1,25-Dihydroxyvitamin D and the Vitamin D Receptor Gene Polymorphism Apa1 Influence Bone Mineral Density in Primary Hyperparathyroidism

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Abstract

Objective: Parathyroid hormone (PTH) and vitamin D are the most important hormones regulating calcium metabolism. In primary hyperparathyroidism (PHPT) excessive amounts of PTH are produced. Bone turnover is enhanced, leading to reduced bone mineral density and elevated levels of serum calcium. The aim of this study was to investigate relations between serum levels of 25-hydroxyvitamin D (25(OH)D), 1,25-dihydroxyvitamin D (1,25(OH)₂D) and bone mineral density, as well as known genetic polymorphisms in the vitamin D receptor and enzymes metabolising vitamin D in patients with PHPT.

Design/Subjects: We conducted a cross-sectional study of 52 patients with PHPT.

Results: Mean level of 25(OH)D was 58.2 nmol/L and median 1,25(OH)₂D level was 157 pmol/L. Among our patients with PHPT 36.5% had 25(OH)D levels below 50 nmol/L. Serum 1,25(OH)₂D was inversely correlated to bone mineral density in distal radius (p = 0.002), but not to bone mineral density at lumbar spine or femoral neck. The vitamin D receptor polymorphism Apa1 (rs7975232) was associated with bone mineral density in the lumbar spine.

Conclusions: The results suggest that PHPT patients with high blood concentrations of 1,25(OH)₂D may have the most deleterious skeletal effects. Randomized, prospective studies are necessary to elucidate whether vitamin D supplementation additionally increases serum 1,25(OH)₂D and possibly enhances the adverse effects on the skeleton in patients with PHPT.

Introduction

Primary hyperparathyroidism (PHPT) is characterized by elevated serum levels of parathyroid hormone (PTH) and calcium. In most cases PHPT is caused by a benign adenoma or glandular hyperplasia producing excessive amounts of PTH [1]. Elevated levels of PTH increase calcium resorption in the kidneys, leading to hyper-calcemia, while phosphate excretion is enhanced. Bone turnover is enhanced, reducing bone mineral density (BMD) and elevating levels of bone markers [2]. Increased fracture risk is described in PHPT patients [3].

To become metabolically active, vitamin D has to be hydroxylated by cytochrome P450 enzymes (Figure 1). The major circulating form of vitamin D, 25-hydroxyvitamin D (25(OH)D), is formed in the liver. 25(OH)D is the most commonly used indicator of vitamin D status, reflecting the storage of the vitamin [4]. Further hydroxylation to 1,25-dihydroxyvitamin D (1,25(OH)₂D) is catalysed by the 1α-hydroxylase. This enzyme is mainly localised in the kidneys but is also found in other tissues [5]. The activity of the 1α-hydroxylase is controlled by PTH and 1,25(OH)₂D keeping serum levels of 1,25(OH)₂D fairly constant [6,7]. In PHPT-patients, high levels of PTH increase the activity of 1α-hydroxylase. Vitamin D inadequacy seems to be more frequent in PHPT patients than in the background population [8,9]. Low levels of 25(OH)D in these patients may be explained through increased activity of both the 1α-hydroxylase and the 24-hydroxylase [7,10,11].

Environmental factors like exposure to sunlight, nutrition, and seasonal variation influence vitamin D status [12,13,14], though, individual variations in vitamin D-concentrations are not fully explained by external factors. Twin studies have shown individual differences in bone formation, bone resorption, calcium homeostasis, concentrations of PTH and vitamin D due to genetic variation [15]. Influences of single nucleotide polymorphisms (SNPs) are independent of lifestyle and are life long. Differences in BMD, vitamin D-metabolism and calcium levels in patients with

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PHPT could be explained by genetic variation in enzymes metabolising vitamin D. Furthermore, 1,25(OH)₂D₃ is a ligand for the intra-nuclear vitamin D receptor (VDR) that regulates gene transcription of vitamin D responsive genes [16]. Accordingly, polymorphisms in the VDR-gene may have an influence on vitamin D-sensitive tissues, such as bone [17].

The aim of this study was to investigate serum levels of 25(OH)D and 1,25(OH)₂D in patients with PHPT. We also investigated whether common SNPs in enzymes important in the vitamin D metabolism and the VDR could explain individual differences in disease severity, especially regarding BMD. We observed an inverse correlation between BMD in distal radius and concentrations of 1,25(OH)₂D₃. Additionally, we found that BMD in lumbar spine was associated with the Apa1 polymorphism in the VDR gene.

