Original Article

Hylonome

Sclerostin and DKK1 levels during 14 and 21 days of bed rest in healthy young men

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Abstract

Objectives: Wnt signaling pathway may be crucial in the pathogenesis of disuse-induced bone loss. Sclerostin and DKK1, antagonists of the Wnt signaling pathway, were assessed during immobilization by bed rest in young, healthy people. **Methods:** Two bed rest studies were conducted at the German Aerospace Center in Cologne. 14 days of 6° head-down-tilt bed rest were applied to eight healthy young male test subjects in study 1 and 21 days of head-down-tilt bed rest to seven healthy male subjects in study 2. **Results:** Sclerostin levels increased in both studies during bed rest (study 1, 0.64±0.05 ng/ml to 0.69±0.04 ng/ml, P=0.014; study 2, 0.42±0.04 ng/ml to 0.47±0.04 ng/ml, P=0.008) and they declined at the end of the 14- and 21-day bed rest periods. DKK1 decreased during the bed rest period in study 1 (P<0.001) but increased during bed rest in study 2 (P=0.006). As expected, bone formation marker PINP decreased (study 1, P=0.013; study 2, P<0.001) and bone resorption marker NTX increased during bed rest (P<0.001). **Conclusion:** Data suggest that the Wnt signaling pathway is involved in disuse-induced bone loss in young, healthy humans.
Keywords: Sclerostin, DKK1, Bone Metabolism, Immobilization, Wnt Signaling

Introduction

Reductions in physical activity and weight-bearing, such as those associated with mechanical unloading in spaceflight or bed rest, are known to induce considerable bone loss, characterized by loss of bone mass and alterations in biochemical markers of bone metabolism[1-4]. One important pathway in the control of bone formation that is currently receiving ample attention is the Wnt signaling pathway. Activation of Wnt signaling occurs upon binding of Wnt glycoproteins to the co-receptors osteoblast membrane-bound Frizzled receptor and low-density lipoprotein receptor-related proteins 5 and 6 (LRP5, 6). Signals inside the cell are generated through β-catenin, which regulates transcription of genes that promote osteoblastic bone formation[5-8]. This

pathway is involved in the bone's response to both loading and unloading[9]. Osteocytes, which are usually considered to be the "mechanosensors" in the control of bone adaptation[10,11], produce a glycoprotein called sclerostin, coded by the SOST gene, which binds to LRP5 and 6 to prevent formation of the Frizzled/LRP receptor complex and thus Wnt-mediated signaling[12]. A series of animal studies on rats and mice have confirmed that sclerostin is an inhibitor of bone formation, whereas sclerostin expression is increased in response to unloading and depressed due to mechanical loading[13,14]. Accordingly, physical activity in humans seems to elicit decreases in sclerostin levels as demonstrated by Ardawi et al.[15] in premenopausal women.

To the best of our knowledge, only a very few studies on sclerostin response to unloading have been conducted in humans. Gaudio et al.[16] reported that postmenopausal women affected by a single episode of stroke, 6 months before they were included in the study, had higher serum levels of sclerostin than their age-matched peers. This change was paralleled by

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Abbreviations: DKK1, Dickkopf-related protein, LRP5,6 low-density lipoprotein receptor-related protein 5 and 6, ELISA, enzyme-linked immunosorbent assay.

high bone resorption and low bone formation rates in the patient group. In a recently published study by Spatz et al.[4] 90 days of bed rest led to elevated sclerostin levels after 28 days of bed rest with a peak on day 60 in young healthy subjects. However, and in apparent contrast, sclerostin levels in patients with spinal cord injury were found to be lower than in an ambulating control group[17,18].

Another inhibitor of the Wnt signaling pathway is Dickkopf-related protein 1 (DKK1)[19]. Butler et al.[20] have shown that serum DKK1 expression is highly correlated with bone mass variables; inverse associations have been found between serum DKK1 expression and lumbar and femur T-scores. Nevertheless, immobilization-induced increases in serum DKK1 level have not been detected until recently[16]. It would be desirable to obtain more data in a younger and healthy population, to elucidate the physiological role of Wnt signaling in immobilized humans.

We hypothesized that elevations in serum sclerostin and DKK1 levels would occur as a result of immobilization during 14 and 21 days of bed rest in healthy young men, and we were particularly interested in the time course of the expected changes.

