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Year: 2020

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DOI: <https://doi.org/10.1002/jbmr.4033>

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ZORA URL: <https://doi.org/10.5167/uzh-187505>

Journal Article

Published Version




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Originally published at:

Braun, Leah T; Fazel, Julia; Zopp, Stephanie; Benedix, Sarina; Osswald-Kopp, Andrea; Riestler, Anna; Rubinstein, German; Seidensticker, Max; Beuschlein, Felix; Drey, Michael; Bidlingmaier, Martin; Schmid-

maier, Ralf; Reincke, Martin (2020). The effect of biochemical remission on bone metabolism in Cushing's syndrome: a two-year follow-up study. *Journal of Bone and Mineral Research*, 35(9):1711-1717.  
DOI: <https://doi.org/10.1002/jbmr.4033>

# The Effect of Biochemical Remission on Bone Metabolism in Cushing's Syndrome: A 2-Year Follow-Up Study

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## ABSTRACT

Endogenous Cushing's syndrome (CS) is a rare cause of secondary osteoporosis. The long-term consequences for bone metabolism after successful surgical treatment remain largely unknown. We assessed bone mineral density and fracture rates in 89 patients with confirmed Cushing's syndrome at the time of diagnosis and 2 years after successful tumor resection. We determined five bone turnover markers at the time of diagnosis, 1 and 2 years postoperatively. The bone turnover markers osteocalcin, intact procollagen-1N-propeptide (PINP), alkaline bone phosphatase, CTX-I, and TrAcP 5b were measured in plasma or serum by chemiluminescent immunoassays. For comparison, 71 sex-, age-, and body mass index (BMI)-matched patients in whom Cushing's syndrome had been excluded were studied. None of the patients received specific osteoanabolic treatment. At time of diagnosis, 69% of the patients had low bone mass (mean  $T$ -score =  $-1.4 \pm 1.1$ ). Two years after successful surgery, the  $T$ -score had improved in 78% of patients (mean  $T$ -score 2 years postoperatively  $-1.0 \pm 0.9$ ). The bone formation markers osteocalcin and intact PINP were significantly decreased at time of diagnosis ( $p \leq 0.001$  and  $p = 0.03$ , respectively), and the bone resorption marker CTX-I and TrAcP 5b increased. Postoperatively, the bone formation markers showed a three- to fourfold increase 1 year postoperatively, with a moderate decline thereafter. The bone resorption markers showed a similar but less pronounced course. This study shows that the phase immediately after surgical remission from endogenous CS is characterized by a high rate of bone turnover resulting in a striking net increase in bone mineral density in the majority of patients. © 2020 The Authors. *Journal of Bone and Mineral Research* published by American Society for Bone and Mineral Research.

**KEY WORDS:** CUSHING'S DISEASE; HYPERCORTISOLISM; OSTEOCALCIN; OSTEOPOROSIS; OSTEOPOROTIC FRACTURES

## Introduction

Cushing's syndrome (CS) is a rare disease with approximately 0.7 to 2.4 new cases per 1 million per year.<sup>(1)</sup> Osteoporosis and osteopenia are typical comorbidities of patients with endogenous and exogenous CS. Depending on the study, 60% to 80% of patients have evidence for a reduced bone mineral density<sup>(2)</sup> characteristically affecting the entire skeleton.<sup>(3)</sup> About 5% of all cases of secondary osteoporosis are caused by hypercortisolism.<sup>(4)</sup> However, data from prospective, well-powered studies are rare, and few risk factors that would predict bone health have been identified so far. Guidelines for the management of osteoporosis due to endogenous CS are still missing.<sup>(5)</sup> In terms of risk assessment, the subtype of CS does not seem to influence

osteoporosis risk,<sup>(6)</sup> whereas the morning cortisol levels are negatively correlated with lumbar bone mineral density.<sup>(6)</sup> The duration of endogenous Cushing's syndrome (or the duration of exogenous replacement therapy after successful surgery) obviously affects bone mineral density.<sup>(7)</sup> Whether the  $T$ -score is the best predictor for fracture risk is not quite clear.<sup>(2)</sup>

Another area of uncertainty is the natural course of osteoporosis and bone turnover markers once the diagnosis of Cushing's syndrome has been established. A number of studies have addressed this topic, but the interpretation of the results is hampered because of limited patient numbers, concomitant osteoanabolic treatment, or both.<sup>(8–10)</sup> In-depth insight on bone remodeling in CS might come from bone turnover marker studies. For example, the bone formation marker osteocalcin is

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Received in original form January 8, 2020; revised form April 1, 2020; accepted April 13, 2020. Accepted manuscript online April 21, 2020.