Materials and Methods

Ethics Statement

The study was performed according to the principles expressed in the declaration of Helsinki. All enrolled subjects signed an informed written consent. The Western Norway Regional Committee for Medical Research Ethics (REK) approved the study.

Subjects and Study Design

52 patients (42 females and 10 males) scheduled to undergo surgery for PHPT participated in the study. Patients included had PHPT caused by either single adenomas (85%) or multi-glandular hyperplasia (15%). PHPT was histologically confirmed in all patients. The diagnosis of PHPT was based on serum levels of ionised calcium above the reference range (reference range: 1.15–1.30 mmol/L) and PTH-levels that were elevated or in the upper third of the reference range (reference range: 1.3–6.8 pmol/L). The recruitment period was from September 2007 to May 2010. Inclusion was consecutive and equally distributed throughout the year. Exclusion criteria were known inflammatory bowel disease, rheumatoid disease or lack of BMD-measurements. Patients with genetically confirmed multiple endocrine neoplasia type 1 (MEN-1 syndrome) or type 2a (MEN-2a syndrome) based on verified mutations in the MENVI gene or RET gene, respectively, were excluded. During the study period, all included patients lived in the Western coastal part of Norway and underwent surgery at the Department of Endocrine Surgery, Haukeland University Hospital, Bergen, Norway. Blood samples, anthropometric data and medical history were collected the day before parathyroidectomy. BMD was measured within 4 months prior to surgery.

Biochemical Analysis and Anthropometric Data

Blood samples from patients were centrifuged and frozen at -80°C within 2 hours after collection. Ionised calcium, albumin, phosphatase, alkaline phosphatase (ALP), alanine transaminase (ALT), and C-reactive protein (CRP) were analysed immediately at the Laboratory of Clinical Biochemistry, Haukeland University Hospital, Bergen, Norway, using the Modular P-system from Roche Diagnostics, Basel, Switzerland. PTH was measured at the Hormone Laboratory, Haukeland University Hospital, Bergen, Norway, using a two-site chemiluminescent immunoassay for intact PTH (Immulite 2000, Siemens Healthcare Diagnostics, Deerfield, IL, USA). The inter-assay variations were 6.3% at a concentration of 5.6 pmol/L and 8.8% at 40 pmol/L. Plasma concentrations of 25(OH)D were analysed with a liquid chromatography/tandem mass spectrometry (LC-MS/MS) method developed at the Hormone Laboratory, Haukeland University Hospital, Bergen, Norway [18]. 25(OH)D₃ and 25(OH)D₂ were measured separately using this method. A radioimmunoassay kit from Immunodiagnostic system, Boldon, UK, was used for analysing 1,25(OH)₂D₃. This assay had a sensitivity of 100% for concentrations of 20.5 pmol/L, 135.2 pmol/L, respectively. Creatinine was determined by including it and its deuterated internal standard (d3-creatinine) in an established LC-MS/MS assay using the ion pairs 114/44.2 and 117/47.2, respectively [19]. Creatinine was analysed at Bevital A/S, Bergen, Norway. The isotope dilution mass spectrometry (IDMS) traceable formula developed by the Modification of Diet in Renal Disease (MDRD) Study Group was used for estimated glomerular filtration rate (eGFR) calculations: eGFR = 175 × (s-creatinine/88.4) × 1.154 × (Age)⁻₀.₂₀₃ × 0.742 if female [20]. Body mass index (BMI) was calculated as the weight divided through the square of the body length (kg/m²).

Bone Mineral Density

BMD was measured at the Department of Rheumatology, Haukeland University Hospital, Bergen, Norway, using a stationary, dual energy X-ray absorptiometry (DXA) (Lunar Prodigy, GE...
Purification of DNA
Genomic leucocyte DNA was purified from EDTA-anticoagulated whole blood using the MagNa PURE LC instrument and the MagNa PURE LC DNA isolation kit (Roche Diagnostics, GmbH, Mannheim, Germany). Quality and concentration of DNA were measured using the NanoDrop® ND-100 spectrophotometer (NanoDrop Technologies, Wilmington, USA).

