XIII.

Effects of up to 15 years of recombinant human GH (rhGH) replacement on bone metabolism in adults with Growth Hormone Deficiency (GHD): The Leiden Cohort Study

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ABSTRACT

BACKGROUND: Growth Hormone Deficiency (GHD) in adulthood may be associated with a decreased bone mineral density (BMD), a decreased bone mineral content (BMC) and an increased fracture risk. Recombinant human GH (rhGH) replacement induces a progressive increase in BMD for up to 5-7 years of treatment. Data on longer follow-up are however scarce.

METHODS: Two-hundred and thirty adult GHD patients (mean age 47.1 years, 52.6% female), of whom 88% patients had adult-onset (AO) GHD, receiving rhGH replacement for ≥5 years were included in the study. Most patients had multiple pituitary hormone deficiencies. Bone turnover markers, BMC and BMD and T-scores at the lumbar spine and femoral neck were evaluated at baseline, and after 5, 10 and 15 years of rhGH replacement. In addition, clinical fracture incidence was assessed.

RESULTS: Mean lumbar spine BMD, lumbar spine BMC and T-scores gradually increased during the first 10 years of rhGH replacement and remained stable thereafter. Largest effects of rhGH supplementation were found in men. In the small subset of patients using bisphosphonates, use of bisphosphonates did not impact additional beneficial effects in the long-term. Low baseline BMD positively affected the change in BMD and BMC over time, but there was a negative effect of high GH dose at 1 year on the change in BMD and BMC over time. Clinical fracture incidence during long-term rhGH replacement was 20.1/1000 py.

CONCLUSIONS: Fifteen years of rhGH replacement in GHD adults resulted in a sustained increase in BMD values at the lumbar spine, particularly in men, and stabilization of BMD values at the femoral neck. Clinical fracture incidence was suggested not to be increased during long-term rhGH replacement.

INTRODUCTION

Growth Hormone (GH) and Insulin-like Growth Factor-1 (IGF-1) are important regulators of bone growth and metabolism during the life span (1). IGF-1 mediates most of the effects of GH on skeletal metabolism via the IGF-1 receptor (2), although GH also exerts direct effects on bone (3). GH and IGF-1 act as anabolic hormones on bone by stimulating proliferation, and to some extent, differentiation of osteoblasts. Furthermore, osteoclastic bone resorption is stimulated, resulting in an overall increase in bone remodeling (3;4).

Untreated Growth Hormone Deficiency (GHD) is characterized by low bone turnover, decreased bone mineral density (BMD) and bone mineral content (BMC)(5-7). Treatment with recombinant human GH (rhGH) replacement has shown to increase bone turnover as reflected by an increase in bone formation and bone resorption markers. Consequently, there is an initial decline in (or unchanged) BMD, followed by a small increase of about 1-2% in the first 2 years of rhGH replacement due to a net positive balance in bone formation (3;8).

Data on the skeletal effects of long-term rhGH replacement are, however, scarce. Previous studies reported that rhGH replacement induces a progressive increase in BMD and BMC up to 5-7 years of treatment, which is followed by a plateau phase (9-12). Götherström *et al.* reported an increase of total body and lumbar spine BMD and BMC even up to 10 years of rhGH replacement, whereas femoral neck BMD and BMC reached a peak value after 5-7 years (13). Only one study reported the effects of 15 years of rhGH supplementation, showing a sustained increase in total body and lumbar BMD and BMC over 15 years, whereas femoral neck BMD and BMC returned towards baseline values after 7 years (14).

The primary aim of our study was to assess the effects of 5, 10 and 15 years of rhGH replacement on bone metabolism in the Leiden Cohort Study, a well-characterized cohort of adult GHD patients. Furthermore, we studied potential influencing factors on the bone response to rhGH replacement and studied clinical fracture incidence in these patients.

PATIENTS AND METHODS

PATIENTS: Consecutive adult patients with an established diagnosis of childhood-onset (CO) or adulthood-onset (AO) GHD followed up at the Department of Endocrinology and Metabolic Diseases of the Leiden University Medical Center (LUMC) were collected in a database from 1994 onwards. In the present study, we selected all GHD patients who started rhGH replacement therapy in or before 2007, and, therefore, had the potential to receive rhGH replacement for ≥5 years. We arbitrarily defined chronic treatment as replacement for ≥5 years, since such a period will reflect a stable situation without confounding by ongoing changes induced by the start and titration of rhGH replacement (15;16). This strategy resulted in inclusion of a total of 230 eligible GHD patients, of which part of the included patients has been previously described (17).