Material and Methods

The described analyses were done as part of the so called "Vibration Bed Rest" (VBR) study (in the following named study 1) and the "Nutritional Countermeasures" (NUC) bed rest study (in the following named study 2) conducted at the DLR, German Aerospace Center in Cologne, Germany, at the Institute of Aerospace Medicine, in 2004/5 (study 1) and 2010 (study 2).

The studies were approved by the ethics committee of the Aerztekammer Nordrhein (North Rhine Medical Association), Duesseldorf, Germany, and were designed and performed in compliance with the Declaration of Helsinki. Studies are registered in the German Clinical Trial Register (http://register.germanctr.de) (study 1: DRKS00000140) or on http://www.clinicaltrials.gov (study 2: NCT01509456).

Study design

Subjects were immobilized for 14 days (HDT1 - HDT14) in 6° head-down-tilt bed rest in study 1 and for 21 days (HDT1 - HDT21) in head-down-tilt bed rest in study 2. Subjects were ambulatory during the baseline (study 1: 4 days, BDC-4 to BDC-1; study 2: 7 days, BDC-7 to BDC-1) and recovery (study 1: 5 days, R+0 to R+4; study 2: 6 days, R+0 to R+5) data collection periods. Reambulation occurred in the morning of R+0.

Both studies originally had a crossover design and aimed at investigating different countermeasures (vibration training and nutrition) was the primary purpose. Description of the primary goals are or will be described elsewhere since it is not relevant for the data given in this paper (study 1[21,22], study 2[23,24]). In both studies the control condition was bed rest only without applying countermeasures. As only the control conditions of both studies are scientifically comparable for the purpose of this publication, sample analysis as described in this paper was restricted only to the control conditions (bed rest without countermeasures) for both studies.

Subjects in a head–down-tilt bed rest study stay in bed for 24 hours per day with the main body axis inclined by -6°, that is, with the head below the level of the feet. All leisure activities and hygienic procedures were performed in this position. It has to be mentioned that test subjects in study 1 walked twice a day 25 steps from their sleeping room to a training room in which the training of the intervention group was performed.

While they were in bed, volunteers were not allowed to raise their heads >30° from normal or to perform dynamic or static muscle contractions. Subjects were instructed to turn over in bed mainly by moving their arms. Compliance with the protocol was ascertained by supervised nursing staff and video control.

Subjects

Respectively eight (study 1) and seven (study 2) healthy young male test subjects (study 1: age, 26.4±4.9 years; body mass, 78.1±9.5 kg; study 2: age, 27.6±3.3 years; body mass, 78.6±6.4 kg) took part in the studies after they had given written informed consent. For both studies, candidates had been excluded if they had chronic hypertension, diabetes, obesity, arthritis, hyperlipidemia, any hepatic disease, or a disorder of calcium or bone metabolism. All volunteers were nonsmokers, were not taking any medication, and were exercising less often than four times a week before study onset. Moreover, heritable blood clotting disorders (AT III, S-Akt, Lupus-PTT, ferritin, Factor V Leiden, Factor IV, and Factor II) were screened for in study 2, and candidates were excluded if they had positive test results. A psychological examination to ensure reliability of test subjects was part of the inclusion process as well.

Diet

The diet in both studies was constant and controlled for macro- and micronutrients matching the dietary reference intakes[25].

All ingredients, food items, and beverages for each test subject were weighed on a laboratory scale with a precision of 0.1 g. To confirm standardized nutrient intake, test subjects were encouraged to eat and drink only and to the full extent the meals provided to them, and their compliance was checked by the facility supervisor.

Sample collection

Fasting blood samples were taken in the supine position under standardized conditions at 7:00 a.m. shortly after subjects awakened and before they had breakfast. Blood was drawn on days BDC-1; HDT2, 6, 8, 11, and 14; and R+2, and +4 in study 1 and on BDC-2; HDT2, 6, 10, 14, and 21; and R+2 and +5 in study 2. Whole blood was centrifuged (3000 rpm, 4°C, 10 minutes) after coagulation, and serum was distributed in small aliquots and immediately frozen at -80°C until analysis.

