Bone mineral density and expression of vitamin D receptor-dependent calcium uptake mechanisms in the proximal small intestine after bariatric surgery

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Background: Roux-en-Y gastric bypass may lead to impaired calcium uptake. Therefore, operation-specific effects of gastric bypass and vertical banded gastroplasty on bone mineral density (BMD) were examined in a randomized clinical trial. Bone resorption markers and mechanisms of decreased calcium uptake after gastric bypass were investigated using blood and endoscopic samples from two additional patient cohorts.

Methods: Total BMD and non-weight-bearing skull BMD were measured by dual-energy X-ray absorptiometry at baseline, and 1 and 6 years after gastric bypass or vertical banded gastroplasty in patients who were not receiving calcium supplements. Bone resorption markers in serum and calcium uptake mechanisms in jejunal mucosa biopsies were analysed after gastric bypass by proteomics including radioimmunoassay, gel electrophoresis and mass spectrometry.

Results: One year after surgery, weight loss was similar after gastric bypass and vertical banded gastroplasty. There was a moderate decrease in skull BMD after gastric bypass, but not after vertical banded gastroplasty (P < 0.001). Between 1 and 6 years after gastric bypass, skull BMD and total BMD continued to decrease (P = 0.001). C-terminal telopeptide levels in serum had increased twofold by 18 months after gastric bypass. Proteomic analysis of the jejunal mucosa revealed decreased levels of heat-shock protein 90β, a co-activator of the vitamin D receptor, after gastric bypass. Despite increased vitamin D receptor levels, expression of the vitamin D receptor-regulated calcium transporter protein TRPV6 decreased.

Conclusion: BMD decreases independently of weight after gastric bypass. Bone loss might be attributed to impaired calcium absorption caused by decreased activation of vitamin D-dependent calcium absorption mechanisms mediated by heat-shock protein 90β and TRPV6.

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Introduction

Bariatric surgery is currently the most effective treatment for obesity. Roux-en-Y gastric bypass (RYGBP) has been shown to reduce mortality and obesity-related metabolic complications compared with non-surgical obesity treatment1. Although calorie malabsorption is not believed to be the primary mechanism explaining weight loss after RYGBP, there is some concern regarding the risk of decreased uptake of specific vitamins and minerals owing to the rerouting of the proximal gastrointestinal tract. RYGBP results in exclusion of the major part of the stomach and the entire duodenum as well as diversion of bile and pancreatic juices from the alimentary limb (proximal jejunum). Uptake of certain nutrients that are absorbed primarily in the proximal small intestine could therefore be affected.

Intestinal calcium uptake is dependent on the amount of calcium ingested and the efficiency of intestinal calcium absorption.
uptake; it is partly an active transcellular process that is saturable, and partly a passive paracellular process that is non-saturable. Active calcium absorption is an energy-dependent process that is localized to the proximal small intestine (duodenum and proximal jejunum), and its efficacy is regulated by activated vitamin D via the vitamin D receptor. The exact mechanism by which the vitamin D receptor regulates calcium uptake is unclear, but several studies have shown that the activated receptor regulates transcriptionally several proteins believed to participate in active calcium absorption, such as the specific calcium transporter protein, transient receptor potential cation channel, subfamily V, member 6 (TRPV6).

The authors hypothesized that exclusion of the duodenum and diversion of bile and pancreatic juices from the proximal jejunum could affect intestinal calcium absorption, and possibly result in decreased bone mineral density (BMD).

Methods

Data and materials from three different clinical studies, one randomized clinical trial and two patient cohorts, were used for the present analysis. The local ethics committee of the University of Gothenburg approved the study protocols (reference numbers 380-06, 583-07 and 647-05) and the studies were conducted according to the principles of the Helsinki declaration. All patients gave written informed consent to participate.

For the randomized trial, which commenced in 1999, patients were allocated to either Roux-en-Y gastric bypass (RYGBP) or vertical banded gastroplasty (VBG). The primary endpoint was weight loss.

The first prospective cohort study comprised a hormone analysis of bone resorption markers after RYGBP. Consecutive patients with a body mass index (BMI) over 35 kg/m² were recruited between 2008 and 2009 at Sahlgrenska University Hospital, Gothenburg, Sweden. Exclusion criteria were type II diabetes, cardiovascular disease, other severe co-morbidities, and age below 18 or above 60 years. Fasting samples taken before and 18 months after surgery were used for the analysis of bone resorption markers.