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The peer review history for this article is available at <https://publons.com/publon/10.1002/jbmr.4033>.

*Journal of Bone and Mineral Research*, Vol. 00, No. 00, Month 2020, pp 1–7.

DOI: 10.1002/jbmr.4033

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suppressed in untreated CS,<sup>(3)</sup> a consistent observation making it useful as a diagnostic marker for CS.<sup>(2)</sup>

Based on the paucity of data, the lack of evidence for treatment guidelines, and the pressing open questions regarding risk assessment and management of osteoporosis, we performed a sufficiently powered study to analyze the natural course of bone turnover and bone mineral density in a monocentric cohort of patients with endogenous Cushing's syndrome. To the best of our knowledge, this is the first such study, and the data obtained will be instrumental for clinicians who care for patients with Cushing's syndrome.

## Materials and Methods

### Patients

This study was performed as part of the prospective German Cushing registry, which has included 450 consecutive patients referred to our department for suspected CS since 2012. Structure and general characteristics of the registry have been described in detail previously.<sup>(11–14)</sup> All patients included in the registry underwent a standardized biochemical screening and clinical examination at time of diagnosis and a yearly follow-up after treatment to treat comorbidities and diagnose recurrence of the disease early.

In all patients, standard screening for CS with a 1 mg low-dose overnight dexamethasone suppression test (LDDST), collection of 24-hour urine (UFC), and sampling of midnight salivary cortisol were performed. When the diagnosis of CS was confirmed, further subtyping was based on plasma adrenocorticotrophic hormone (ACTH), corticotropin-releasing hormone (CRH) test, high-dose dexamethasone suppression test, imaging, and inferior petrosal sinus sampling (in case of ACTH dependence). Final diagnosis was CS in 156 patients and exclusion of CS in the remaining 294 patients. Patients with excluded CS were a quite heterogenic group with lead symptoms such as obesity (73%), arterial hypertension (50%), or hirsutism (33%). Final diagnoses in these subjects were metabolic syndrome, polycystic ovary syndrome (PCOS), obesity, depression, or primary hyperaldosteronism. Patient selection is shown in Fig. 1.

In our analysis, we excluded patients for whom no densitometry data were available ( $n = 63$ ) and patients receiving pharmacologic treatment for osteoporosis following diagnosis ( $n = 4$ ). Densitometry data were not available for multiple reasons (very young age, external densitometry in a different clinic, missing consent to perform densitometry).

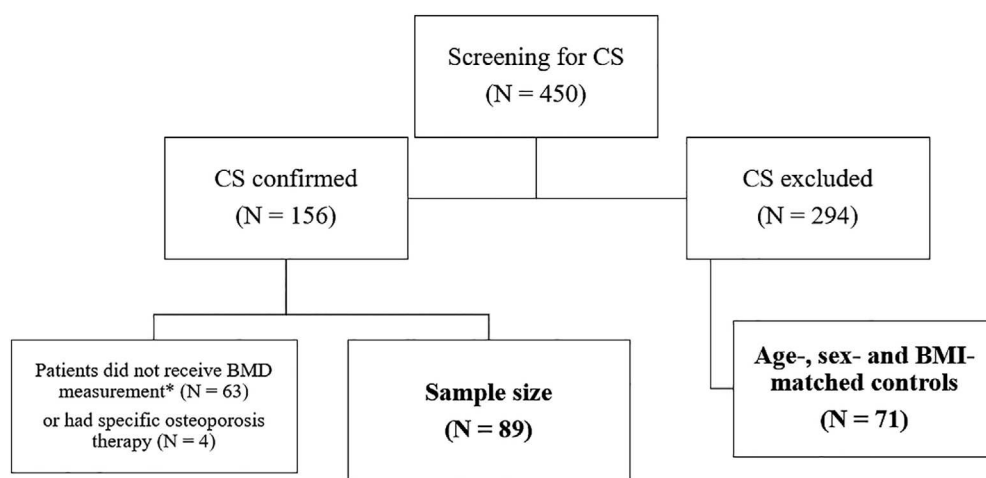
We matched the remaining 89 patients with 71 controls subjects selected from those subjects in whom CS was excluded. Matching was done according to sex, age, and body mass index (BMI). None of the patients and controls received specific osteoanabolic or antiresorptive treatment, but 47% of patients with CS received vitamin D supplementation after remission. At time of diagnosis, 11% of controls and 17% of patients with CS received vitamin D supplementation.