Single Nucleotide Polymorphisms
Based on previous studies we selected 10 SNPs located in genes coding for 25-hydroxylation/CYP2R1 (rs10741657 and rs2060793), 24-hydroxylase/CYP24A1 (rs6013897 and rs2762939), 1α-hydroxylase/CYP27B1 (rs10877012 and rs703895) and VDR (rs1544410 (Bsm1), rs731236 (Taq1), rs7975232 (Apal), and rs2285730 (Fok1)) [21,22,23,24,25]. The nucleic acid sequences of the selected SNPs were found through the database http://snpper.chip.org/. SNPs and alleles were verified using the database http://hapmap.ncbi.nlm.nih.gov/. 

Statistical Analysis
Continuous variables are reported as mean (standard deviation) or median (25th to 75th percentile) and categorical variables as counts (percentages). P-values, ALP, ALAT, and 1,25(OH)2D did not show normal distribution and values were logarithmically transformed before used in statistical tests. Differences across the tertiles of 25(OH)D, 1,25(OH)2D and PTH were calculated using Spearman’s rho test. Associations between SNP-variants and BMD at the forearm, lumbar spine and right femoral neck were assessed using linear regression. These tests were performed once assuming that minor allele was dominant and once assuming that minor allele was recessive. All analysis including BMD in distal radius, lumbar spine and femoral neck was performed using the Bonferroni method. Thus, where multiple SNPs were analysed a correction factor of 7 (7 SNPs included in the analyses) was used. After Bonferroni-correction a p-value <0.007 was required for statistical significance. Statistical analyses were performed using SPSS Statistics 19 for Mac (IBM Corporation, New York, USA).

Results

Table 1. Patient characteristics at inclusion (n=52).

| Characteristics                  | Values are given as mean (SD) or median (25th to 75th percentile) |
|----------------------------------|---------------------------------------------------------------------|
| Age (years)                      | 60.8 (12.7)                                                         |
| 25(OH)D (mmol/L)                 | 58.2 (21.5)                                                         |
| 1,25(OH)2D (pmol/L)              | 157 (130-197.5)                                                    |
| BMI (kg/m²)                      | 26.4 (4.20)                                                         |
| PTH (pmol/L)                     | 13.1 (9.63–18.5)                                                   |
| iCa (mmol/L)                     | 1.48 (0.092)                                                        |
| Phosphate (mmol/L)               | 0.84 (0.17)                                                         |
| Albumin (g/L)                    | 45.6 (2.66)                                                         |
| ALP (U/L)                        | 85.5 (73.3–117)                                                    |
| eGFR (mL/min/1.73 m²)            | 84.0 (22.1)                                                         |
| ALAT (U/L)                       | 26.0 (18.3–31.8)                                                   |
| CRP (mg/L)                       | 1.0 (0.10–2.0)                                                     |

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Healthcare, Diegem, Belgium). Calibration of the scanner was performed against a standard calibration block each day, and was stable during the whole measurement period. The in vitro long-term coefficient of variation was 0.86%, and the in vivo short-term precision for femoral neck was 1.47%. BMD in distal radius, lumbar spine (L2-L4) and femoral neck was available for 52 patients and BMD was measured within four months prior to surgery. Left distal radius was measured in all except four patients, where right radius was measured. Right femoral neck was used in all cases.
1,25(OH)2D was significantly associated with BMD in distal sites. 1,25(OH)2D did not contribute to differences in BMD at any of the neck and lumbar spine. Phosphate, ionised calcium, and eGFR whereas only age and BMI were associated with BMD in femoral investigated sites. Phosphate, ionised calcium, eGFR, 25(OH)D, 1,25(OH)2D, and PTH as covariates did not show an influence of 25(OH)D, 1,25(OH)2D, and PTH was not significantly correlated (r = 0.287, p = 0.062). We did not observe a correlation between 25(OH)D age (r = 0.293, p = 0.035).

Multiple linear regression with age, BMI, gender, phosphate, ionised calcium, eGFR, 25(OH)D, 1,25(OH)2D, and PTH as covariates did not show an influence of 25(OH)D, 1,25(OH)2D or PTH on BMD in femoral neck and lumbar spine. 1,25(OH)2D was significantly associated with BMD in distal radius (standardised beta (effect size) = 0.28, p = 0.010). In this model PTH was not associated with BMD in distal radius (standardised beta = 0.024, p = 0.84). Age, gender, and BMI contributed significantly to differences in BMD in distal radius, whereas only age and BMI were associated with BMD in femoral neck and lumbar spine. Phosphate, ionised calcium, and eGFR did not contribute to differences in BMD at any of the investigated sites.