Urine was collected as 24-h urine pools on all study days. The first morning sample was scheduled each day at 7:00 a.m. Each void was kept dark and cold until final pooling in the 24-h urine pool. Aliquots were stored at -20°C.
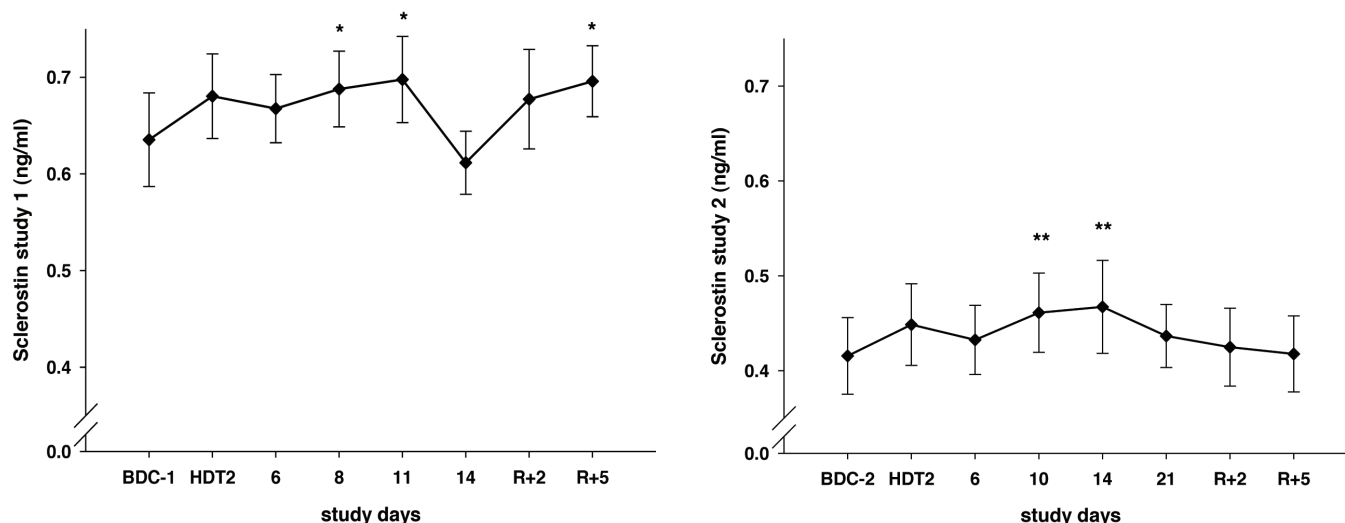
Figure 1. Serum sclerostin levels in the time course of study 1 (left) and study 2 (right). Shown are mean values ± SEM. Sclerostin levels increased in both studies during bed rest and declined at the end of the bed rest period. *P*-values refer to change from baseline. *$P<0.05$, **$P<0.01$ (Linear mixed-effect models).

All samples for analysis from study 2 were analyzed after a storage period at -80°C of nearly 1 year. Samples from study 1 for the analysis of PINP and NTX were analyzed after a storage period of 1.5 years. Samples from study 1 were stored for nearly 8 years at -80°C before they were analyzed for sclerostin and DKK1.

Laboratory methods

Serum concentrations of sclerostin, DKK1, PTH (only measured in study 2, bone resorption marker urinary NTX) and bone formation marker N-terminal propeptide type I (PINP) were determined with commercially available assays in the in-house laboratory of the Institute of Aerospace Medicine, Germany.

Sclerostin and DKK1 were measured by enzyme-linked immunosorbent assays (ELISA) (sclerostin, TECO®; DKK1; both TECOmedical GmbH Bünde, Germany).

Interassay variation was as follows: study 1: sclerostin, 3.4%; DKK1, 3.0%; study 2: sclerostin, 0.75%; DKK1, 2.7%. Intraassay variation was as follows: study 1: sclerostin, 4.5%; DKK1, 1.7%; study 2: sclerostin, 1.4%; DKK1, 2.3%. Intra- and interassay variation based on the following concentrations: Sclerostin: 0.42-0.69 ng/ml, DKK1: 5.5-10.3 pmol/l. The bone formation marker PINP was analyzed by a radioimmunoassay (Orion Diagnostica, Finland), the bone resorption marker NTX was analyzed by ELISA (Osteomark®, TECOmedical GmbH, Bünde, Germany) and parathyroid hormone (PTH) was determined with kits from Immunotech, Beckmann Coulter GmbH, Krefeld, Germany. Interassay variation was as follows: study 1: PINP, 2.2%; NTX, 5.8%; study 2: PINP, 3.8%, NTX, 4.9%, PTH, 7.9% Intraassay variation was as follows: study 1: PINP, 2.2%; NTX, 5.0%; study 2: PINP, 1.9%, NTX, 1.3%. Intra- and inter assay variation based on the following concentrations:

PINP: 35.7-53.8; 86-177 µg/l, NTX: 334-566; 1010-1689 nmol/l, PTH: 65-98; 234-352 pg/ml.