### Methods

In patients with confirmed CS, a bone mineral densitometry was conducted. Bone mineral density (BMD) was determined at the lumbar spine and the femur (neck and total femur).

If a reduced bone mineral density was diagnosed, a follow-up densitometry was performed 2 years after surgery. If bone mineral density was normal initially or during follow-up, only one further densitometry was performed 2 or 3 years after initial diagnosis. An improvement or decrease of bone mineral density was defined according to the least significant change ( $LSC = 2.8 \times 1.8\%$ ).<sup>(15)</sup> Accordingly, an alteration of more than 5.04% of BMD was rated as significant. A detailed fracture history was taken and X-ray of the spine was performed when clinical suspicion for fractures was high.

In all patients, blood samples (serum and plasma) were taken at time of diagnosis and also 1 and 2 years after successful transphenoidal surgery or adrenalectomy. Blood was taken in the fasting state between 8:00 and 10:00 a.m. Samples were centrifuged within 20 minutes at 4°C and stored at -80° until assayed. Three bone formation markers and two bone resorption markers were measured: osteocalcin, intact procollagen I-N-propeptide (PINP), and bone alkaline phosphatase (BAP) as bone formation markers, and CrossLaps (CTX-I) and tartrate-resistant acid phosphatase (5b TrAcP5b) as bone resorption marker, on basis of



**Fig. 1.** Patient selection. \*Very young age; patient conducted densitometry in a different clinic/outpatient clinic; patient refused densitometry. CS = Cushing's syndrome; BMD = bone mineral density; BMI = body mass index. Bold text indicates actual cohort of the study.

published data demonstrating their usefulness in CS and primary osteoporosis.<sup>(2,16)</sup>

Samples were measured at the Endocrine Laboratory of the Department of Internal Medicine IV on the iSYS automated analyzer (IDS-iSYS, Boldon, UK) by well-validated assays.<sup>(17,18)</sup> Published, method-specific reference intervals are available from a large healthy population.<sup>(19,20)</sup> For the determination of osteocalcin, an N-MID assay was used, as pre-analytics are less critical in this assay.<sup>(21)</sup> TrAcP 5b is a new marker, which, in contrast to CTX-1, can also reliably be measured in the non-fasting state.<sup>(22)</sup>

## Statistical analysis

In a priori power analysis, we calculated that a total sample size of 102 would be sufficient to identify significant differences between groups, assuming a medium effect size (0.5), a power of  $1 - \beta = 0.80$  and a type I error of  $\alpha = 0.05$ , with 51 subjects having Cushing's syndrome and 51 subjects being control subjects after excluding Cushing's syndrome.

For statistical analysis, SPSS 25 (IBM Corp., Armonk, NY, USA) was used. Clinical characteristics are shown as mean and standard deviation when data is normal distributed; otherwise as median and ranges. Because of the lack of normal distribution of bone turnover markers, nonparametric tests were used to test differences between groups. Differences between bone turnover markers at different times were tested by Friedman test. Multiple regression analysis was used to investigate differences between CS and the control group regarding bone turnover markers adjusted for sex, age, and BMI. Any  $p$  values  $< 0.05$  were considered to indicate statistical significance.

## Results

### Patient characteristics

The clinical and biochemical characteristics of the patient sample are summarized in Table 1. Sixty-five percent of patients had

pituitary CS, 28% adrenal, and 7% suffered from ectopic CS. Patients and controls were well-matched regarding sex, age, and vitamin D levels and supplementation, but differed in terms of diabetes prevalence.