### Discussion

The main finding of the present study was the inverse correlation between serum 1,25(OH)2D and BMD in distal radius in patients with PHPT, indicating that high serum levels of 1,25(OH)2D may have deleterious skeletal effects. Moreover, we observed an association between BMD in the lumbar spine and the VDR polymorphism Apa1.

The observed inverse correlation between 1,25(OH)2D and BMD in distal radius is in accordance with a study from Denmark, where high levels of 1,25(OH)2D in PHPT-patients were associated with increased bone turnover and decreased BMD [29]. PTH also seemed to influence BMD in lumbar spine after Bonferroni correction. When considering the minor allele (allele A) as dominant, we observed significant lower lumbar spine BMD in patients with genotype AA/CA vs. CC. We did not observe a relation between SNP genotypes and any of the biochemical parameters analysed (data not shown).

### Table 2. Patients divided into tertiles according to serum levels of 25(OH)D.

| Tertiles of 25(OH)D | Range: 22–47 nmol/L (n = 17) | Range: 47–65 nmol/L (n = 18) | Range: 67–115 nmol/L (n = 17) | p-value |
|---------------------|-----------------------------|-----------------------------|-----------------------------|---------|
| Females (%)<sup>a</sup> | 13 (76.5) | 16 (88.9) | 13 (76.5) | 0.56 |
| Age | 55.9 (14.7) | 61.4 (11.3) | 64.9 (10.8) | 0.038 |
| BMI (kg/m<sup>2</sup>) | 28.0 (4.12) | 26.1 (3.47) | 24.9 (4.47) | 0.013<sup>b</sup> |
| 1,25(OH)2D (pmol/L) | 151 (124–205) | 138 (122–198) | 172 (146–193) | 0.32<sup>c</sup> |
| PTH (pmol/L) | 15.0 (8.55–23.4) | 12.8 (9.55–18.9) | 12.7 (10.4–16.6) | 0.28<sup>d</sup> |
| iCa (mmol/L) | 1.52 (0.09) | 1.47 (0.09) | 1.44 (0.09) | 0.069<sup>e</sup> |
| Phosphate (mmol/L) | 0.81 (0.15) | 0.90 (0.20) | 0.80 (0.13) | 0.23<sup>f</sup> |
| eGFR (ml/min/1.73 m<sup>2</sup>) | 91.7 (19.8) | 83.0 (22.4) | 77.4 (22.9) | 0.096<sup>g</sup> |
| ALP (U/L) | 94.0 (75.5–124) | 89.0 (68.5–108) | 86.0 (72.5–114) | 0.35<sup>h</sup> |
| BMD radius (g/cm<sup>2</sup>) | 0.50 (0.11) | 0.46 (0.11) | 0.45 (0.10) | 0.55<sup>i</sup> |
| BMD lumbar spine (g/cm<sup>2</sup>) | 1.12 (0.17) | 1.03 (0.19) | 1.06 (0.19) | 0.42<sup>j</sup> |
| BMD femoral neck (g/cm<sup>2</sup>) | 0.86 (0.13) | 0.77 (0.12) | 0.79 (0.10) | 0.62<sup>k</sup> |

Values are given as mean (standard deviation) or median (25th–75th percentile). PTH, 1,25(OH)2D and ALP were log-transformed before used in the analyses. BMI, body mass index; PHPT, primary hyperparathyroidism; PTH, parathyroid hormone; iCa, ionised calcium; ALP, alkaline phosphatase; 25(OH)D, 25-hydroxyvitamin D; 1,25(OH)2D, 1,25-dihydroxyvitamin D; eGFR, estimated glomerular filtration rate; BMD, bone mineral density. P-values for linear trend over tertiles.

<sup>a</sup>Values are numbers (percentages), differences across tertiles are assessed with a chi-square test.

<sup>b</sup>Age was used as covariate.

<sup>c</sup>Age and BMI were used as covariates.

<sup>d</sup>Age was used as covariate.

<sup>e</sup>Age and BMI were used as covariates.

