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. 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

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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 hypoacidity, 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 (Goeris *et al.* 1992, Merriman *et al.* 2010). This suggests that the effect of PPIs on bone could be related to gastric hypoacidity and its consequences, rather than a direct drug effect on bone.

While the role of hypoacidity 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: AGACAATCGGCTGCTCTGAT and reverse primer: ATACTTTCTCGGCAGGAGCA, 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 $24\pm1^{\circ}\text{C}$ 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 $\mu\text{mol/kg}$) 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 initiation and 2% during the scan, with O₂ and N₂ in a ratio of 3:2 as carrier gas with total gas flow of 1 L/min.

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). Scans were performed with a pixel size of 5 μm , with 0.5 mm aluminum filter, current of 163 μA , voltage 61 kV, rotation step of 0.4 and frame averaging of 3. The datasets were reconstructed using the Skyscan NRecon software (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 κ B 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 (1000g, 10 s). The bone marrow pellets from all animals were pooled and resuspended in culture medium (MEM- α) (Gibco BRL, Life Technologies) 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.

	WT/PEG	WT/NTZ	KO/PEG	KO/NTZ
Baseline measures				
Total mass (g)	19.05 $\pm$ 1.17	19.76 $\pm$ 1.60	19.92 $\pm$ 1.58	18.09 $\pm$ 1.37 ^a
Fat (%)	12.0 $\pm$ 6.3	12.2 $\pm$ 3.1	11.4 $\pm$ 2.8	8.7 $\pm$ 2.8 ^b
Lean mass (%)	86.1 $\pm$ 6.2	85.8 $\pm$ 3.1	86.8 $\pm$ 3.0	89.4 $\pm$ 2.9
BMC (g)	0.38 $\pm$ 0.05	0.42 $\pm$ 0.07	0.34 $\pm$ 0.03 ^c	0.34 $\pm$ 0.06
BMD (g/cm ²)	0.063 $\pm$ 0.005	0.063 $\pm$ 0.004	0.056 $\pm$ 0.00 ^d	0.058 $\pm$ 0.006
Terminal measures				
Total mass (g)	29.63 $\pm$ 5.67	30.19 $\pm$ 2.10	28.33 $\pm$ 2.80	24.51 $\pm$ 1.43 ^e
Fat (%)	19.0 $\pm$ 14.3	12.2 $\pm$ 5.7	14.4 $\pm$ 5.7	12.5 $\pm$ 3.1
Lean mass (%)	78.3 $\pm$ 14.4	85.1 $\pm$ 5.7	83.5 $\pm$ 5.7	85.0 $\pm$ 3.0
Femur length (mm)	16.7 $\pm$ 0.5	16.6 $\pm$ 0.5	16.9 $\pm$ 0.4	16.5 $\pm$ 0.5
Body length (cm)	10.1 $\pm$ 0.6	10.1 $\pm$ 0.5	10.2 $\pm$ 0.9	9.9 $\pm$ 0.3
BMC (g)	0.83 $\pm$ 0.18	0.80 $\pm$ 0.09	0.61 $\pm$ 0.09 ^f	0.60 $\pm$ 0.05
BMD (g/cm ²)	0.080 $\pm$ 0.005	0.082 $\pm$ 0.002	0.070 $\pm$ 0.004 ^g	0.070 $\pm$ 0.003

All variables are presented as mean $\pm$ 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.

^aSignificantly lower than KO/PEG, $P=0.012$; ^bSignificantly lower than KO/PEG, $P=0.009$; ^cSignificantly lower than WT/PEG, $P=0.037$; ^dSignificantly lower than WT/PEG, $P=0.003$; ^eSignificantly lower than KO/PEG, $P=0.033$; ^fSignificantly lower than WT/PEG, $P=0.004$; ^gSignificantly 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 oligodeoxythymidine (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 assays for gastrin/CCK2 receptor (assay ID Mm00432329_ml) 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 $\pm$ 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/NTZ groups and 9 in

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

	WT/PEG	WT/NTZ	KO/PEG	KO/NTZ
Cortical bone morphology				
Tt.Ar (mm ²)	1.980 $\pm$ 0.171	2.088 $\pm$ 0.100	1.810 $\pm$ 0.044 ^a	1.669 $\pm$ 0.123 ^c
Ct.Ar (mm ²)	1.043 $\pm$ 0.075	1.064 $\pm$ 0.047	0.822 $\pm$ 0.069 ^b	0.830 $\pm$ 0.050
Ct.Th (mm)	0.131 $\pm$ 0.008	0.131 $\pm$ 0.002	0.114 $\pm$ 0.009 ^b	0.121 $\pm$ 0.004 ^d
Ct.Ar/Tt.Ar (%)	0.528 $\pm$ 0.024	0.510 $\pm$ 0.016	0.454 $\pm$ 0.044 ^b	0.498 $\pm$ 0.019 ^e
Cort.BMD (g/cm ³)	1.938 $\pm$ 0.087	1.896 $\pm$ 0.083	1.948 $\pm$ 0.045	1.961 $\pm$ 0.053
Trabecular bone morphology				
BV/TV (%)	1.730 $\pm$ 0.618	1.663 $\pm$ 1.019	0.629 $\pm$ 0.210 ^b	0.768 $\pm$ 0.620
Tb.N (1/mm)	0.482 $\pm$ 0.156	0.448 $\pm$ 0.249	0.230 $\pm$ 0.071 ^b	0.234 $\pm$ 0.150
Tb.Th (mm)	0.035 $\pm$ 0.004	0.036 $\pm$ 0.003	0.027 $\pm$ 0.002 ^b	0.032 $\pm$ 0.005 ^f
Tb.Sp (mm)	0.425 $\pm$ 0.045	0.482 $\pm$ 0.120	0.524 $\pm$ 0.033 ^g	0.610 $\pm$ 0.143
Trab.BMD (g/cm ³)	1.338 $\pm$ 0.098	1.310 $\pm$ 0.104	1.227 $\pm$ 0.078 ^h	1.321 $\pm$ 0.108 ⁱ
Histomorphometry				
MAR (μ m/day)	0.22 (0.15, 0.29)	0.19 (0.10, 0.39)	0.28 (0.20, 0.37)	0.23 (0.22, 0.29)
MS/BS (%)	22.4 (15.5, 26.0)	23.4 (13.6, 40.2)	32.8 (26.2, 39.5)	38.6 (18.5, 51.3)
BFR/BS (μ m ³ /mm ² /day)	0.92 (0.78, 1.15)	0.81 (0.67, 0.97)	0.85 (0.77, 0.93)	0.71 (0.47, 1.35)
N.Oc/B.Pm (1/mm)	1.81 (0.63, 2.48)	0.73 (0.48, 1.07)	0.60 (0.59, 1.82)	0.84 (0.56, 0.96)