GHD was diagnosed on the basis of a GH peak response to the Insulin Tolerance Test (ITT) <3μg/l (glucose nadir <2.2mmol/l) according to current guidelines or, in case of contraindications for ITT, to the combined Growth Hormone Releasing Hormone/Arginine-test (GHRH/Arg) with BMI-adjusted GH cut-offs (18;19). All patients receiving rhGH replacement during childhood were re-tested at the time of transition to the adult outpatient clinic, after treatment cessation for >3 months. GHD was replaced according to a standardized Leiden protocol, in which patients received rhGH replacement (Genotropin, Pharmacia/Pfizer, Capelle a/d Ijssel, The Netherlands; Zomacton, Ferring, Hoofddorp, The Netherlands; Norditropin, NovoNordisk, Alphen a/d Rijn, The Netherlands), injected subcutaneously in the evening. GH starting dose was 0.2mg/day, which was individually adjusted each month in the first half year to achieve serum IGF-1 concentrations within the age-dependent laboratory reference range, aimed at SDS between 0 and +2. When stable plasma concentrations were reached, this individualized dose was continued during the entire study period and adjusted according to the IGF-1 standard deviation score (SDS). After dose titration, patients were evaluated at least yearly at the outpatient clinic, according to a standard protocol.

Adrenocorticotropic hormone (ACTH) deficiency was defined as an insufficient increase in cortisol levels (absolute value 0.55umol/l) after an ITT or a corticotropin-releasing hormone stimulation test in case that an ITT was contra-indicated. Standard hydrocortisone replacement was 20 mg divided in 3 doses, individually adjusted when necessary. Premenopausal women were classified as gonadotropin-deficient when secondary amenorrhea persisted for >1year. In men, gonadotropin

deficiency was defined as a testosterone level <8.0nmol/l. Thyroid-stimulating hormone (TSH) deficiency was defined as total thyroxine (T_4) or free T_4 levels below the normal laboratory reference range (<10pmol/l). Hypopituitarism was supplemented by hydrocortisone, L-thyroxine, testosterone in men, and/or estrogen in combination with progestagens (in premenopausal women only) and adequacy of supplementation was regularly controlled and adjusted as required.

Patients were treated with bone-modifying medication according to the discretion of their attending physicians.

METHODS: Data were collected from the patients' electronic hospital records on the following efficacy parameters, which were assessed on a yearly basis after establishment of diagnosis and start of treatment at the outpatient clinic:

1. Anthropometric parameters: body weight was measured to the nearest 0.1kg, and body height was measured barefoot to the nearest 0.001m. BMI was calculated as weight in kilograms divided by the square of height in meters (kg/m^2).
2. Biochemical parameters: data were collected on serum concentrations of IGF-1, GH, TSH, fT4, LH, FSH, estradiol, testosterone and prolactin, which were collected after an overnight fast. Assays were presented in *Supplementary File 1*.
3. Markers of bone metabolism: Data on serum creatinine, calcium, albumin, alkaline phosphatase, vitamin D, PTH, procollagen type 1 amino-terminal propeptide (P1NP, bone formation) and β-crosslaps concentrations (bone resorption) (the latter two markers available from 2006 onwards). Assays were presented in *Supplementary File 1*.
4. BMD and BMC: Data on BMD and BMC measurements were collected. BMD was measured yearly at the lumbar spine (L1-L4) and femoral neck using dual energy X-ray absorptiometry (DXA) (Hologic QDR 1000 (until 2008) and Hologic QDR 4500 (thereafter), Hologic Inc., Waltham, MA, USA) equipped with reference values based on the National Health and Nutrition Examination Survey (NHANES III) from the time of start of rhGH replacement onwards (20). Coefficient of variation of lumbar spine and femoral neck BMD measurements was 1%. An in-house comparison using 300 measurements provided a conversion formula for all Hologic 1000 measurements, which were converted for comparison for the laterally acquired Hologic 4500 BMD measurements. BMD T-scores were used to reflect the number of standard deviations (SD) above or below the mean for a healthy age- and sex-matched adult. World Health Organization (WHO) criteria were used to define osteopenia (T-score between -1.0 and -2.5) and osteoporosis (T-score ≤-2.5). BMC was measured at the lumbar spine using the same DXA apparatus.
5. Fracture data: Specific information on clinical fractures was retrieved from the medical charts. No routine spine radiographs were performed to assess the presence of vertebral fractures; however, radiographs were made in case of height loss >3cm or specific complaints at the discretion of the treating physician (21).Vertebral fractures were defined as clinical fractures (*i.e.* pain or decrease in height) and/or radiographic fractures in case of available radiographs, defined as the presence of Genant≥2(22).
6. Data on medication use and co-morbidity was obtained from electronic files.