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calcium, phosphate, eGFR, 25(OH)D, 1,25(OH)2D, and PTH as covariates, the influence of PTH on BMD in distal radius disappeared, whereas the influence of 1,25(OH)2D was still significant. Goltzman et al. showed that the 1,25(OH)2D/VDR-system is required for an appropriate osteoclast response to elevated PTH [6]. Both 1,25(OH)2D and PTH increase the expression of Receptor Activator NF-κB Ligand (RANKL) from osteoblasts [30,31]. In turn, RANKL stimulates the maturation of osteoclasts resulting in enhanced bone resorption [31,32]. Physiological concentrations of 1,25(OH)2D inhibit PTH-induced release of RANKL in osteoblasts, while supra-physiological levels of 1,25(OH)2D further increase the RANKL-release [30]. This suggests that serum concentrations of 1,25(OH)2D above a certain threshold may result in adverse effects on bone [33]. Bone loss in PHPT-patients occurs mainly at sites with high proportion of cortical bone, such as distal radius, while bone loss in trabecular bone, such as lumbar spine, is less prominent [34,35,36]. An effect on cortical bone prior to trabecular bone is in line with our findings.

### Table 3. Patients divided into tertiles according to serum levels of 1,25(OH)2D.

| Tertiles of 1,25(OH)2D | Range: 48–137 pmol/L n = 17 | Range: 138–176 pmol/L n = 18 | Range: 179–350 pmol/L n = 17 | p-value |
|------------------------|-----------------------------|-----------------------------|-----------------------------|---------|
| Females (%)a           | 13 (76.4)                   | 14 (77.8)                   | 15 (88.2)                   | 0.63    |
| Age                    | 60.5 (12.3)                 | 61.1 (10.6)                 | 60.6 (15.5)                 | 0.99    |
| BMI (kg/m²)            | 26.1 (3.66)                 | 26.8 (5.09)                 | 26.2 (3.79)                 | 0.96    |
| 25(OH)D (nmol/L)       | 47.7 (15.7)                 | 67.6 (21.2)                 | 58.6 (23.2)                 | 0.14    |
| PTH (pmol/L)           | 11.4 (8.70–15.2)            | 11.2 (8.60–14.1)            | 18.2 (12.5–24.8)            | 0.011   |
| iCa (mmol/L)           | 1.48 (0.09)                 | 1.46 (0.08)                 | 1.51 (0.10)                 | 0.29    |
| Phosphate (mmol/L)     | 0.50 (0.17)                 | 0.83 (0.13)                 | 0.80 (0.18)                 | 0.062   |
| eGFR (ml/min/1.73 m²)  | 76.8 (21.3)                 | 81.9 (20.7)                 | 93.3 (22.3)                 | 0.029   |
| ALP (U/L)              | 80.0 (70.0–97.0)            | 99.0 (73.3–124)             | 89.0 (79.5–125)             | 0.30    |
| BMD radius (g/cm²)     | 0.48 (0.11)                 | 0.50 (0.80)                 | 0.41 (0.11)                 | 0.002d  |
| BMD lumbar spine (g/cm²) | 1.04 (0.19)            | 1.09 (0.15)                 | 1.07 (0.22)                 | 0.66d   |
| BMD femoral neck (g/cm²)| 0.77 (0.12)              | 0.82 (0.10)                 | 0.82 (0.14)                 | 0.11d   |

Values are given as mean (standard deviation) or median (25th–75th percentile). PTH, 1,25(OH)2D and ALP were log-transformed before used in the analyses. BMI, body mass index; PHPT, primary hyperparathyroidism; PTH, parathyroid hormone; iCa, ionised calcium; ALP, alkaline phosphatase; 25(OH)D, 25-hydroxyvitamin D; 1,25(OH)2D, 1,25-dihydroxyvitamin D; eGFR, estimated glomerular filtration rate; BMD, bone mineral density. P-values for linear trend over tertiles. 

Values are numbers (percentages), differences across tertiles are assessed with a chi-square test.

aValues are numbers (percentages), differences across tertiles are assessed with a chi-square test.

dAge, BMI and gender were used as covariates.