The second prospective cohort study included consecutive diabetic and non-diabetic patients with a BMI over 35 kg/m². Jejunal samples were retrieved during surgery, and gastroscopy was performed 6–8 months after operation for sampling of jejunal mucosa for proteomics and/or western blot analysis. Exclusion criteria were cardiovascular disease or other severe co-morbidities, and age below 18 or above 60 years.

Weight, height, body mass index and body composition

Weight and height were measured with the patient in light underwear after an overnight fast. Height was measured to the closest 0–5 cm using a wall-mounted standard stadiometer with the patient standing. Weight was measured to the nearest 0–1 kg on an electronic scale, which was calibrated at regular intervals. BMI was calculated (kg/m²). Weight outcome at 2 years after surgery has been reported previously from this study.

Body composition was assessed using a LUNAR DPX-IQ dual-energy X-ray absorptiometry scanner (Lunar, Madison, Wisconsin, USA). Software version 4.7, 4.7c or 4.7e was used at baseline, and version 4.7e at the 6-year follow-up. An extended analysis program for total body analysis (Lunar) was used. Body fat, lean tissue mass, bone mineral content, BMD and bodyweight were assessed as described previously. As RYGBP induces weight loss that by itself may affect whole-body BMD, the non-weight-bearing BMD of the skull was also studied.

Dietary intake

All patients were recommended to take non-prescription multivitamin/mineral supplements after primary surgery, and those in the gastric bypass group were prescribed vitamin B12 supplement. When the randomized study commenced additional calcium and vitamin D supplementation was not prescribed routinely. Therefore, patients in the randomized trial did not systematically receive calcium and vitamin D supplementation. However, patients in the cohort studies were prescribed calcium/vitamin D supplementation (500 mg/400 units twice daily). Patients in the randomized trial were asked to fill in a recall questionnaire in which they were instructed to describe their food intake over the previous 3 months. The questionnaire has been validated for assessment in obese and lean subjects. Total energy intake, and intake of macronutrients (fat, carbohydrates and protein) and different food groups (fruit and vegetables, desserts, candy or prepared food) were analysed.

Measurement of bone resorption markers in serum

25-Hydroxy (25(OH)) vitamin D was measured by means of a Liaison® analyser (DiaSorin, West Boylston, Massachusetts, USA) (reference range 25–100 nmol/l).
1,25-Dihydroxy (1,25(OH)₂) vitamin D was measured using a commercially available radioimmunoassay kit (IDS, Boldon, UK). The intra-assay and interassay coefficients of variation (CVs) were 8.7 per cent (n = 20) and 12.6 per cent (n = 20) respectively. The 95 per cent c.i. for normal adults for the 1,25(OH)₂ vitamin D assay was 43 to 168 pmol/l. Intact parathyroid hormone (PTH) was measured using an ADVIA Centaur® system (Siemens Healthcare Diagnostic, Frimley, UK). The interassay CV for PTH was 3.4–5.2 per cent and the intra-assay CV was 4.3–9.1 per cent. The reference range for PTH was 10–70 ng/ml. A serum CrossLaps® enzyme-linked immunosorbent assay (ELISA) kit (IDS) was used for the measurement of C-terminal telopeptide (CTX), with an interassay CV of below 6 per cent and an intra-assay CV of less than 10 per cent. The reference range (95 per cent c.i.) for CTX was 0.122 to 0.738 ng/ml in premenopausal women and 0.142 to 1.351 ng/ml in postmenopausal women. A Quantikine® ELISA kit (R&D Systems, Abingdon, UK) was used to measure vitamin D-binding protein.