### Baseline evaluation

At time of diagnosis, the mean levels of bone formation markers osteocalcin and intact PINP were significantly decreased compared with the controls, and the bone formation marker bone alkaline phosphatase was increased (Table 1; Fig. 2). Both bone degradation markers CTX and TrAcP were increased (Table 1). Taken together, this demonstrates increased bone resorption and decreased bone formation in florid CS. Results of multiple linear regression analysis comparing Cushing's syndrome patients and controls are shown in Table 2. Bone markers were similar in patients with a reduced bone mass versus those with a normal bone mass (data not shown).

Overall, bone mineral density was decreased with an average lowest  $T$ -score of  $-1.4 (\pm 1.1)$ . BMD was significantly lower ( $p = 0.001$ ) at the femoral neck ( $T$ -score =  $-0.9 \pm 1.0$ ) and the spine ( $T$ -score =  $-1.0 \pm 1.5$ ) compared with the total femur ( $T$ -score =  $-0.5 \pm 1.2$ ). Twenty-eight patients (32%) had a normal bone mineral density, 46 (52%) osteopenia, and the other 15 patients (17%) osteoporosis with a  $T$ -score lower than  $-2.5$ .

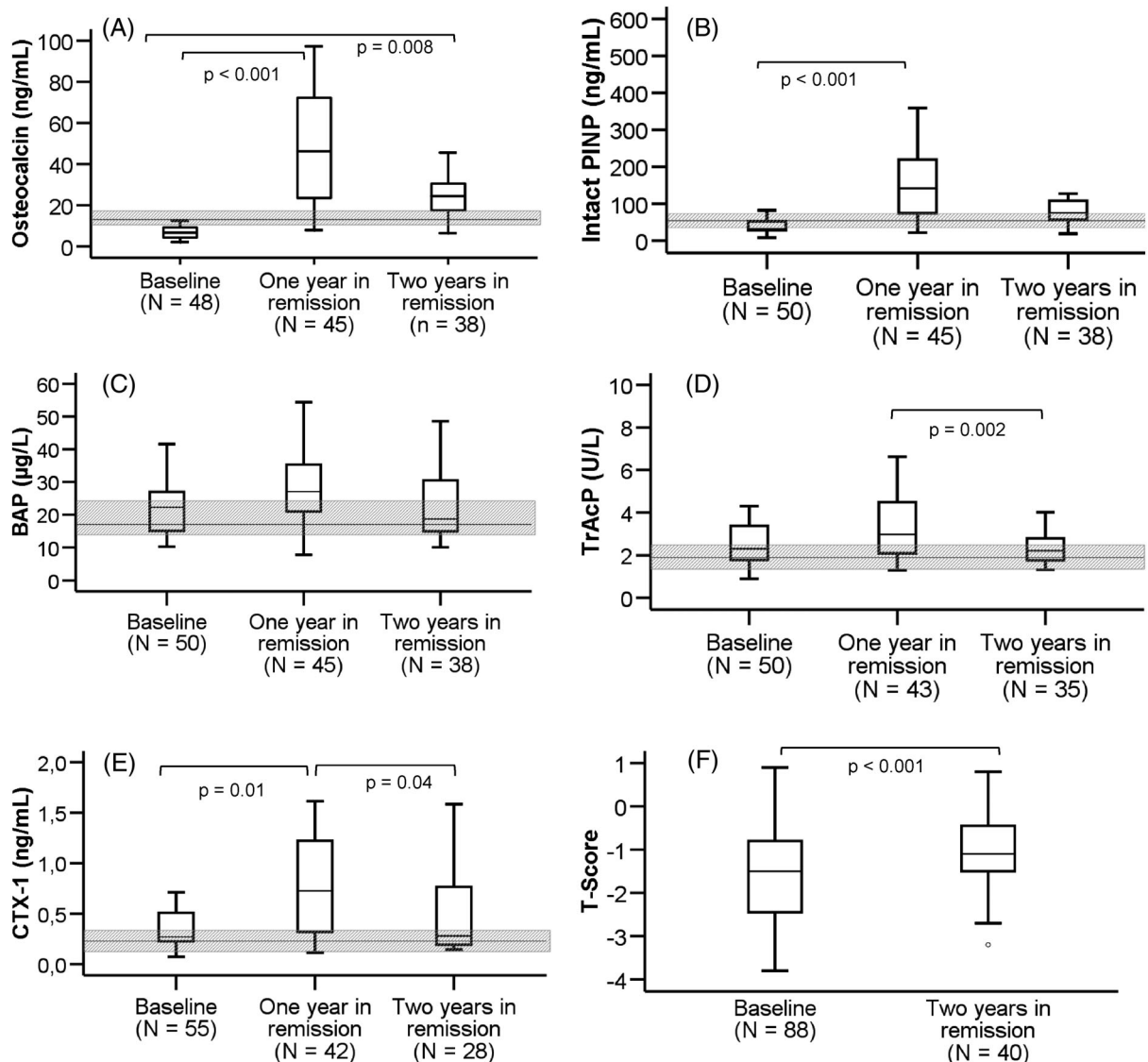
Seventeen of the patients (19%) had a history of low-trauma osteoporotic fractures (9 vertebral fractures, 8 nonvertebral fractures). The fractures took place shortly before diagnosis (58%) or more than 2 years before diagnosis of the CS (42%). Patients with osteoporotic fractures had a significantly lower  $T$ -score than patients without fractures ( $T$ -score =  $-1.9 \pm 0.8$  versus  $-1.3 \pm 1.1$ ,  $p = 0.03$ ) but did not differ in the values of the bone turnover markers or standard biochemical screening. Subtype, age, or BMI also did not differ between groups. However, men were significantly at higher risk of having fractures than women (35% of men had fractures versus 14% of women,  $p = 0.03$ ). Both severity of hypercortisolism and duration of CS did not contribute to fractures rates (data not shown), but UFC was significantly higher in patients with a  $T$ -score lower than  $-1.5$  (Table 3).