STATISTICAL ANALYSIS: SPSS for Windows, Version 20.0 (SPSS, Inc., Chicago, IL, USA) was used for statistical analysis. Although 19 patients did not complete 5 years of rhGH replacement for various reasons (*vide infra*), these patients were included using the last observation carried forward method according to the intention-to-treat approach used. Results are presented as mean±SD, unless stated otherwise. ANOVA repeated measurements with Bonferroni correction for multiple comparisons were used to compare anthropometric parameters, bone markers, BMD, T-scores and BMC between baseline and after rhGH treatment. Analyses were repeated after stratification for bisphosphonate use. For the subgroup of patients followed ≥10 years, we only analyzed the baseline data of this specific subgroup.We identified factors influencing the response to long-term rhGH supplementation on bone by incorporating age, sex, childhood-onset *versus* adult-onset GHD, hydrocortisone use, GH dose, hypogonadism, number of pituitary deficiencies (<2 *vs* ≥2), bisphosphonate use, baseline Vitamin D levels and baseline BMD values in a linear regression model. Finally, clinical fracture incidence rate was calculated during rhGH replacement.

RESULTS

DESCRIPTION OF PATIENTS: Baseline characteristics of the 230 adult GHD patients (202 AO/28 CO) are shown in *Table 1*. Pituitary insufficiency was mainly caused by pituitary tumors or their treatment. Most patients had multiple pituitary hormone deficiencies. Two-hundred and eleven patients completed 5 years of rhGH replacement (*Supplementary File 2*). Reasons for earlier discontinuation (19 patients, 8.3% of total) are shown in *Supplementary File 2*. There were no differences between patients completing 5 years of rhGH replacement and non-completers with respect to age, sex, pituitary hormone deficiencies or co-morbidity. Ninety-eight and 43 patients, respectively, completed10 and 15 years of rhGH replacement.

At baseline, 10 patients (4.3%) received bisphosphonates, and, respectively, 84 (36.5%) and 85 patients (37.0%) received calcium and Vitamin D supplements. After respectively 5, 10 and 15 years of rhGH replacement 28 (12.2%), 19 (19.4%) and 8 (18.6%) patients used bisphosphonates. Thirty-eight patients used bisphosphonates at any moment during the study, for a mean duration of 6.9±4.3 years. Indications for bisphosphonate use were osteoporosis (N=23), clinical fractures (N=10), osteoporosis and clinical fractures (N=8), and other (N=7). Mean age at start of bisphosphonate-therapy was 52.6±13.5 years.

GH dose, IGF-1 SDS and hydrocortisone dose during rhGH replacement: Mean GH doses after dose titration (*i.e.* after 1year) and 5 years of rhGH replacement were, respectively, 0.39±0.20mg/day and 0.43±0.27mg/day (p=0.016 compared to 1year dose). Mean doses after, respectively, 10 and 15 years of rhGH replacement were 0.44±0.26mg/day and 0.39±0.21 mg/day (both NS compared to 1 year dose).

Mean IGF-1 SDS was within the normal range during the entire study period, and increased significantly from -1.09±2.46 at baseline to 0.66±2.01 after 5 years and was 1.24±2.45 after 10 years (both p<0.01 compared to baseline). In men, mean IGF-1 SDS increased significantly from -0.60±2.21 at baseline to 0.95±1.88 after 5 years (p=0<001) and to 1.64±2.57 after 10 years (p=0.011). In women, mean IGF-1 SDS increased significantly from -1.73±2.63 at baseline to 0.35±2.11 after 5 years of rhGH replacement (p<0.001) and to 0.83±2.29 after 10 years (p=0.003) (*Figure 1*).

Mean hydrocortisone doses were 24.1±7.3mg/day, 21.4±4.10mg/day and 21.1±4.3mg/day at baseline, after 10 and after 15 years of rhGH replacement, respectively, indicating no significant change in hydrocortisone dose during rhGH replacement.

Table 1. Baseline characteristics of 230 adult GHD patients

Patient characteristics	**GHD patients (N = 230)**
Sex, female / male (N)	121 / 109
Age at start rhGH replacement, years (range)	47.1 ± 14.9 (15 – 81)
AO / CO (N)	202 / 28
BMI, kg/m^2 (range)	27.3 ± 5.1 (16.9 – 57.1)
Etiological diagnosis of GHD (N)	
NFA	76
Functioning adenoma	68
Craniopharyngeoma	21
Cerebral malignancy	12
Congenital	27
Other causes	26
Surgery, TS / TC (N)	136 / 38
Radiotherapy (N)	102
Pituitary deficiencies (N)	
TSH	190
ACTH	197
FSH / LH	173
ADH	51
Isolated GHD	10
Bisphosphonates (N)	10
Calcium supplementation (N)	84
Vitamin D supplementation (N)	85

Data are presented as mean ± SD, unless specified otherwise.