Protein expression in the alimentary limb mucosa after Roux-en-Y gastric bypass

For the proteomics analysis, tissue samples were collected as described previously⁹ from patients who had a first-time laparoscopic RYGBP or a conversion from VBG to RYGBP. The operative technique included an antecolic–antegastric Roux-en-Y construction with a 10–20-ml gastric pouch. The gastroenteric anastomosis was constructed with a straight 45-mm stapler and complementary hand-suturing. A tissue sample was removed from the jejunum between the gastroenteric and the enteroenteric anastomosis as the 75-cm loop was divided to create the Roux-en-Y construction. After excision the mucosal/submucosal biopsy was retrieved using an endoscopic biopsy forceps, in order to make the sampling depth as similar as possible to that during postoperative sampling by gastroscopy. Between 6 and 8 months after surgery the patients underwent an endoscopic examination of the alimentary limb. Mucosal biopsies were collected approximately 8 cm distal to the stoma. Mucosal tissue specimens were snap-frozen in liquid nitrogen and kept frozen (–70 °C) for later analysis of protein expression.

Global protein expression analysis of proximal jejunal mucosal epithelium was performed by two-dimensional gel electrophoresis and mass spectrometry by nanoflow liquid chromatography–mass spectrometry (LC-MS/MS). Biopsy samples were lyophilized and ground to a coarse powder. Protein was dissolved in sample solubilization buffer consisting of 7 mol/l urea, 2 mol/l thiourea, 50 mmol/l sodium dodecyl sulphate (SDS), 50 mmol/l CHAPS and 50 mmol/l Tris-hydrochloric acid (pH 8.0). Interfering substances were removed by using the ProteoExtract Protein Precipitation Kit (Calbiochem, Darmstadt, Germany) in accordance with the manufacturer’s instructions. The pellet was resolved in labelling buffer (7 mol/l urea, 2 mol/l thiourea, 4 per cent CHAPS, 30 mmol/l Tris-hydrochloric acid; pH 8.5) and protein concentration was determined by means of the Non-Interfering Protein Assay Kit (Calbiochem). All samples were diluted to a final protein concentration of 2 μg/μl. Each sample (50 μg) was labelled with 400 pmol of respective CyDye and pooled following a standard protocol (GE Healthcare, Uppsala, Sweden). Isoelectric focusing was done in 24-cm Nonlinear Immobiline™ DryStrip, pH 3–11 on an Ettna IPGphore (GE Healthcare). The second dimension was run on an Ettna DALT II system (GE Healthcare) in 1-mm acrylamide (T = 11 per cent, C = 2.6 per cent) Bis–Tris gel with standard MOPS cathode buffer and acetic acid/diethanol amine anode buffer.

Gel images were analysed using DeCyder 2-D Differential Analysis Software version 6.0 (GE Healthcare). Spots were selected for picking and further MS analysis. Selected protein spots, on a preparative gel of pooled samples to a total protein concentration of 450 μg stained with SYPRO Ruby, were picked and trypsinized in an Ettna Spot-handling Workstation (GE Healthcare). The method for in-gel protein digestion with trypsin described by Shevchenko and colleagues¹⁰ was applied with some minor modifications. Briefly, the gel pieces were destained by washing three times in 25 mmol/l ammonium bicarbonate in 50 per cent methanol and once in 70 per cent acetonitrile. Gel pieces were dried and incubated with digestion buffer (50 mmol/l ammonium bicarbonate, 10 ng/μl trypsin) at 37 °C for 3 h. Peptides were extracted in 50 per cent acetonitrile/0.5 per cent trifluoroacetic acid and the supernatant was evaporated to dryness. Before MS analysis, the peptides were reconstituted in 0.2 per cent methanoic acid.

Sample injections were made with an HTC-PAL autosampler (CTC Analytics, Zwingen, Switzerland) connected to an Agilent 1100 binary pump (Agilent Technologies, Palo Alto, California, USA). The peptides were trapped on a precolumn (45×0.075-mm internal diameter) and separated on a reversed phase column (200×0.050 mm). Both columns were packed with 3-μm Reprosil-Pur® C₁₈-AQ particles (Dr Maisch, Ammerbuch, Germany). The flow through the analytical column was reduced by a split to approximately 100 nl/min. A 40-min gradient of 10–50 per cent acetonitrile in 0.2
per cent carboxylic acid was used for separation of the peptides. Nanoflow LC-MS/MS was performed on a hybrid linear ion trap–Fourier transform ion cyclotron resonance (FTICR) mass spectrometer equipped with a 7-T ICR magnet (LTQ-FT; Thermo Electron, Bremen, Germany). The spectrometer was operated in data-dependent mode, automatically switching to MS/MS mode. MS spectra were acquired in the FTICR part, whereas MS/MS spectra were acquired in the linear quadruple trap of the instrument. For each FTICR scan, the three most intense, doubly or triply charged, ions were fragmented sequentially in the linear trap by collision-induced dissociation. All the tandem mass spectra were searched by Mascot (Matrix Science, London, UK) against all species in the National Centre for Biotechnology Information database. The search parameters were set to: MS accuracy 5 p.p.m., MS/MS accuracy 0-5 Da, one missed cleavage by trypsin allowed, fixed propionamide modification of cysteine and variable modification of oxidized methionine. For protein identification the minimum criterion was one tryptic peptide matched. Only proteins that were regulated in the same direction in all patients, and at least 50 per cent from peptide matched. Only proteins that were regulated in the same direction in all patients, and at least 50 per cent from baseline, were selected for further analysis.