**Table 1.** Clinical and Biochemical Baseline Characteristics of Patients with Cushing's Syndrome (CS) and Control Subjects in Whom CS Has Been Excluded

	CS at time of diagnosis ( $n = 89$ )	CS excluded ( $n = 71$ )	$p$ Value
Sex	66 women (74%), 23 men (26%)	53 women (75%), 18 men (25%)	0.94
Age (years)	44 $\pm$ 13	43 $\pm$ 14	0.56
BMI	30 $\pm$ 7	31 $\pm$ 6	0.11
Vitamin D (ng/mL)	24 $\pm$ 10	24 $\pm$ 12	0.59
Vitamin D supplementation	17%	11%	0.37
Diabetes mellitus	30% (26)	11% (7)	<b>0.007</b>
Morning serum cortisol ( $\mu$ g/dL)	18 (11.7–24.9)	8.4 (5.9–11.6)	<b><math>\leq 0.001</math></b>
LDDST ( $\mu$ g/dL)	14.7 (7.7–23.7)	1.0 (0.8–1.2)	<b><math>\leq 0.001</math></b>
UFC ( $\mu$ g/24 h)	587 (331–843)	140 (78–216)	<b><math>\leq 0.001</math></b>
ACTH (pg/mL)	47 (9–76)	13 (9–18)	<b><math>\leq 0.001</math></b>
Late-night salivary cortisol (ng/mL)	7.9 (3.3–11.8)	1.2 (0.6–1.8)	<b><math>\leq 0.001</math></b>
Bone turnover markers			
Osteocalcin (ng/mL)	8 (5–13)	13 (10–17)	<b><math>&lt; 0.001</math></b>
PINP (ng/mL)	35 (29–62)	52 (35–73)	<b>0.025</b>
BAP ( $\mu$ g/L)	23 (16–31)	17 (14–24)	<b>0.006</b>
CTX-I (ng/mL)	0.28 (0.17–0.42)	0.23 (0.12–0.32)	<b>0.033</b>
TrAcP (U/L)	2.3 (1.7–3.4)	1.9 (1.3–2.4)	<b>0.009</b>

Data are shown as mean  $\pm$  standard deviation or median and ranges.