### Table 4. Patients divided into tertiles according to serum levels of PTH.

| Tertiles of PTH | Range: 5.6–10.5 pmol/L n = 17 | Range: 10.8–15.8 pmol/L n = 18 | Range: 17.5–51.3 pmol/L n = 17 | p-value |
|-----------------|-----------------------------|-----------------------------|-----------------------------|---------|
| Females (%)a   | 13 (76.4)                   | 15 (83.3)                   | 14 (82.4)                   | 0.86    |
| Age             | 59.8 (11.7)                 | 60.7 (10.8)                 | 61.8 (15.8)                 | 0.65    |
| BMI (kg/m²)    | 27.3 (4.41)                 | 25.0 (3.58)                 | 27.0 (4.50)                 | 0.75    |
| 25(OH)D (nmol/L) | 55.5 (18.1)              | 62.4 (23.4)                 | 56.3 (23.2)                 | 0.92    |
| 1,25(OH)2D (pmol/L) | 140 (99.5–159)           | 159 (128–179)               | 204 (138–246)               | <0.001  |
| iCa (mmol/L)   | 1.43 (0.07)                 | 1.47 (0.08)                 | 1.55 (0.08)                 | <0.001  |
| Phosphate (mmol/L) | 0.90 (0.16)              | 0.85 (0.17)                 | 0.77 (0.15)                 | 0.017   |
| eGFR (ml/min/1.73 m²) | 87.1 (19.0)           | 81.9 (19.6)                 | 83.1 (27.8)                 | 0.598   |
| ALP (U/L)       | 89.0 (69.0–105)            | 81.0 (72.5–123)             | 90.0 (81.5–127)             | 0.42    |
| BMD radius (g/cm²) | 0.50 (0.08)             | 0.47 (0.10)                 | 0.43 (0.13)                 | 0.027d  |
| BMD lumbar spine (g/cm²) | 1.06 (0.14)           | 1.09 (0.22)                 | 1.05 (0.19)                 | 0.67d   |
| BMD femoral neck (g/cm²) | 0.81 (0.13)           | 0.80 (0.9)                  | 0.80 (0.15)                 | 0.19d   |

Values are given as mean (standard deviation) or median (25th–75th percentile). PTH, 1,25(OH)2D and ALP were log-transformed before used in the analyses. BMI, body mass index; PHPT, primary hyperparathyroidism; PTH, parathyroid hormone; iCa, ionised calcium; ALP, alkaline phosphatase; 25(OH)D, 25-hydroxyvitamin D; 1,25(OH)2D, 1,25-dihydroxyvitamin D; eGFR, estimated glomerular filtration rate; BMD, bone mineral density. P-values for linear trend over tertiles. 

Values are numbers (percentages), differences across tertiles are assessed with a chi-square test.

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Values are given as mean (standard deviation) or median (25th–75th percentile). PTH, 1,25(OH)2D and ALP were log-transformed before used in the analyses. BMI, body mass index; PHPT, primary hyperparathyroidism; PTH, parathyroid hormone; iCa, ionised calcium; ALP, alkaline phosphatase; 25(OH)D, 25-hydroxyvitamin D; 1,25(OH)2D, 1,25-dihydroxyvitamin D; eGFR, estimated glomerular filtration rate; BMD, bone mineral density. P-values for linear trend over tertiles.
The optimal concentration of vitamin D for beneficial health outcome is still subject for discussion [37,38]. Based on associations between 25(OH)D, BMD, bone turnover, muscular function and falls, serum levels of 25(OH)D below 50 nmol/L has been characterised as vitamin D inadequacy [39]. However, levels up to 75 nmol/L may still increase the beneficial effects of vitamin D on risk of falls, extremity strength, and prevention of cancer and hypertension [40]. It has been suggested that patients with mild PHPT and vitamin D insufficiency should receive supplementation of vitamin D to ensure 25(OH)D above 50 nmol/L [41,42].

We observed levels of 1,25(OH)2D, the ligand for the VDR, above the reference range in the patients with PHPT. The influence of SNPs in the VDR-gene on bone metabolism could therefore be stronger in patients with PHPT than in a healthy population. Our analyses of the most common SNPs in the VDR revealed an association for the different genotypes of the Apal polymorphism (rs7975232) and BMD in lumbar spine [48]. In twin studies a stronger influence of genetic factors on BMD in the trabecular than the cortical skeleton is observed [49]. This is in accordance with the findings in our study where BMD in lumbar spine, consisting mainly of trabecular bone, but not distal radius, consisting mainly of cortical bone, was influenced by the Apa1 polymorphism. The levels of 1,25(OH)2D were not associated with the Apa1 polymorphisms. Thus, it may appear that the effect of the Apa1 polymorphism on BMD in trabecular bone is different from the bone-resorbing effect of 1,25(OH)2D on cortical bone. The association between BMD in lumbar spine and the Apa1 polymorphism has to our knowledge not been observed in PHPT patients before. However, further studies are needed to assess if patients with the minor allele of the Apa1 polymorphism have an increased fracture risk and if surgery would be indicated at an earlier disease stage in these patients. Of note, SNPs in the vitamin D binding protein also influence circulating vitamin D levels [22]. Rs10877012 in the 1α-hydroxylase gene has also been associated with 25(OH)D levels [21].