Western blot

Total protein samples were diluted in SDS buffer and heated at 70°C for 10 min before being loaded on a NuPAGE® 10 per cent Bis–Tris gel (Invitrogen, Lidingö, Sweden), and electrophoresed using a MOPS buffer (Invitrogen). One lane of each gel was loaded with a prestained molecular weight standard (SeeBlue; Invitrogen). A positive control was loaded when available. After electrophoresis the proteins were transferred to a polyvinylidifluoride transfer membrane (Hybond™, 0.45 μm; GE Healthcare Life Sciences, Little Chalfont, UK) using an iBlot® system (Invitrogen). Membranes were then incubated with polyclonal specific primary antibodies against heat-shock protein 90β, vitamin D receptor or TRPV6. A secondary alkaline phosphatase-conjugated goat antirabbit IgG antibody was used with CDP-Star® (Tropix, Bedford, Massachusetts, USA) as a substrate, to identify immunoreactive proteins by chemiluminescence. Glycerinaldehyde 3-phosphate dehydrogenase (GAPDH; Imgenex, BioSite, San Diego, California, USA) was used as control for equal loading, and for each tested sample the optical density of primary antibody/GAPDH represents the result. The membrane was stripped for reprobing with other primary antibodies using a stripping buffer (ReBlot Plus Mild Solution (10×); Millipore, Temecula, California, USA). Images were captured by a Chemidoc™ XRS cooled charged-coupled device camera (Bio-Rad Laboratories, Hercules, California, USA), and semiquantification was done using Quantity One® software (Bio-Rad).

Statistical analysis

Statistical analyses of dual-energy X-ray absorptiometry and western blot data were carried out using non-parametric tests (Wilcoxon signed rank test and Mann–Whitney U test). A multiple linear regression model was used to evaluate the impact of operation type and weight loss on changes in skull BMD from baseline to year 1. Paired-samples t test was used for analysis of intra-individual changes in hormone levels of CTX, 25(OH) vitamin D, 1,25(OH)₂ vitamin D, PTH and vitamin D-binding protein, as well as for analysis of changes in protein levels measured in the proteomics analysis. All tests were two-tailed and P < 0.050 was considered statistically significant. SPSS® version 16 (IBM, Armonk, New York, USA) was used for statistical analysis.

Results

For the randomized trial, of 100 patients assessed for eligibility, a total of 82 were recruited, of whom 37 patients underwent RYGBP and 45 VBG. Baseline statistics have been published previously6. There were no significant differences in preoperative BMI, age, sex or total BMD between the RYGBP and VBG groups. Of 37 patients randomized to gastric bypass and 45 to VBG, 36 and 39 respectively remained in the study after 1 year, and only 28 and 35 underwent dual-energy X-ray absorptiometry owing to space limitation of the machine. After 6 years, only 17 and 14 patients respectively remained in their original groups and were assessed by dual-energy X-ray absorptiometry. Data from these patients were used for the present analyses of BMD.

The first cohort study included 46 consecutive patients (28 women and 18 men). Mean(s.d.) BMI decreased from 44.0(5.8) kg/m² before operation to 30.3(6.1) kg/m² 18 months after RYGBP in this cohort (P < 0.001). The second cohort study included seven diabetic and ten non-diabetic patients (11 women and 6 men). Their mean BMI was 41.3(4.1) kg/m² before surgery and 32.0(3.0) kg/m² at 6–8 months after gastric bypass.