N, number of patients; GHD, Growth Hormone Deficiency; rhGH, recombinant human Growth Hormone replacement; AO, adult-onset GHD; CO, childhood-onset GHD; BMI, body mass index; NFA, non-functioning adenoma; TS, transsphenoidal; TC, transcranial; TSH, thyroid stimulating hormone; ACTH, adrenocorticotropic hormone; FSH, follicle-stimulating hormone; LH, luteinizing hormone; ADH, anti-diuretic hormone; Vitamin D, vitamin D.

BIOCHEMICAL BONE MARKERS: Baseline calcium and Vitamin D levels were, respectively, 2.40±0.24mg/dl and 60.3±26.7nmol/l. After 5 years, mean calcium and Vitamin D levels were 2.40±0.11mg/dl (NS compared to baseline) and 63.3±24.5nmol/l (p=0.02 compared to baseline), respectively, and 2.39±0.26mg/dl and 61.8±26.0nmol/l (both NS compared to baseline) after 10 years of rhGH replacement. At baseline, P1NP and β-crosslaps concentrations were only available in 12 and 30 patients and were therefore not reported. After 5 years or rhGH replacement, mean P1NP and β-crosslaps concentrations were 45.3±26.6ng/mL and 0.42±0.24ng/mL, respectively, and 48.4±25.4ng/mL and 0.71±2.19ng/mL after 10 years. After 15 years of rhGH replacement, mean P1NP and β-crosslaps concentrations were 42.1±24.7ng/mL and 0.37±0.20ng/mL. These data indicate normal bone turnover throughout the entire study period with stable rhGH replacement.

BMC and BMD: Mean height, weight and BMI at the different time points during rhGH replacement are shown in *Table 2*. Mean lumbar spine BMD gradually increased during the first 10 years of rhGH replacement, from 0.98±0.15g/cm^2 at baseline to 1.03±0.16g/cm^2 after 10 years (p<0.001, 5% above the baseline value) and stabilized thereafter (*Table 2*). Mean lumbar spine T-scores increased during the first 10 years of rhGH replacement from -0.69±1.30 to -0.43±1.33 (p<0.001), and remained constant thereafter. Lumbar spine BMC increased throughout the first 10 years of rhGH replacement being 4% above the baseline level after 10 years (from 63.2±15.4kg to 65.8±18.0kg), and then reached a plateau. Similar results were obtained after stratification for the use of bisphosphonates, indicating that in the long-term there was no beneficial effect impacted from additional bisphosphonate use (*Figure 2*).

Left femoral neck BMD did not change during rhGH replacement (*Table 2)*, neither in the small subgroup of patients using bisphosphonates (*Figure 2*).

In *Supplementary File 3*, the mean BMC, BMD and T-score values at the different time points during rhGH supplementation were shown for the subgroup of GHD adults with 15 years of rhGH replacement (N=43).Within this subgroup, mean lumbar spine BMD and BMC scores increased during the first 10 years of rhGH supplementation and stabilized thereafter. With respect to left femoral neck, no changes during rhGH supplementation were found.

Table 2. Effects of 15 years of rhGH replacement in 230 adults with GHD on BMC, BMD and T-scores measured using DXA, and anthropometric parameters

	Baseline (N=230)	**5 years rhGH (N=230)**	**10 years rhGH (N=98)**	**15 years rhGH (N=43)**
DXA lumbar (L1-L4) spine				
BMC (kg)	63.2 ± 15.4	65.5 ± 15.4**	65.8 ± 18.0*	63.6 ± 13.9
BMD (g/cm^2)	0.98 ± 0.15	1.01 ± 0.14**	1.03 ± 0.16**	1.01 ± 0.14*
T-score (SD)	-0.69 ± 1.30	-0.58 ± 1.29**	-0.43 ± 1.33**	-0.54 ± 1.11
DXA left femoral neck				
BMD (g/cm^2)	0.83 ± 0.14	0.83 ± 0.15	0.84 ± 0.16	0.83 ± 0.15
T-score (SD)	-0.94 ± 1.30	-0.94 ± 1.06	-0.77 ± 1.18	-0.82 ± 1.04
Height (cm)	171.1± 13.0	171.6± 11.2	170.8± 11.1	168.4±14.7
Weight (kg)	81.7± 19.1	82.4±18.6	84.6 ±20.6	81.9±17.1
BMI (kg/m^2)	27.3±5.1	27.9 ±5.3*	28.8± 5.9*	29.6±8.4*

Statistical analyses were done by performing a Repeated Measurements ANOVA. To take into account multiple testing, p<0.01 was considered statistically significant.