Serum and urinary calcium concentrations were analyzed in duplicate by flame photometry (EFOX 5053, Eppendorf, Germany).

Statistical analysis

Subjects' age and weight data are presented as means ± SD, and all other data are given as means ± SEM.

Linear mixed-effect models were constructed, with time as a fixed factor and subject as a random factor, as described in [26]. Models were checked for heteroskedacity and with quantile-quantile plots. Where necessary Box-Cox transformation was performed before *P*-values were derived. These computations were done with the R statistical environment (www.r-project.org) in its version 2.13.1 (dated 2011-07-08).

Comparison of serum calcium, urinary calcium and PTH levels between baseline and the mean of the intervention levels values was done with Student's t-test for dependent variables.

An alpha level of 0.05 was set for all tests to indicate significance.

Results

Sclerostin

In study 1 sclerostin levels increased from 0.64±0.05 ng/ml to maximum values of 0.69±0.04 ng/ml on HDT11 (*P*=0.014). Sclerostin levels were back to baseline on HDT14, that is, on the last day of the bed rest period (Figure 1), and they were again increased during the recovery period (*P*=0.012).

In study 2, sclerostin levels showed a steady increase, starting from 0.42±0.04 ng/ml (before bed rest) and reaching max-
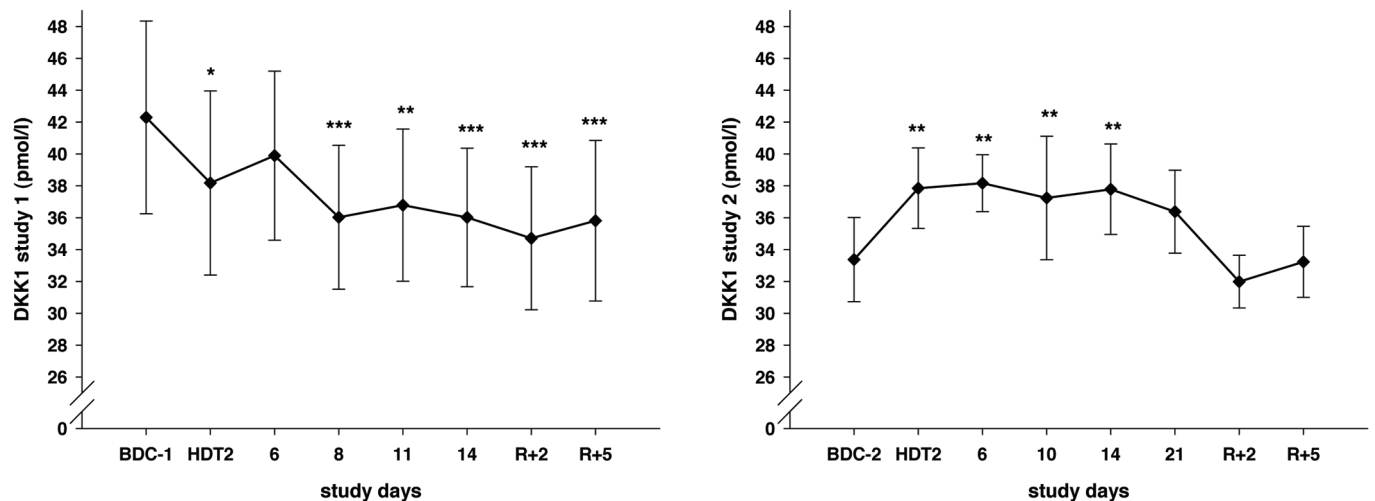
Figure 2. Serum DKK1 levels in the time course of study 1 (left) and study 2 (right). Shown are mean values ± SEM. DKK1 decreased during bed rest in the study 1. DKK1 was elevated during bed rest in study 2. *P*-values refer to change from baseline. *$P<0.05$, **$P<0.01$, ***$P<0.001$ (Linear mixed-effect models).
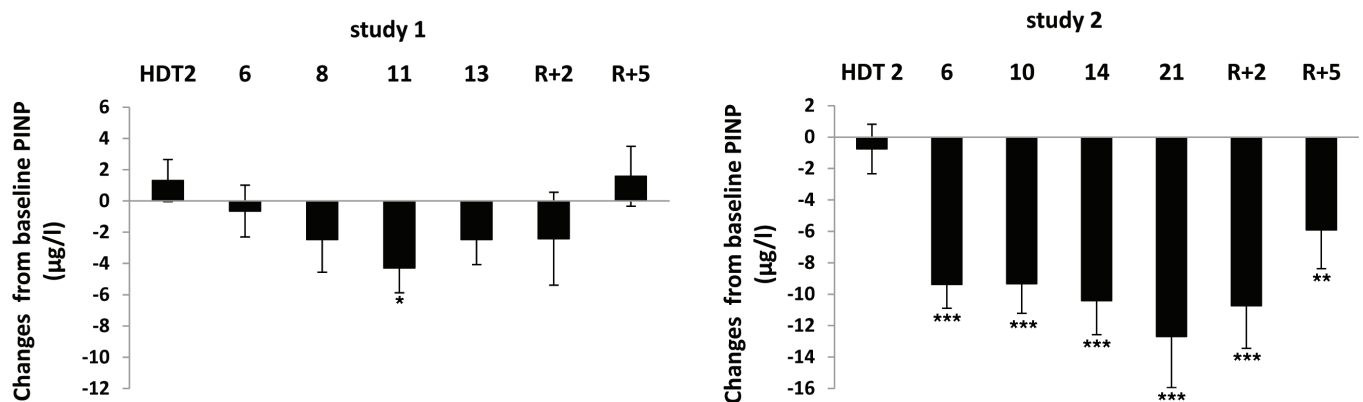