Weight-loss phase: first year after surgery

Weight loss

At 1 year after surgery, bodyweight was significantly lower after RYGBP than VBG (Table 1). The weight loss was
Bone mineral density and calcium uptake in small intestine after bariatric surgery

**Table 1** Bodyweight and bone density during the weight-loss phase from baseline to 1 year after surgery

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>1 year postop.</th>
<th>Change after 1 year</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean(s.d.)</td>
<td>Mean(s.d.)</td>
<td>Mean(s.d.)</td>
</tr>
<tr>
<td>Bodyweight (kg)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RYGBP</td>
<td>114.9(10.4)</td>
<td>80.9(10.2)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>VBG</td>
<td>118.3(9.9)</td>
<td>89.7(13.2)</td>
<td>-33.9(7.6)</td>
</tr>
<tr>
<td>Total BMD (g/cm²)</td>
<td>2.53(0.08)</td>
<td>1.24(0.08)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>VBG</td>
<td>2.4(0.22)</td>
<td>2.3(0.25)</td>
<td>-0.08(0.10)</td>
</tr>
</tbody>
</table>

RYGBP, Roux-en-Y gastric bypass (28 patients); VBG, vertical banded gastroplasty (35 patients); BMD, bone mineral density. *RYGBP versus VBG (Mann–Whitney U test); †baseline versus 1 year (Wilcoxon signed rank test).

**Table 2** Bodyweight and bone mineral density during the weight-stable phase after surgery

<table>
<thead>
<tr>
<th></th>
<th>1 year postop.</th>
<th>6 years postop.</th>
<th>Change from year 1 to year 6</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean(s.d.)</td>
<td>Mean(s.d.)</td>
<td>Mean(s.d.)</td>
</tr>
<tr>
<td>Bodyweight (kg)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RYGBP</td>
<td>81.8(7.9)</td>
<td>84.6(12.1)</td>
<td>2.3(10.6)</td>
</tr>
<tr>
<td>VBG</td>
<td>87.0(13.0)</td>
<td>97.3(14.5)</td>
<td>9.6(15.4)</td>
</tr>
<tr>
<td>Total BMD (g/cm²)</td>
<td>1.24(0.08)</td>
<td>1.15(0.07)</td>
<td>-0.08(0.06)</td>
</tr>
<tr>
<td>VBG</td>
<td>1.28(0.07)</td>
<td>1.27(0.08)</td>
<td>-0.03(0.05)</td>
</tr>
<tr>
<td>Skull BMD (g/cm²)</td>
<td>2.37(0.23)</td>
<td>2.26(0.20)</td>
<td>-0.12(0.13)</td>
</tr>
<tr>
<td>VBG</td>
<td>2.39(0.22)</td>
<td>2.38(0.23)</td>
<td>-0.05(0.14)</td>
</tr>
</tbody>
</table>

RYGBP, Roux-en-Y gastric bypass (17 patients); VBG, vertical banded gastroplasty (14 patients); BMD, bone mineral density. Values were missing at 1 year for one patient with RYGBP and two patients with VBG, and at 6 years for two patients with RYGBP for the skull BMD measurements. *RYGBP versus VBG (Mann–Whitney U test); †baseline versus 6 years (Wilcoxon signed rank test).

not, however, significantly different between the groups, although both groups decreased significantly in weight compared with baseline.

**Total bone mineral density**

Total BMD was similar in the two groups before surgery (Table 1). After 1 year, there was no significant difference in total BMD or change in total BMD between the groups. Patients who underwent VBG had a small but significant increase in total BMD (Table 1).

**Skull bone mineral density**

There was no significant difference in skull BMD between the RYGBP and VBG groups at baseline or at 1 year after surgery (Table 1). Skull BMD was, however, reduced significantly at 1 year after RYGBP compared with baseline, but not in patients who had VBG. There was a significant difference in the change in skull BMD at 1 year between the groups.

In a multiple linear regression analysis of factors predicting changes in skull BMD during the first year after surgery, there was no significant difference in skull BMD at 1 year between the RYGBP and VBG groups.