N, number of patients on rhGH replacement at different time points; rhGH, recombinant human Growth Hormone; GHD, Growth Hormone Deficiency; BMC, bone mineral content; BMD, bone mineral density; DXA, dual energy X-ray absorptiometry; BMI, body mass index; SD, standard deviation.

**, p<0.01 (versus baseline)*
***, p<0.001 (versus baseline)*

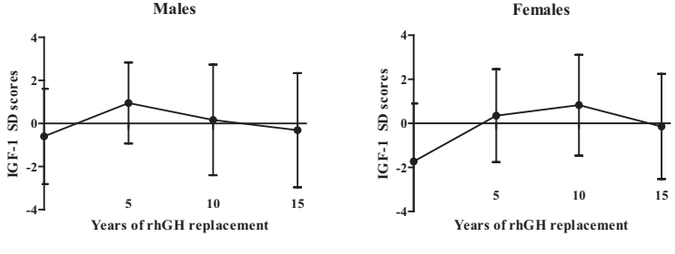

Figure 1. *Mean IGF-1 SD scores during 15 years of rhGH replacement, stratified for men (A) and women (B)*

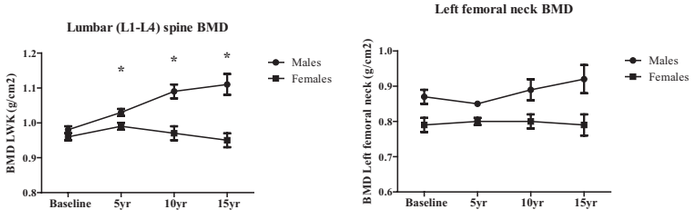

Figure 2. *BMD changes during 15 years of rhGH replacement at the lumbar spine and the left femoral neck, stratified for the use of bisphosphonates in male (A) and female GHD adults (B)*

Data are presented as mean BMD values (g/cm²), stratified by the use of bisphosphonates. Vertical bars indicate SD values.

rhGH, recombinant human Growth Hormone therapy; GHD, Growth Hormone Deficiency; BMD, bone mineral density; SD, standard deviation.
**, p<0.01 (versus baseline)*

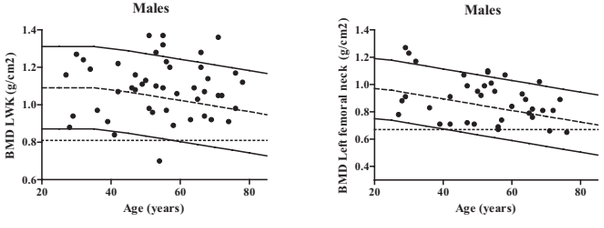

Figure 3A

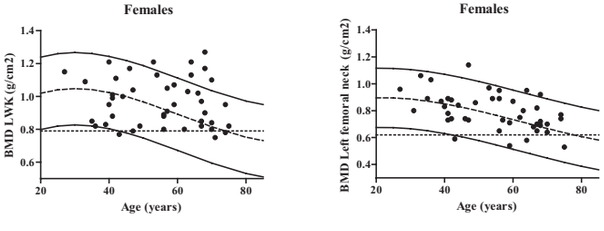

Figure 3B

Scatterplots of individual BMD values at the lumbar spine and left femoral neck in female (A) and male (B) GHD patients after 10 years of rhGH supplementation

Closed circles indicate individual scores, the interrupted line indicates mean BMD value for age and the solid lines indicate, respectively, +2 S.D. and -2 S.D. Dashed line indicates T-score -2.5 S.D.

BMD, bone mineral density; rhGH, recombinant human Growth Hormone supplementation; LWK, lumbar spine.

OSTEOPENIA AND OSTEOPOROSIS: At baseline, 29.1% and 31.7% of the patients had osteopenia (T-score<-1.0SD) in the lumbar spine and femoral neck, respectively. After 10 years of rhGH replacement, 37.8% of the patients had osteopenia at the lumbar spine and 35.7% at the femoral neck. The patients with persisting osteopenia at the femoral neck and/or lumbar spine at the study end, were older (68.5 years *vs* 59.2 years, p=0.014) and included more women (66.7% *vs* 46.7%, although NS). No differences were found with respect to 1 year GH dose or number of pituitary deficiencies between patients with and without persisting osteopenia and/or osteoporosis at the study end.

