources (Weitzzmann 2013).

There are some limitations to our study. We have not investigated potential changes in the organic matrix of the bone after netazepide treatment. Differences in advanced glycation end products (AGEs) or crosslinking could possibly explain why we observed increased Young’s modulus in the KO/NTZ group in spite of no differences in the µCT-derived cortical tissue mineral density. Secondly, the lack of trabecular bone in the histomorphometry samples precluded our ability to conclude on whether netazepide administration induced changes in bone formation or resorption. Finally, we cannot rule out that snap freezing and µCT scanning of the bones before mechanical testing might have influenced the mechanical properties of the bones. However, others have found that deep freezing does not influence the mechanical strength of rat bones (Pelker et al. 1984). Lee and coworkers also found that µCT irradiation had a negligible effect on the Young’s modulus of trabecular bone (Lee & Jasiuk 2014). All the bones in our study were handled in the same way, and possible alterations should, therefore, be equal in all groups.

In conclusion, our findings suggest that gastrin, or processes mediated by gastrin, might contribute to the deterioration of bone quality and increased bone fragility in H+/K+-ATPase beta subunit KO mice. The mechanism behind this, however, remains unclear, and other processes seem to play a more important role.

Declaration of interest
Malcolm Boyce is the Medical and Managing Director of Hammersmith Medicines Research and holds the license for netazepide. The other authors have nothing to disclose.

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Skeletal effect of gastrin receptor antagonist

Research

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