**Table 3** Bone resorption markers before and after Roux-en-Y gastric bypass

<table>
<thead>
<tr>
<th></th>
<th>No. of patients</th>
<th>Baseline</th>
<th>18 months postop.</th>
<th>P†</th>
<th>Reference range</th>
</tr>
</thead>
<tbody>
<tr>
<td>C-terminal telopeptide (pg/ml)</td>
<td>39</td>
<td>363(165)</td>
<td>811(331)</td>
<td>&lt; 0.001</td>
<td>See methods</td>
</tr>
<tr>
<td>Parathyroid hormone (pg/ml)</td>
<td>37</td>
<td>634(35-5)</td>
<td>521(29-4)</td>
<td>0.010</td>
<td>10–70</td>
</tr>
<tr>
<td>25(OH) vitamin D (nmol/l)</td>
<td>38</td>
<td>389(19-1)</td>
<td>586(17-9)</td>
<td>&lt; 0.001</td>
<td>25–100</td>
</tr>
<tr>
<td>1,25(OH)2 vitamin D (pmol/l)</td>
<td>37</td>
<td>135(49)</td>
<td>231(98)</td>
<td>&lt; 0.001</td>
<td>43–168</td>
</tr>
<tr>
<td>Ratio 25(OH)1,25(OH)2 vitamin D</td>
<td>37</td>
<td>281(194)</td>
<td>258(135)</td>
<td>0.643</td>
<td>–</td>
</tr>
<tr>
<td>Vitamin D-binding protein (μg/ml)</td>
<td>38</td>
<td>290(104)</td>
<td>334(122)</td>
<td>0.062</td>
<td>–</td>
</tr>
</tbody>
</table>

Values are mean(s.d.). 25(OH)2, 25-hydroxy; 1,25(OH)2, 1,25-dihydroxy. *Paired-samples t test.
operation type (VBG or RYGBP) had a significant influence on the changes in skull BMD \((P = 0.002)\), whereas weight loss (over the first year) did not \((P = 0.210)\).

**Weight-stable phase: 1–6 years after surgery**

**Bodyweight**

Bodyweight did not change significantly between 1 and 6 years after RYGBP \((Table 2)\). At 6 years bodyweight was significantly higher in the VBG group than in the RYGBP group. Although bodyweight was greater 6 years after VBG, there was no significant increase compared with 1 year, and no statistical difference in weight change between the groups \((Table 2)\).

**Total bone mineral density**

In the weight-stable phase, total BMD decreased significantly in the RYGBP group \((Table 2)\). It also decreased slightly from year 1 to year 6 after VBG, but these patients still had significantly higher BMD than those in the RYGBP group after 6 years. The decrease in BMD between 1 and 6 years after operation was significantly greater among patients who had RYGBP.
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**Skull bone mineral density**
Skull BMD continued to decrease between 1 and 6 years after RYGBP, and was significantly lower at 6 years (Table 2). There was no statistically significant change in skull BMD between 1 and 6 years after operation in the VBG group. The change in skull BMD was significantly greater among patients who had gastric bypass.

**Calcium intake at baseline, and 1 and 6 years after surgery**
Dietary questionnaires were sent to all patients randomized to VBG or RYGBP and deemed eligible for inclusion (50 in each group) before operation and 1 year later. After 6 years dietary questionnaires were sent to included patients who remained in their original group. Before surgery, mean(s.d.) reported daily calcium intake was 1-5(0-8) g in the RYGBP group (43 patients) and 1-9(0-9) g in the VBG group (48 patients). Respective values at 1 year were 1-0(0-5) g (46 patients) and 1-3(0-7) g (44 patients). These data indicated that calcium intake at 1 year after surgery was well above the recommended daily intake of calcium for both groups (National Food Agency; http://www.slv.se). No analyses were performed for the 6-year data as few patients filled out the dietary questionnaires (RYGBP 7, VBG 9).

**Bone resorption after Roux-en-Y gastric bypass**
CTX levels were increased significantly at 18 months after surgery compared with baseline (Table 3). Both 25(OH) vitamin D and 1,25(OH)₂ vitamin D levels also increased. However, the ratio between 25(OH) and 1,25(OH)₂ vitamin D did not change. There was a small but significant decrease in PTH. The level of vitamin D-binding protein did not change.