### Table 5. Selected SNPs analysed in 52 individuals with primary hyperparathyroidism.

| rs number | Genotype major/minor homozygote | Frequency major/minor homozygote | Included samples | HWE p-value |
|-----------|-------------------------------|----------------------------------|-----------------|-------------|
| VDR       |                               |                                  |                 |             |
| rs1544410 (Bsm1) | GG/AA                        | 0.37/0.13                        | 52              | 0.44        |
| rs7975232 (Apa1) | CC/AA                        | 0.21/0.18                        | 51              | 0.12        |
| rs2228570 (Fok1) | CC/TT                        | 0.35/0.13                        | 52              | 0.27        |
| 25-hydroxylase (Cyp2R1) |                               |                                  |                 |             |
| rs10741657 | GG/AA                        | 0.44/0.21                        | 52              | 0.14        |
| 24-hydroxylase (Cyp24a1) |                               |                                  |                 |             |
| rs6013897 | TT/AA                        | 0.36/0.08                        | 52              | 0.19        |
| rs2762939 | GG/CC                        | 0.46/0.06                        | 52              | 0.21        |
| 1-alfa hydroxylase (Cyp27B1) |                               |                                  |                 |             |
| rs10877012 | GG/TT                        | 0.53/0.12                        | 51              | 0.25        |

Only samples without missing values were included in the analyses. P-values for Hardy Weinberg equilibrium are based on a chi-squared test. SNP, single nucleotide polymorphism; HWE, Hardy Weinberg equilibrium; VDR, vitamin D receptor. doi:10.1371/journal.pone.0056019.t005

Both PTH and 1,25(OH)2D regulate cytochrome P450 enzymes metabolising vitamin D. SNPs in genes encoding these enzymes are of particular interest in patients with PHPT, where the production of both PTH and 1,25(OH)2D is enhanced. According to Wang et al [25], rs10741657 is the locus in the 25-hydroxylase gene with strongest association to 25(OH)D levels. Wang et al also showed an association between 25(OH)D levels and the SNP rs6013897 in the 24-hydroxylase gene. Furthermore, an association with the SNP rs2762939 in the 24-hydroxylase gene and risk of coronary artery calcification was shown. However, this SNP was not correlated to 25(OH)D levels [22]. Rs10877012 in the 1α-hydroxylase gene has also been associated with 25(OH)D levels [21]. In the present study none of these SNPs were associated with levels of 25(OH)D, 1,25(OH)2D, PTH, serum calcium or BMD. We observed levels of 1,25(OH)2D, the ligand for the VDR, above the reference range in the patients with PHPT. The influence of SNPs in the VDR-gene on bone metabolism could therefore be stronger in patients with PHPT than in a healthy population. Our analyses of the most common SNPs in the VDR revealed an association for the different genotypes of the Apa1 polymorphism (rs7975232) and BMD in the lumbar spine. The minor allele was associated with lower BMD in the lumbar spine. A previous study on osteoporotic women has also shown a relationship between the minor allele of the Apal polymorphism and lower BMD in lumbar spine [48]. In twin studies a stronger influence of genetic factors on BMD in the trabecular than the cortical skeleton is observed [49]. This is in accordance with the findings in our study where BMD in lumbar spine, consisting mainly of trabecular bone, but not distal radius, consisting mainly of cortical bone, was influenced by the Apa1 polymorphism.

The 1,25(OH)2D has been shown to affect several different biological processes, including cell proliferation, differentiation, and gene expression. In addition, it has been suggested that 1,25(OH)2D may have a role in the regulation of the immune system, with potential implications for the prevention of autoimmune diseases. However, further studies are needed to clarify the precise mechanisms by which 1,25(OH)2D regulates these processes, and to determine the clinical relevance of these findings. Overall, these results highlight the importance of understanding the role of vitamin D in the regulation of cellular processes and disease prevention.
low 25(OH)D has been recommended. Randomized, prospective studies are necessary to conclude on safety of vitamin D supplementation and the effect on bone in PHPT.