Micro-CT variables are presented as mean $\pm$ 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/Tt.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 periosteal envelope; WT/NTZ, WT mice receiving netazepide; WT/PEG, wild-type mice receiving only vehicle.

^aSignificantly lower values than WT/PEG, $P = 0.010$; ^bSignificantly lower values than WT/PEG $P < 0.001$; ^cSignificantly lower values than KO/PEG, $P = 0.004$;

^dSignificantly higher values than KO/PEG, $P = 0.039$; ^eSignificantly higher values than KO/PEG, $P = 0.019$; ^fSignificantly higher values than KO/PEG,

$P = 0.017$; ^gSignificantly higher than WT/PEG, $P < 0.001$; ^hSignificantly lower values than WT/PEG, $P = 0.006$; ⁱSignificantly higher than KO/PEG, $P = 0.042$.

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.

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

WT/PEG, whereas no differences were found between the netazepide and PEG groups (Table 1).

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

Bone microarchitecture of the femur evaluated by μ CT

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).

Figure 2

Load versus displacement curves from three-point bending tests. 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 (polyethylene glycol).

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 ($146.0 \pm 27.3 \text{ N/mm}$ vs $101.7 \pm 14.5 \text{ N/mm}$, $P < 0.001$) and lower failure displacement ($0.113 \pm 0.029 \text{ mm}$ vs $0.153 \pm 0.125 \text{ mm}$, $P = 0.013$) in WT/PEG compared with KO/PEG and a trend toward higher values in KO/NTZ compared with KO/PEG ($119.3 \pm 24.4 \text{ N/mm}$ vs $101.7 \pm 14.5 \text{ N/mm}$, $P = 0.063$). Breaking force tended to be higher in WT/PEG than in KO/PEG ($16.22 \pm 2.92 \text{ N}$ vs $13.98 \pm 2.38 \text{ N}$, $P = 0.081$), 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

Figure 3

Plasma gastrin, intragastric pH and stomach weight. 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. Administration of the gastrin receptor antagonist netazepide elevates plasma gastrin levels (A) and intragastric pH (B) in WT mice, and counteracts the trophic effect of gastrin on gastric mucosa in KO mice (C). a: elevated compared with all other groups, $P < 0.001$. b: elevated compared with both WT groups, $P < 0.001$. c: elevated compared with WT/PEG, $P < 0.05$. Mann-Whitney *U* test was used for comparison. Bars are presented with mean values and s.d.

Table 3 Plasma analyses of KO and WT mice.

	WT/PEG	WT/NTZ	KO/PEG	KO/NTZ
OPG (pg/mL)	1256 ± 796	1121 ± 370	2737 ± 2252 ^a	1576 ± 432
RANKL (pg/mL)	116.3 ± 81.44	87.75 ± 59.93	74.37 ± 57.61	68.80 ± 25.81
OPG/RANKL	36.0 ± 78.7	19.3 ± 13.1	89.7 ± 163.8 ^b	25.2 ± 9.3
Leptin (pg/mL)	6002 ± 6719	4839 ± 2336	3171 ± 1957	2588 ± 1229
Sclerostin (pg/mL)	73.7 ± 18.4	66.3 ± 8.7	74.8 ± 64.9	63.6 ± 27.1
Osteocalcin (pg/mL)	16,185 ± 1900	15,767 ± 1101	16,669 ± 2928	16,367 ± 2116

Data are presented as mean ± s.d.

KO/NTZ, KO mice receiving netazepide; KO/PEG, KO mice receiving only vehicle; OPG, osteoprotegerine; RANKL, receptor activator of nuclear factor κ-B ligand; WT/NTZ, WT mice receiving netazepide; WT/PEG, wild-type mice receiving only vehicle (polyethylene glycol).

^aSignificantly higher than WT/PEG $P=0.006$; ^bSignificantly higher than WT/PEG ($P=0.043$).

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 3 pb 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

et al. 2009, Corley *et al.* 2010, Gray *et al.* 2010, Khalili *et al.* 2012) and patients with chronic atrophic gastritis (Goerss *et al.* 1992, Merriman *et al.* 2010). Histing and coworkers demonstrated delayed fracture healing in mice given the PPI pantoprazole (Histing *et al.* 2012), and our group has previously found reduced BMC, BMD and mechanical bone strength in H⁺/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⁺/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⁺/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 H⁺/K⁺ATPase beta subunit 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⁺/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 (Weitzmann 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 μ CT-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 μ CT 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

μ CT 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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