**Protein expression in the alimentary limb**
To explore the mechanisms underlying the potentially decreased active calcium uptake in proximal jejunum, global protein expression analysis of jejunal biopsies taken from seven patients before and 6–8 months after RYGBP surgery was carried out. This showed, among several other protein changes, a significant decrease in signal intensity in a spot that MS identified as containing heat-shock protein 90β (average ratio -1.64, P = 0.006, n = 7). After searching relevant literature, this was the only protein change identified that was related to active intestinal calcium absorption.

**Heat-shock protein 90β**
Western blot analysis of paired jejunal mucosal samples obtained before and after gastric bypass from an additional ten patients was performed. This confirmed a significant decrease in heat-shock protein 90β levels after RYGBP (P = 0.047) (Fig. 1d). The finding was consistent for all 17 patients analysed individually before and after surgery by proteomics or western blotting (7 and 10 respectively).

**Transient receptor potential cation channel, subfamily V, member 6**
Protein expression levels of the calcium transporter protein TRPV6 in the jejunum decreased after RYGBP (P = 0.007) (Fig. 1b). Changes were analysed within the same patient before and after surgery. The change in TRPV6 levels correlated significantly with the change in heat-shock protein 90β levels (R = 0.78, P = 0.008) (Fig. 1c).

**Vitamin D receptor**
Jejunal vitamin D receptor protein levels increased significantly after RYGBP (P = 0.002) (Fig. 1d).

**Discussion**
The effects of bariatric surgery on BMD in the weight-loss phase (during the first year after surgery) and the long-term weight-stable phase indicate that the type of operation rather than weight loss predict the changes in skull BMD. Weight loss induced by the two procedures did not differ significantly after the first year, yet BMD data showed two distinct patterns. Skull BMD did not change in the year after VBG and, somewhat surprisingly, there was even a slight increase in total BMD, possibly reflecting increased physical activity after the rapid weight loss. On the other hand, total BMD did not change in the first year after RYGBP, but skull BMD showed a significant decrease. During the weight-stable period 1–6 years after surgery, weight remained stable among patients who had RYGBP; however, skull BMD decreased, as did total BMD.

Several previous studies have reported reduced intestinal calcium absorption after RYGBP. Insufficient calcium uptake could eventually lead to demineralization of the skeleton, subsequent osteoporosis, and increased fracture risk. The present study aimed to characterize changes in BMD after two mechanistically different bariatric procedures: RYGBP and VBG. After RYGBP, the foregut is excluded from passage of ingested food and bile, which is diverted from the majority of the jejunum. Active intestinal calcium absorption is located in the proximal intestine and exclusion of the duodenum will abolish calcium uptake there. There is
also a possibility that diversion of bile from the proximal jejunum (alimentary limb) might inhibit normal calcium absorption. The strengths of this article are that the data originate from a randomized trial and that the patients did not receive oral calcium supplementation, which was not routine at this centre when the study was initiated in 1999. Calcium and vitamin D are now prescribed routinely after bariatric surgery, which would preclude a similar study being performed. Weaknesses include the lack of blood samples from the randomized trial and the relatively short follow-up of 6 years after operation. Furthermore, the risk of osteoporosis cannot be evaluated directly from these data as only BMD data from the femoral neck and lumbar spine have been shown to correlate with the development of osteoporotic fractures. However, it was also shown recently that dual-energy X-ray absorptiometry measurements of areal BMD during profound weight loss, for example at the hip, may show artefactual changes compared with quantitative CT measurements. Skull BMD, on the other hand, may be less affected by the weight loss per se. A large number of patients originally assigned to VBG had undergone conversion to RYGBP by the 6-year follow-up. Only patients who remained in their original group were analysed at 6 years, hence the small number of subjects at this time point. The nature of per-protocol analysis in the present study will clearly have affected the weight loss data, especially in the VBG group, because only those patients whose gastroplasty was relatively successful, in terms of weight loss and absence of complications, remained in this group as late as 6 years after operation. However, this may be a strength of the study from another perspective (BMD changes), as patients who had VBG had similar weight changes to those who underwent RYGBP, even at 6 years, and should therefore be more comparable to the RYGBP group in terms of the effect of weight loss on BMD. Owing to the small numbers remaining in the original VBG group, intention-to-treat analysis of osteoporotic fracture risk was not meaningful. A further weakness is that it was not possible to measure intestinal calcium uptake, and the authors are not aware of a method for this that would be useful in such a clinical setting.