BMI = body mass index; LDDST = low-dose dexamethasone suppression test; UFC = urinary free cortisol; ACTH = adrenocorticotropic hormone; PINP = intact procollagen I-N-propeptide; BAP = bone alkaline phosphatase; CTX-I = CrossLaps; TrAcP = tartrate-resistant acid phosphatase. Bold numbers indicate statistical significance.



**Fig. 2.** Bone turnover markers and bone mineral density at baseline and 1 and 2 years after remission. Boxplot = median and ranges of bone turnover marker in patients with Cushing's syndrome. Gray box = median and ranges of bone turnover markers in the control group. PINP = procollagen I-N-propeptide; BAP = bone alkaline phosphatase; TrAcP = tartrate-resistant acid phosphatase; CTX-1 = CrossLaps.

### One- and 2-year follow-up

Surgical tumor resection leading to biochemical remission of CS resulted in a strong increase of bone formation markers tested at 1-year follow-up (Table 4; Fig. 2A, B). After 2 years, the markers had decreased slightly but remained elevated. Bone resorption markers were mildly increased at time of diagnosis, increased further at 1 year post-surgery, and returned almost to normal levels at 2 years (Table 4; Fig. 2D, E). A follow-up bone densitometry conducted in 40 patients showed a parallel increase of the T-score of  $0.6 \pm 0.8$  (Fig. 2F). In particular, BMD of the spine improved (Table 5).

In 78% of patients, bone mineral density improved after 2 years; in 45% of patients, T-score improved more than 0.5. No clinical fractures occurred after successful treatment of the CS. There was no significant correlation between improvement of bone mineral density and any of the bone turnover markers.

### Discussion

This study investigated for the first time to our knowledge a panel of bone formation and resorption markers in a large cohort of patients with CS over the long term. The unique and comprehensive data show that initially bone metabolism is characterized by decreased bone formation and increased bone resorption, in line with the classical action of glucocorticoids. Successful treatment of endogenous Cushing's syndrome leads to a strong activation of bone turnover, characterized by increased bone formation and bone resorption, a process that is continuous beyond year 2 after remission of CS, although at a reduced activity level. In parallel, bone mineral density increases in the majority of patients. Although 19% had low-trauma fractures at baseline, none of the subjects experienced clinical fractures during follow-up. In summary, these data give new insight into bone healing after remission of CS. They strongly suggest that an

**Table 2.** Results of Multiple Linear Regression Analysis Comparing Cushing's Syndrome Patients Versus Controls

Dependent variable	Standardized regression coefficient and <i>p</i> value for group variable	
	Unadjusted	Adjusted for age, sex, and BMI
Osteocalcin (ng/mL)	−0.392, <b>0.006</b>	−0.375, <b>0.010</b>
PINP (ng/mL)	−0.215, 0.204	−0.256, 0.145
BAP (μg/L)	0.404, <b>0.001</b>	0.470, <b>&lt;0.001</b>
CTX-I (ng/mL)	0.111, 0.366	0.065, 0.616
TrAcP (U/L)	0.227, 0.014	0.186, 0.069

PINP = procollagen I-N-propeptide; BAP = bone alkaline phosphatase; CTX-I = CrossLaps; TrAcP = tartrate-resistant acid phosphatase. Bold numbers indicate statistical significance.

observational approach to the bone phenotype is justified as long as remission from CS is secured.

### Reversibility of osteoporosis and bone turnover markers

Although established in osteoporosis research, bone turnover markers are not measured on a routine basis in patients with CS. However, it is a consistent result from different studies that osteocalcin is depressed in patients with CS. In fact, this finding is so reliable that it was even suggested to use osteocalcin in the diagnosis of CS.<sup>(2)</sup> PINP and procollagen carboxy-terminal propeptide (P1CP) have also been studied in several studies, with contradictory results.<sup>(23)</sup> In a retrospective study with 21 patients

with CS, it was shown that osteocalcin is depressed; this applies also for PINP, whereas CTX is increased.<sup>(24)</sup>

Some studies already have focused on the reversibility of osteoporosis after treatment of CS. In the majority of patients, bone mineral density increased within 2 years after successful treatment<sup>(8–10,25)</sup> Hermus and colleagues showed in a study with 20 patients that bone mineral density did not change 3 or 6 months after surgery but increased thereafter in almost all patients.<sup>(8)</sup> In a study with 68 patients, the patients were followed up for 4 years. Bone mineral density increased over lumbar spine and femur but decreased at the forearm.<sup>(25)</sup> The authors concluded that bone minerals were redistributed from the peripheral to the axial skeleton.