### Table 6. Relations between the genotype variations of the SNPs studied and bone mineral density in lumbar spine, femoral neck and distal radius.

| rs number | Genotype | Bone mineral density (g/cm²) | | | |
|---|---|---|---|---|---|
| VDR | Frequency n (%) | Radius | Lumbar spine | Femoral neck | |
| rs1544410 | GG | 0.48 (0.09) | 1.14 (0.17) | 0.85 (0.13) | |
| | GA | 0.46 (0.12) | 1.03 (0.19) | 0.79 (0.12) | |
| | AA | 0.46 (0.08) | 1.00 (0.16) | 0.72 (0.07) | |
| p-value¹ | 0.39 | 0.046 | 0.036 | |
| p-value² | 0.88 | 0.41 | 0.065 | |
| rs7975232 | CC | 0.50 (0.08) | 1.21 (0.16) | 0.87 (0.12) | |
| | CA | 0.47 (0.12) | 1.04 (0.17) | 0.80 (0.13) | |
| | AA | 0.44 (0.10) | 1.01 (0.19) | 0.74 (0.09) | |
| p-value¹ | 0.21 | 0.003 | 0.032 | |
| p-value² | 0.79 | 0.47 | 0.26 | |
| rs2228570 | CC | 0.47 (0.10) | 1.05 (0.16) | 0.80 (0.10) | |
| | CT | 0.47 (0.11) | 1.07 (0.21) | 0.81 (0.15) | |
| | TT | 0.48 (0.10) | 1.10 (0.16) | 0.79 (0.09) | |
| p-value¹ | 0.35 | 0.49 | 0.95 | |
| p-value² | 0.87 | 0.57 | 0.70 | |
| 25-hydroxylase (Cyp2R1) | | | | | |
| rs10741657 | GG | 0.49 (0.11) | 1.09 (0.17) | 0.81 (0.13) | |
| | GA | 0.48 (0.10) | 1.11 (0.21) | 0.83 (0.14) | |
| | AA | 0.42 (0.10) | 0.95 (0.12) | 0.75 (0.08) | |
| p-value¹ | 0.10 | 0.38 | 0.84 | |
| p-value² | 0.79 | 0.47 | 0.26 | |
| 24-hydroxylase (Cyp24a1) | | | | | |
| rs6013897 | TT | 0.50 (0.10) | 1.15 (0.16) | 0.82 (0.11) | |
| | TA | 0.46 (0.10) | 1.02 (0.18) | 0.80 (0.11) | |
| | AA | 0.42 (0.14) | 1.03 (0.23) | 0.78 (0.27) | |
| p-value¹ | 0.26 | 0.018 | 0.83 | |
| p-value² | 0.40 | 0.83 | 0.86 | |
| rs2762939 | GG | 0.48 (0.10) | 1.05 (0.17) | 0.80 (0.10) | |
| | GC | 0.47 (0.11) | 1.08 (0.21) | 0.83 (0.14) | |
| | CC | 0.42 (0.10) | 1.04 (0.07) | 0.67 (0.03) | |
| p-value¹ | 0.74 | 0.57 | 0.60 | |
| p-value² | 0.52 | 0.99 | 0.13 | |
| 1-alfa hydroxylase (Cyp27B1) | | | | | |
| rs10877012 | GG | 0.46 (0.11) | 1.09 (0.19) | 0.79 (0.11) | |
| | GT | 0.47 (0.10) | 1.03 (0.17) | 0.82 (0.14) | |
| | TT | 0.49 (0.07) | 1.05 (0.19) | 0.80 (0.12) | |
| p-value¹ | 0.77 | 0.24 | 0.95 | |
| p-value² | 0.52 | 0.97 | 0.46 | |

SNP, single nucleotide polymorphism; VDR, vitamin D receptor.

1p-values are based on linear regression with age, gender and body mass index as covariates. Minor allele considered as dominant.

2p-values are based on linear regression with age, gender and body mass index as covariates. Minor allele considered as recessive. After Bonferroni-correction a p-value <0.007 is considered to be statistically significant.

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Author Contributions
Conceived and designed the experiments: MHEC JEV GM EAL. Performed the experiments: MHEC. Analyzed the data: MHEC EMA.
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