As blood samples were not available from the first series of patients who underwent RYGBP and VBG, bone resorption markers were measured in blood samples from another series of patients who had RYGBP at this centre. The difference between these studies was that all patients in this latter series were prescribed calcium and vitamin D supplements. Most importantly, CTX levels in serum doubled by 18 months after gastric bypass. This should reflect increased bone turnover, and may well be in line with increased bone resorption and the decreased BMD levels observed in the first series of patients. Alternatively, it could merely reflect a response to the decreased body weight leading to increased bone turnover, although the lack of BMD decrease among patients who had VBG with similar weight loss during the first year is not consistent with increased bone turnover as a response primarily to the weight loss.

Levels of 25(OH) and 1,25(OH)₂ vitamin D increased after gastric bypass, and were high compared with the reference range, probably reflecting the vitamin D substitution therapy given to these patients. The ratio between 25(OH) and 1,25(OH)₂ vitamin D levels did not change, however, suggesting unchanged 1α-hydroxylase activity. There was also a small but significant decrease in PTH level by 18 months after operation, which could have been a response to the increased vitamin D levels. This may reflect a non-functional regulation as vitamin D signalling in the proximal small intestine seemed to be decreased based on the downregulation of heat-shock protein 90β and TRPV6, despite increased vitamin D receptor levels. Other explanations are also possible, as it has been reported that obese patients in general tend to have low vitamin D levels, and these seem to be normalized after gastric bypass. The decreased PTH levels could represent a secondary response to that.

To find possible mechanisms leading to decreased active calcium uptake in the proximal intestine after RYGBP, a proteomic analysis of jejunal mucosa before and after surgery was carried out to determine changes in protein expression levels that might influence active intestinal calcium absorption. Among the proteins regulated was heat-shock protein 90β, which has been reported to influence vitamin D receptor activity. Western blot analysis confirmed decreased levels of heat-shock protein 90β in alimentary limb mucosa after gastric bypass in an additional group of patients; however, the total number of patients analysed was still small (proteomics 7, western blot 10). The intraoperative and postoperative sampling techniques were not completely identical, which may have introduced bias related to, for example, sampling thickness. Thus, although all mucosal protein analyses were carried out within the same patient before and after surgery, the data should still be interpreted with some caution.

To analyse this pathway further, protein levels of TRPV6, as a marker for vitamin D receptor activity, were measured. TRPV6 has been shown to be transcriptionally regulated by vitamin D receptor in human proximal intestine. The significant decrease in TRPV6 levels after surgery is suggested to reflect a decrease in vitamin D receptor activity.
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**Fig. 2** Regulation of active vitamin D-induced calcium absorption mechanisms in the proximal jejunum after Roux-en-Y gastric bypass (RYGBP). 

**a** Active calcium absorption at baseline; mediating mechanisms are encircled. 

**b** Changes to active calcium mechanisms (encircled) after RYGBP. Active calcium absorption normally occurs in the duodenum (blue) and jejunum (green). After RYGBP the duodenum is bypassed and cannot absorb calcium (pale blue). Levels of heat-shock protein (HSP) 90β, a co-activator of vitamin D receptor (VDR), are decreased in the jejunum (green) after RYGBP. VDR regulates transcriptionally several proteins believed to participate in active calcium absorption, such as transient receptor potential cation channel, subfamily V, member 6 (TRPV6). After RYGBP, despite increased VDR expression, TRPV6 expression is decreased, and may lead to decreased calcium uptake in the jejunum and, ultimately, decreased bone mineral density (BMD).

A large proportion of patients undergoing RYGBP receive oral calcium supplementation as well as vitamin D substitution. However, if intestinal vitamin D-induced uptake of calcium is impaired, oral supplementation could be insufficient. There are recent data indicating that calcium uptake may be decreased after RYGBP, even with calcium and vitamin D supplementation, and lead to increased PTH activity. Patients who have had RYGBP are at increased risk of recalcitrant symptomatic hypocalcaemia after thyroidectomy. This is well in line with the present findings and could be explained by decreased vitamin D receptor activity in the small intestine. Whether the present findings may be an indication of an eventually increased risk of osteoporosis after RYGBP remains to be investigated.

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