In our study, bone mineral density also improved in the majority of patients but remained reduced in some. We did not find any difference in bone turnover markers between patients with improvement and without improvement.

### Current treatment guidelines and treatment suggestions

As observed in our study, bone formation markers increase significantly after surgical cure, whereas bone degradation markers are mildly elevated at baseline and increase slightly at 1 year, returning within the normal range at 2 years. So far, there is no international guideline on the treatment of osteoporosis induced by endogenous CS and very few controlled interventional studies. In an opinion paper, Scillitani and colleagues recommended to treat all patients with vitamin D and calcium but not with bisphosphonates.<sup>(5)</sup> In a randomized open-label study by Di Somma and colleagues,<sup>(26)</sup> 39 patients (18 patients with active CS and 21 patients with CS in remission) received alendronate or no medication. Patients with active CS also received ketoconazole to control hypercortisolism. Bone mineral density improved and serum levels of osteocalcin increased in

**Table 3.** Biochemical Markers in Patients With Cushing's Syndrome With a *T*-Score Lower Than −1.5 and Above −1.5 Shown in Median and Ranges

Variable	<i>T</i> -score < −1.5 ( <i>n</i> = 39)	<i>T</i> -score ≥ −1.5 ( <i>n</i> = 42)	<i>p</i> Values
LDDST (μg/dL)	16.6 (10.3–28.3)	11.9 (6.1–21.9)	0.12
UFC (μg/24 h)	706 (410–906)	398 (285–787)	<b>0.03</b>
Late-night salivary cortisol (ng/mL)	8.3 (3.5–13.6)	5.7 (2.9–11.7)	0.39
ACTH (pg/mL)	53 (16–73)	42 (6–82)	0.88

LDDST = low-dose dexamethasone suppression test; UFC = urinary free cortisol; ACTH = adrenocorticotropic hormone. Bold numbers indicate statistical significance.

**Table 4.** Bone Turnover Markers and Bone Mass in Patients With Cushing's Syndrome at Time of Diagnosis and During 2 Years of Follow-Up

	Time of diagnosis ( <i>n</i> = 50)	1 year in remission ( <i>n</i> = 45)	2 years in remission ( <i>n</i> = 38)	<i>p</i> (0 versus 1)	<i>p</i> (0 versus 2)	<i>p</i> (1 versus 2)
<i>T</i> -score	−1.5 (−2.0 to −0.8)	–	−1.1 (−1.5 to −0.4)	–	<b>&lt;0.001</b>	–
Osteocalcin (ng/mL)	8 (5–13)	30 (14–60)	21 (13–31)	<b>&lt;0.001</b>	<b>0.008</b>	0.3
PINP (ng/mL)	35 (29–62)	117 (52–221)	69 (46–113)	<b>&lt;0.001</b>	0.1	0.1
BAP (μg/L)	23 (16–31)	26 (19–38)	22 (15–31)	0.2	0.4	0.1
CTX-I (ng/mL)	0.28 (0.17–0.42)	0.51 (0.22–0.91)	0.25 (0.18–0.73)	<b>0.01</b>	0.1	<b>0.04</b>
TrAcP (U/L)	2.3 (1.7–3.4)	2.8 (1.8–4.0)	2.3 (2–3.2)	0.1	0.6	<b>0.002</b>

PINP = procollagen I-N-propeptide; BAP = bone alkaline phosphatase; CTX-I = CrossLaps; TrAcP = tartrate-resistant acid phosphatase. Bold numbers indicate statistical significance.