At baseline, 11 GHD patients (4.8%) had osteoporosis at the lumbar spine (4 women/7 men), with a mean age of 54.1±15.2 years. Six of these patients used bisphosphonates and respectively 7 and 5 patients had calcium and Vitamin D supplementation. Seventeen patients (7.4%) had baseline osteoporosis at the femoral neck (7 women/10 men), of whom nine patients used bisphosphonates, 11 calcium and 9 Vitamin D supplementation. In this small subgroup of patients with osteoporosis, no change in femoral neck BMD was found during rhGH supplementation. However, lumbar spine BMD increased during the first 5 years of rhGH supplementation, without a further increase afterwards. After 10 years of rhGH supplementation, osteoporosis was found in, respectively, 7.1% and 1.1% of patients at the femoral neck and the lumbar spine. BMD values of individual GHD patients after 10 years of rhGH supplementation were shown in *Figure 3A+3B*.

Influence of sex: Mean GH dose was significantly higher in women compared to men for the first 10 years of rhGH replacement, resulting in a higher GH dose/IGF-1 SDS ratio in female GHD patients. After 5 years of rhGH treatment, mean GH dose in men and women was respectively 0.37±0.26mg/day and 0.49±0.28mg/day (p=0.001) and at 10 years respectively 0.36±0.18mg/day and 0.53±0.30mg/day (p=0.001).

Men had a greater increase in lumbar spine BMD than women at all time points (all p<0.01 compared to baseline), but not in femoral neck BMD (*Figure 4A+2B*). These data were similar in patients with and without bisphosphonate use (*Figure 2A-D*). Similar sex differences, with highest response to rhGH replacement in men, were seen for lumbar spine BMC. Also in lumbar spine T-scores highest increase was seen in men after 10 and 15 years of rhGH replacement, although this was not shown for femoral neck T-scores (*data not shown*).

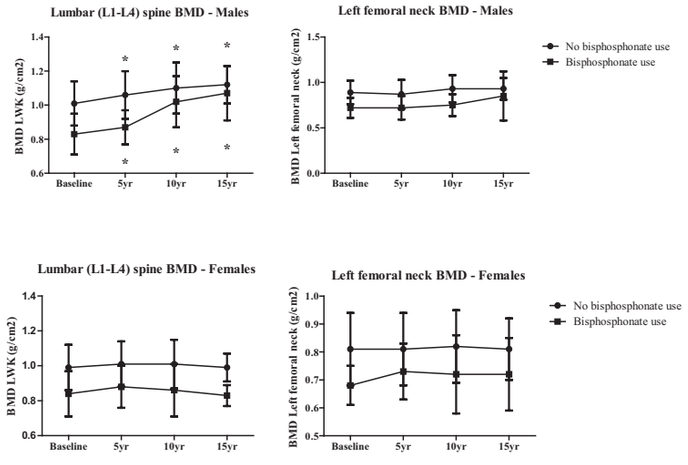

Figure 4. *Sex differences in the effects of 15 years of rhGH replacement in 230 adult GHD patients on lumbar (L1-L4) spine BMD (A) and left femoral neck BMD (B)*

Data are presented as mean BMD values (g/cm^2), stratified by sex. Vertical bars indicate SEM values. Men had a greater increase in lumbar spine BMD than women at all time points when compared to baseline (all p<0.001).

rhGH, recombinant human Growth Hormone therapy; GHD, Growth Hormone Deficiency; BMD, bone mineral density; SEM, standard error of the mean.

**, p<0.001 (versus baseline)*

OTHER POTENTIAL INFLUENCING FACTORS : Subsequently, we identified potential factors influencing the response to rhGH replacement on BMD and BMC. One hundred seventy-seven patients (77%) were <60 years and 53 patients (23%) were ≥60 years of age at baseline. The individualized rhGH dose used in older patients was significantly lower than in younger patients (0.41±0.21mg/day *vs* 0.32±0.13mg/day, p<0.001). After adjustment for the difference in rhGH dose, the rhGH response did not differ between younger and older patients with respect to IGF-1, BMD, BMC and T-scores at the lumbar spine or femoral neck. Univariate analyses including GH dose at 1year, low Vitamin D levels, hydrocortisone use, use of bisphosphonates, use of L-thyroxine, childhood- *vs* adulthood-onset, presence of ≥2 pituitary hormone deficiencies, or hypogonadism showed that these factors did not affect the effects of rhGH replacement on femoral neck BMD, lumbar spine BMD or BMC. A lower baseline lumbar BMD was found to be associated with a higher increase in lumbar BMD during 15 years of rhGH replacement, in both sexes. Bone response to rhGH replacement did not differ between patients with a history of functioning *vs* non-functioning adenoma or between patients with childhood- *vs* adulthood-onset GHD.