Figure 3. Changes in serum PINP levels from baseline of the different time points of study 1 (left) and study 2 (right). Shown are mean values ± SEM. The bone formation marker PINP decreased during the 21-day study, whereas a significant decrease in the 14-day bed rest study was detected only on day 11 of HDT. *P*-values refer to change from baseline. *$P<0.05$, **$P<0.01$, ***$P<0.001$ (Linear mixed-effect models).

imum values of 0.47±0.04 ng/ml on HDT14 (*P*=0.008). As in study 1, sclerostin levels decreased on the last day of the bed rest phase (HDT21), when they were approaching baseline levels. However, unlike in study 1, sclerostin levels did not increase again, but rather showed a further decrease in the recovery period, and baseline levels were reached on R+5 (0.42±0.04 ng/ml) (Figure 1).

DKK1

In study 1, DKK1 levels decreased from a high baseline level (42.3±6.1 pmol/l) to a minimum level of 36.0±4.4 pmol/l (*P*<0.001) during bed rest, decreasing further until day R+2 in the recovery period (34.7±4.5 pmol/l) (*P*<0.001) (Figure 2).

In study 2, conversely, DKK1 levels were increased over

baseline levels (33.4±2.6 pmol/l vs. 37.9±2.5 pmol/l) as early as HDT2 (*P*=0.006). During bed rest, DKK1 levels remained constantly elevated, and they returned to baseline levels of 32.0±1.7 pmol/l within 3 days after bed rest ended (Figure 2).

Bone formation and resorption

Part of the results, but focusing on the primary study intention, are presented by our working group elsewhere[22]. In study 1 a significant decrease in the bone formation marker PINP could be detected on HDT 11 (*P*=0.013) (Figure 3). The urinary bone resorption marker NTX was increased on the third day of bed rest compared to the mean of the baseline levels, and increased further over time during bed rest (*P*<0.001) (Figure 4).