**Table 5.** Overview: T-Scores, Z-Scores, and BMD Values With Percent Changes (Mean and Standard Deviation)

Variable	CS at time of diagnosis	CS 2 years in remission	p Values, percent changes (†)
Femoral neck			
T-score femoral neck	-0.81 ± 0.97	-0.59 ± 0.86	0.06
Z-score femoral neck	-0.59 ± 0.98	-0.28 ± 0.79	<b>0.02</b>
BMD (g/cm <sup>2</sup> ) femoral neck	0.91 ± 0.12	0.95 ± 0.12	0.16; 4% ↑
Femur			
T-score femur	-0.49 ± 1.11	-0.42 ± 1.04	0.67
Z-score femur	-0.40 ± 1.04	-0.37 ± 0.85	0.31
BMD (g/cm <sup>2</sup> ) femur	0.95 ± 0.15	0.97 ± 0.14	0.77, 2% ↑
Spine			
T-score spine	-0.96 ± 1.56	-0.55 ± 1.25	<b>&lt;0.001</b>
Z-score spine	-0.85 ± 1.53	-0.58 ± 1.14	<b>&lt;0.001</b>
BMD (g/cm <sup>2</sup> ) spine	1.08 ± 0.22	1.13 ± 0.15	<b>0.001, 0.6% ↑</b>

BMD = bone mineral density; CS = Cushing's syndrome. Bold numbers indicate statistical significance.

patients who received alendronate to a greater extent than those receiving no alendronate.

In a small study by the same research group,<sup>(27)</sup> 15 patients with CS (9 adolescent patients and 6 adults) were observed for 2 years after successful treatment, showing that osteocalcin levels and bone mineral density increased significantly.

### Strengths and limitations

Although this study has several strengths, including the large prospective design and measuring a panel of bone formation and resorption markers, there are a few limitations. Some asymptomatic fractures may have been overlooked because an X-ray was not taken systematically in each patient. Furthermore, a follow-up bone densitometry was not available for all patients. Additionally, patients in the control group suffered from diabetes, overweight, arterial hypertension, or other diseases.

### Novel aspects and outlook

This study analyzes for the first time in a comprehensive way bone turnover markers during the course of CS. The data show that cure from CS leads to increases in bone remodeling and bone mineral density, in line with spontaneous "bone healing." Our data support a wait-and-watch strategy despite a high endogenous risk for additional fractures, based on the baseline assessment. This observation will influence future therapeutic strategies in patients with CS.

Our data suggest that the phase immediately after remission from CS is characterized by a high rate of bone turnover, resulting in a spontaneous net increase in bone mineral density in the majority of patients. Both bone attachment and bone degradation markers increase significantly, leading to an increase in bone mass and to a reduced risk of osteoporotic fractures. This unconstrained increase in bone formation markers after remission should be considered before specific therapy is initiated. Our data do not favor specific pharmacologic interventions with bisphosphonates or denosumab during this phase of remodeling because they may disrupt the osteoblast-mediated bone mass increase.

### Disclosures

All authors state that they have no conflicts of interest.

### Acknowledgments

This work is part of the German Cushing's Registry CUSTODES and has been supported by a grant from the Else Kröner-Fresenius Stiftung to MR (2012\_A103 and 2015\_A228). Additionally, AR, FB, and MR received funding by the Deutsche Forschungsgemeinschaft (CRC/TRR 205/1 "The Adrenal Gland"). Furthermore, funds for this project were provided by the Verein zur Förderung von Wissenschaft und Forschung an der Medizinischen Fakultät der Ludwig-Maximilians-Universität München eV to LB.

The data are stored on the following repository: <https://figshare.com/> and will be made accessible after publication of the article.

Authors' roles: LB served as the principal investigator in this work and was responsible for the study conception and design, the analysis and interpretation of the data, and the drafting of the manuscript. JF, SZ, AO, AR, GR and SB contributed to the collection and analysis of the data. MS, FB, MD, MB substantially contributed to the interpretation of the data and the drafting of the manuscript. RS contributed to the conceptual design of the study, the interpretation of data and the revision of the paper. MR contributed to the conceptual design of the study, the collection, analysis and interpretation of data, and the drafting and revision of the paper. All authors contributed to the critical revision of the manuscript and approved the final version for publication.

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