When incorporating all factors in a linear regression model, only higher GH dose at 1 year was associated with a decreased response to rhGH on BMC over 15 years. No other influencing factors were found.

FRACTURE DATA: Twenty-three patients (10%, 11 women/12 men) sustained fractures during rhGH supplementation, of which 12 at ≥2 sites. Fifteen patients had vertebral fractures (10 patients had ≥2 fractures), 9 patients non-vertebral fractures (hip N=6 (2 patients bilateral hip fracture), toe N=2, clavicula N=1). Consequently, the incidence rate of fractures during rhGH replacement was 46/2288.5 (mean duration of rhGH therapy 9.95 years x 230 patients) = 20.1/1000py in our GHD cohort (incidence rates for, respectively, non-vertebral and vertebral fractures in our cohort were 3.9/1000py and 16.2/1000py).

DISCUSSION

The present study describes the long-term effects of rhGH replacement on bone metabolism in a well-defined cohort of adult GHD patients. We found a sustained increase in BMD at the lumbar spine during the first 10 years of rhGH replacement, and stabilization thereafter, as was found for lumbar spine BMC and T-scores. At all time points, the largest effects of rhGH replacement on lumbar spine BMD and BMC were observed in men. In the small subset of patients using bisphosphonates, bisphosphonate use did not impact additional beneficial effects beyond an initial early effect. Lower baseline BMD and high GH dose at 1year significantly, respectively, positively and negatively influenced the bone response to long-term rhGH replacement. Clinical fracture incidence during long-term rhGH supplementation was 20.1/1000py.

Untreated GHD in adults is characterized by low bone turnover, decreased BMD and BMC (5-7), which increased or even normalized by short-term rhGH replacement (3;5;8). We have previously shown that 2 years of physiological dose of rhGH in GHD adults increased bone turnover in favor of bone formation, as reflected by a significant increase in BMD (23) that sustained after 7 years of treatment (24). In addition, during the first 4 years of rhGH supplementation, we observed a significant greater increase in BMD in GHD patients with osteoporosis with additional bisphosphonate treatment (24). These additional beneficial effects of bisphosphonates were preserved during another 3 years of rhGH replacement (25).

To date, only a single other study has reported the skeletal effects of rhGH replacement in GHD adults beyond 10 years (14). In agreement with this report, we observed a sustained increase of lumbar spine BMD and BMC during 15 years of rhGH replacement with a peak value after 5-7 years, being largest in male patients. In both studies, no changes in femoral neck BMD were found. However, since due to an ageing effect, it would be expected that femoral neck BMD decreases with increasing age over time (26). The observed stabilization of the femoral neck BMD in our study among GHD adults indicates that, in addition to an effect on lumbar spine BMD, rhGH supplementation also exerts an effect at the level of the femoral neck. The present study is the first to report that during long-term rhGH replacement, concomitant bisphosphonate use has no additional beneficial effect on BMD after an initial early effect (25;27), in the presence of stringently controlled Vitamin D and calcium status. In addition, this study also focused on clinical fracture incidence during long-term rhGH replacement.

When stratifying analyses for sex, lumbar spine BMD increases in both sexes during the first 5 years of rhGH replacement. Thereafter, we observed a further increase in BMD in men, whereas in women BMD decreased towards baseline values. Gender-specific differences in the bone response to rhGH supplementation were also demonstrated in several other studies, with largest effects in men (28;29). The lack of response in BMD in adult GHD women is suggested to due to the inhibition of the effect of GH on IGF-1 production by orally administered estrogens (30;31) or by inhibiting direct actions of GH on both osteoblasts and osteoclasts by estrogen (3). However, in this respect, it should be noted that GH doses are adjusted according to sex-adjusted IGF-1 levels. In general, given that IGF-1 levels are comparable between men and women, it is likely that other factors, such as differences in bone structure between both sexes play a role in sex-specific differences in bone response to rhGH supplementation.

Literature on fracture risk in rhGH-treated adult GHD patients is scarce and, to date, has been investigated in only three studies. Holmer *et al.* studied clinical fracture incidence in 832 rhGH-treated patients compared to 2581 matched healthy controls and reported a two-fold increase in fracture risk in CO-GHD women, although in AO-GHD men fracture risk was significantly lowered. No increased risk was found in AO-GHD women and CO-GHD men (32). Elbornsson *et al.* reported clinical fractures in a Swedish GHD cohort, and observed only 1 hip fracture and 1 symptomatic vertebral fracture (14). Mazziotti *et al.* reported a high prevalence of radiographic vertebral fractures of 53.8% in rhGH-treated GHD adults when compared to a prevalence of 78.6% in untreated GHD patients (33), suggesting that rhGH supplementation decreases fracture risk in GHD adults. In our cohort,10% of patients sustained a clinical fracture during long-term rhGH replacement, giving an incidence rate of 20.1/1000py. When compared to the Rotterdam Study in a healthy Dutch population aged ≥55 years, reporting a vertebral fracture incidence rate of 10.9/1000py and a non-vertebral fracture incidence rate of 25.0/1000py in women and 9.6/1000py in men (34;35) and in view of the younger age of our cohort, our data may suggest that fracture incidence is not increased in rhGH-treated GHD patients.