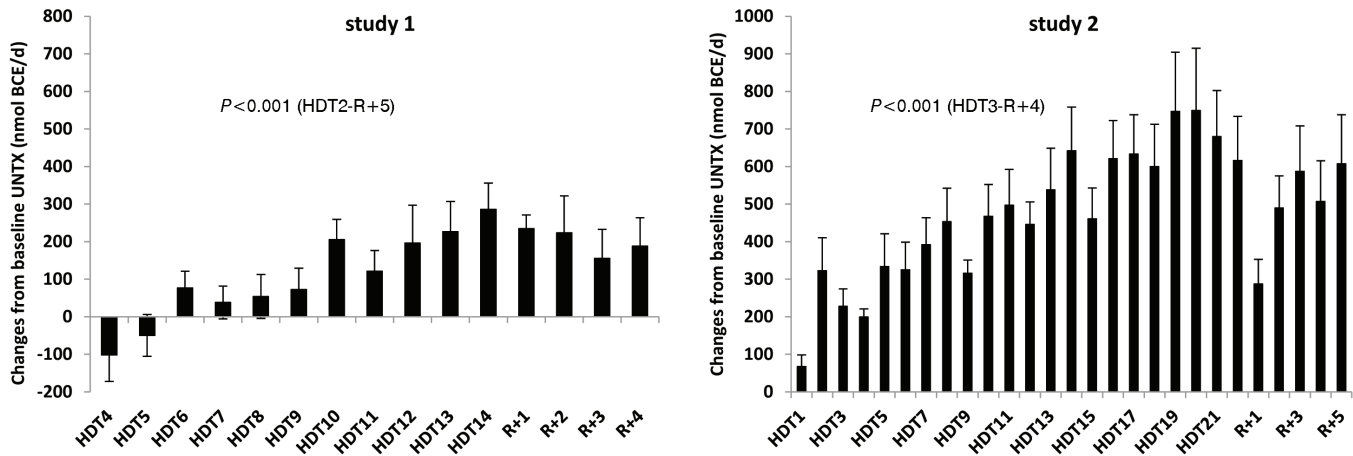In study 2, PINP was reduced during bed rest (*P*<0.001)

Figure 4. Changes in urinary NTX levels from the mean of the baseline values of the different time points of study 1 (left) and study 2 (right). Shown are mean values ± SEM. In both studies, the bone resorption marker NTX started to increase in the first days of bed rest and showed further increases later in the bed rest period. *P*-values refer to change from baseline. *P*<0.001 (Linear mixed-effect models).

from a baseline level of 70.8±7.3 µg/l to a minimum value on HDT21 of 58.1±5.2 (*P*<0.001). PINP levels did not reach baseline levels during the recovery period (Figure 3). NTX was already increased in the first days of bed rest (*P*<0.001) and showed further increases in the course of the bed rest period (*P*<0.001) (Figure 4).

Calcium and PTH

In both studies serum calcium levels were unchanged and urinary calcium increased during bed rest (*P*<0.001) as expected. Study 2 exposed a reduction in PTH during 21 days of immobilisation (*P*<0.001) (Table 1).

	Baseline	Bed rest	*P*-value
serum calcium (mmol/l)			
study 1	2.36 ± 0.05	2.36 ± 0.06	ns
study 2	2.38 ± 0.06	2.38 ± 0.06	ns
urinary calcium (mmol/24h)			
study 1	4.46 ± 0.80	5.2 ± 0.81	*P*<0.001
study 2	5.37 ± 0.84	6.05 ± 1.05	*P*<0.001
serum PTH (pg/ml)			
study 2	36.17 ± 5.09	24.90 ± 3.53	*P*<0.001

Table 1. Serum calcium, urinary calcium and PTH at baseline and during bed rest. Shown are mean values ± SEM.

Discussion

In the present study we assessed alterations in serum levels of sclerostin and DKK1 in response to bed rest-induced immobilization in young male human volunteers. Data from both studies provide clear evidence that sclerostin is elevated during bed rest. In addition to the 90 days bed rest study of Spatz et al.[4], with the first sclerostin measurement on bed rest day 28, these data show that sclerostin is already elevated after eight (study 1) to eleven (study 2) days of disuse. Sclerostin levels seemed to decline toward the end of the bed rest period. The results of the studies are discordant with regard to DKK1 levels. Well-known markers of bone resorption (urinary NTX), bone formation (PINP), serum and urinary calcium and PTH changed as expected in the course of immobilization. In conclusion, the data suggest that sclerostin and Wnt signaling in general are involved in disuse-induced bone loss in young, healthy adult humans.

In both studies we found an unexpected decline in serum sclerostin at the end of the bed rest period on the last day before subjects became ambulatory again. Also Spatz et al.[4] dedicated a lesser increase of sclerostin on day 90, which was the last bed rest day in this study. This is unlikely to reflect a specific temporal pattern of the sclerostin response, because the studies had different durations. Rather, could it be the anticipation of reambulation that leads to the decline at the end of the bed rest period through brain-bone interactions? This may sound like a remote possibility, but some neural mediators, such as leptin and serotonin that may link the brain to the skeleton have already been identified[27]. Experiments on mice demonstrated that sympathetic tone regulates bone mass, mainly by influencing osteoblasts. Additional brain-bone interactions are suggested by results from studies in rats of the effects of β-blockers, very low doses of which are able to increase bone formation markers[28]. In further support of the existence of this mechanism of bone regulation, in humans it is known that children taking sympathomimetics to treat asthma have a low bone mass[29]. As sclerostin interacts with multiple neurotransmitter and other proteins that alter bone formation and resorption, and is likely to function by altering several bi-

ologically relevant pathways in bone, a brain-bone interaction could be considered.

In general, the exact mechanism of sclerostin stimulation and action is not completely understood. There is no doubt that osteocyte sclerostin production is regulated substantially by the mechanical environment of bone, and it seems as if loading-induced down regulation would be an early osteogenic response to strain rather than a consequence of strain itself[30].

Regarding DKK1, to the best of our knowledge this is the first study showing that immobilization leads not only to increased sclerostin, but also to increased DKK1 levels after the onset of bed rest. This was observed as early as bed rest day 2 in study 2. However, study 1 showed an opposite reaction of DKK1 levels. Due to the long storage period of samples from study 1 for DKK1 analysis, considering a possible effect on the quality of samples, we would argue that findings from study 2 give a more realistic picture of DKK1 levels, and we conclude that a clear role exists for DKK1 in the mediation of bone formation in response to unloading in humans. We should not neglect to mention that study 1 test subjects´ short walk twice a day during the bed rest period could have had an impact on DKK1 levels as well. Moreover, subjects of study 1 started with an unexplainable high baseline level.

Serum levels of DKK1 reacted even faster to unloading than sclerostin levels did. One possible reason is that DKK1 could have a higher affinity for the receptor LRP5/6, and the binding of DKK1 to LRP5/6 triggers osteocytes to increase the expression of DKK1 first. Balemans et al.[31] demonstrated that DKK1 and sclerostin do not simultaneously bind to wild-type LRP5, and that DKK1 is able to displace sclerostin from previously formed sclerostin-LRP5 complexes. They conclude that DKK1 and sclerostin are independent, and not synergistic, regulators of LRP5.

Knowledge of sclerostin's actions led rather quickly to the development of sclerostin antibodies in both animals and humans to be used primarily as an anabolic therapeutic agent in the treatment of osteoporosis[32-34]. But, because of the described sclerostin changes in immobilization, anti-sclerostin antibodies seem to also show promise as a new countermeasure to limit bone loss during immobilized conditions, or when astronauts are in space. A host of preclinical animal studies have shown salutary effects on bone formation in animal models such as ovariectomized rats and aging animals. A human antisclerostin antibody, known as AMG 785, developed by Amgen (Thousands Oaks, CA) and UCB Inc. (Smyrna, GA), was associated with an increase in bone formation as well as a decrease in bone resorption, which supports the recent *in vitro* evidence for sclerostin's additional catabolic activity. Also, lumbar spine and total hip bone mineral density increased 5.3% and 2.8%, respectively, in a dose-dependent manner 3 months after administration of AMG 785[35]. Clinical studies performed in healthy humans showed that serum sclerostin levels increase markedly with age[36,37], which makes sclerostin even more important as a target of therapeutic agents against bone loss in the elderly.

The results of our study suggest a therapeutic potential for DKK1 antibodies as well. Some preclinical studies in mice and monkeys have shown promising effects of anti-DKK1 antibodies on bone mineral density[38-41]. Anti-DKK1 therapies in humans are limited to studies with multiple myeloma patients, as osteolytic lesions and fractures, which have positive correlations with serum DKK1 level[42-44], are potential complications in these patients.

Due to only small group of subjects in our studies, more studies are needed to get insight into the activation of sclerostin and DKK1 and their effects on the processes of bone formation and bone resorption as well as on possible interactions between sclerostin and neuronal factors as mediators that connect the brain to the skeleton.

In conclusion, the described data from two bed rest studies show strong evidence for alterations in Wnt signaling during experimental bed rest in young, healthy male human test subjects occurring already after just a bit more than a week and highlight especially the key roles of sclerostin and DKK1 in the regulation of bone metabolism.

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