Our study has strengths as well as limitations. Its main strengths are the duration of follow-up and the size of our adult GHD cohort, who were regularly followed and stringently controlled over the years according to internationally used guidelines. Care for attention was given to Vitamin D status as reflected by normal average levels of Vitamin D and normal bone turnover rates throughout the entire study period. Limitations of the present study, and a general drawback in long-term follow-up studies, are, first, the lack of a non-rhGH treated control group. Since the beneficial effects of rhGH therapy are well-established in the short-term, rhGH replacement became a widely available therapy for adult GHD in the Netherlands, in case of no contra-indications. Consequently, it is no longer possible or ethical to perform long-term randomized placebo-controlled studies in GHD patients. A second limitation is the potential underestimation of the fracture incidence in our patients as spinal X-rays were only performed in case of a clinical fracture. Another feature that needs to be addressed is that due to the presence of multiple pituitary hormone deficiencies in almost all patients, it is difficult to examine to what extent the reported effects can be attributed to rhGH treatment. In this respect, the presence of interactions between glucocorticoids and rhGH during substitution and the negative effects of cortisol excess, either endogenous or supraphysiological supplementation, on bone has to be noted (23;36). However, in our study, there were no signs of hydrocortisone oversupplementation and when incorporating hydrocortisone use in a regression model, we found no significant association with BMD response. Finally, bisphosphonates, calcium and Vitamin D supplementation were initiated at the discretion of the treating physician and not given using a standardized protocol with only a small subset of patients using bisphosphonates. However, in other studies investigating skeletal effects of long-term rhGH supplementation, information on bisphosphonate use is often not available. In this respect, the combined use of bisphosphonates and rhGH supplementation should be a topic for future research.

In conclusion, these findings from our cohort study of GHD patients demonstrate ongoing beneficial effects on bone metabolism of 10 years of rhGH replacement therapy, particularly in men, with stabilization thereafter. Beneficial effects are most marked on BMD at the lumbar spine. In addition, clinical fracture incidence seems low in our adult GHD patients. Sex-specific differences in bone response to rhGH replacement and the long-term beneficial effects of bisphosphonates on fracture risk warrant further studies in adult GHD patients, and therefore, protocolled prolonged follow-up of these patients is mandatory.

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SUPPLEMENTARY FILE 1.

Assays

Serum GH was measured with a sensitive IFMA (Wallac, Turku, Finland), specific for the 22 kDA GH protein (detection limit: 0.01µg/l, interassay coefficient of variation (CV): 1.6-8.4% of 0.01-15.38µg/l) from 1992 onwards. For the conversion of µg/l to mU/l, multiply by 2.6. Before 1992, GH was measured by RIA (Biolab, Serona, Coissins, Switzerland), detection limit: 0.5mU/l, with an interassay CV <5%; for the conversion of µg/l to mU/l, multiply by 2. From 1986 to 2005, serum IGF-1 concentrations were determined by RIA (Incstar, Stillwater, MN) with a detection limit of 1.5nmol/l and an interassay CV less than 11%. IGF-1 is expressed as SD score for age- and gender-related normal levels determined in the same laboratory (33). Since 2005, serum IGF-1 concentrations (nmol/l) were measured using an immunometric technique on an Immulite 2500 system (Siemens Healthcare Diagnostics, Deerfield, IL, USA). The intra-assay variations at mean plasma levels of 8 and 75nmol/l were 5.0 and 7.5%, respectively. IGF-1 levels were expressed as SDS, using lambda-mu-sigma smoothed reference curves based on 906 controls(21;22).

B-crosslaps and P1NP concentrations were measured by an electrochemolumiscent immunoassay with a Modular Analytics E-170 system (Roche Diagnostics, Almere, The Netherlands). References values for β-crosslaps and P1NP concentrations were, respectively, <0.854ng/ml and <59ng/ml. Vitamin D25OH was measured by RIA (Incstar/DiaSorin, Stillwater, MN, USA) (reference range (50 – 250nmol/l).

SUPPLEMENTARY FILE 2.

Flow chart of selection and follow-up of our adult GHD cohort (N=230)