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On the combined effects of normobaric hypoxia and bed rest upon bone and mineral metabolism: Results from the PlanHab study

Jörn Rittweger [a,b,*], Tadej Debevec [c], Petra Frings-Meuthen [a], Patrick Lau [a], Uwe Mittag [a], Bergita Ganse [a], Philip G. Ferstl [d], Elizabeth J. Simpson [e], Ian A. Macdonald [e], Ola Eiken [f], Igor B. Mekjavic [c,g]

[a] Institute of Aerospace Medicine, German Aerospace Center (DLR), 51147 Cologne, Germany
[b] Department of Pediatrics and Adolescent Medicine, University of Cologne, Cologne, Germany
[c] Department of Automation, Biocybernetics and Robotics, Jozef Stefan Institute, Ljubljana, Slovenia
[d] Medizinische Klinik I, University of Frankfurt, Frankfurt, Germany
[e] MRC/Arthritis UK Centre for Musculoskeletal Aging Research, University of Nottingham Medical School, School of Life Sciences, Queen's Medical Centre, Nottingham, United Kingdom
[f] Department of Environmental Physiology, Swedish Aerospace Physiology Centre, Royal Institute of Technology, Stockholm, Sweden
[g] Department of Biomedical Physiology and Kinesiology, Simon Fraser University, Burnaby, British Columbia, Canada

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ABSTRACT

Bone losses are common as a consequence of unloading and also in patients with chronic obstructive pulmonary disease (COPD). Although hypoxia has been implicated as an important factor to drive bone loss, its interaction with unloading remains unresolved. The objective therefore was to assess whether human bone loss caused by unloading could be aggravated by chronic hypoxia.

In a cross-over designed study, 14 healthy young men underwent 21-day interventions of bed rest in normoxia (NBR), bed rest in hypoxia (HBR), and hypoxic ambulatory confinement (HAmb). Hypoxic conditions were equivalent to 4000 m altitude. Bone metabolism (NTX, P1NP, sclerostin, DKK1) and phospho-calcic homeostasis (calcium and phosphate serum levels and urinary excretion, PTH) were assessed from regular blood samples and 24-hour urine collections, and tibia and femur bone mineral content was assessed by peripheral quantitative computed tomography (pQCT).

Urinary NTX excretion increased ($P < 0.001$) to a similar extent in NBR and HBR ($P = 0.69$) and P1NP serum levels decreased ($P = 0.0035$) with likewise no difference between NBR and HBR ($P = 0.88$). Serum total calcium was increased during bed rest by 0.059 (day D05, SE 0.05 mM) to 0.091 mM (day D21, $P < 0.001$), with no additional effect by hypoxia during bed rest ($P = 0.199$). HAmb led, at least temporally, to increased total serum calcium, to reduced serum phosphate, and to reduced phosphate and calcium excretion.

In conclusion, hypoxia did not aggravate bed rest-induced bone resorption, but led to changes in phospho-calcic homeostasis likely caused by hyperventilation. Whether hyperventilation could have mitigated the effects of hypoxia in this study remains to be established.

1. Introduction

The risk of bone loss in response to unloading by experimental bed rest [1,2] and space flight [3] is widely recognized. Such bone losses occur predominantly in the legs and not in the upper extremities [2–5]. Loading forces in the lower extremities are typically low in space [6], but exercises with sufficiently large musculoskeletal loading prevent the well-established bed rest-induced bone losses [7–9]. However, even though the primary origin may be of a mechanical nature, bone losses in astronauts and immobilized patients are likely modulated by the endocrine environment and the milieu intérieur.

The interaction between hypoxia and unloading is under-studied, but has become a relevant topic in space medicine. While originally perceived as a countermeasure [10], the current interest arises from atmosphere definition for next generation spacecrafts: atmospheres equivalent to *e.g.* 3000 m will be used to avoid decompression sickness and thus to facilitate extra-vehicular activity. This perspective raises new questions about potential interactions between hypoxia and unloading with regards to bone and beyond.

Specifically, arterial hypoxemia and hypercapnia, as a consequence of reduced alveolar ventilation and the resultant alveolar hypoxia, are commonly observed in many patient groups that are inactive, and rendered hypoxic by their illness, most prominently patients suffering

* Corresponding author at: Institute of Aerospace Medicine, Linder Höhe 1, 54117 Cologne, Germany.
E-mail address: joern.rittweger@dlr.de (J. Rittweger).

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moderate to severe chronic obstructive pulmonary disease (COPD) [11]. In these patients, arterial hypoxemia leads to pulmonary remodeling, causing emphysema and pulmonary hypertension. Anemia caused by chronic systemic inflammation is associated with poor prognosis [12], and cachexia further increases mortality in these patients. Of note, initial stages of COPD typically present with normocapnia [13].

Osteoporosis is also prevalent in chronic obstructive pulmonary disease (COPD) [14]. The factors that have been implicated in its etiology include lack of physical activity, vitamin-D deficiency, hypogonadism [15] and smoking [16], and use of high-dose corticosteroid therapy, which can be required in severe COPD exacerbations [17,18]. Notably, hypoxia at 1 kPa reduces, *in vitro*, osteoblastic secretion of sclerostin *via* Mef2 and Smad [19], which must be expected to counteract bone loss. On the other hand, reducing PO_2 from 20 kPa to 2 kPa and 0.2 kPa *in vitro* leads to marked increase in osteoclastogenesis and a decrease in osteoblastic differentiation [20,21]. Thus, it seems undetermined from *in vivo* studies alone whether hypoxia promotes bone loss or bone formation.

To address the problem regarding interaction of hypoxia and inactivity, the PlanHab study [22] was implemented as a 21-day bed rest study in cross-over design with a broad spectrum of physiological endpoints. Although the primary aim of the project was to investigate the effects of the anticipated hypoxic environments in future planetary habitats on physiological systems, the results are relevant for Earth-bound patient populations that are inactive and hypoxic. Healthy young male subjects underwent bed rest in normoxic conditions (NBR), bed rest in hypoxic conditions (HBR) at altitude equivalent of 4000 m, and hypoxic ambulatory conditions (HAmb) at the same altitude equivalent as HBR. The HAmb was meant to reveal the effects of hypoxia *per se* by analysis of time-effects. Within the PlanHab project, a sub-study was organized to explore effects upon bone metabolic response to quantify potential aggravation of bed rest-induced bone losses by hypoxia. However, one also has to consider that hypoxia induces hyperventilation and thus respiratory alkalosis. This may positively affect bone metabolism *via* a renal effect [23] and also *via* a direct effect upon osteocytes [19]. Accordingly, no specific hypothesis was made as to whether hypoxia would aggravate or mitigate bed rest-induced bone losses.

2. Materials and methods

The present sub-study within the PlanHab project (registration number NCT02637921 at www.ClinicalTrails.gov) addressed bone metabolism and calcium homeostasis through biochemical analysis of blood and urine samples. In addition, it assessed leg bone structural changes by peripheral quantitative computed tomography (pQCT). The detailed outline of the PlanHab project is given elsewhere [22], and we only briefly explain the project's general features here.

2.1. Study design and setting

The PlanHab study was performed between October 2012 and December 2013 at the hypoxic facility at the Olympic Sport Center Planica in Rateče, Slovenia, located at 940 m of altitude. The study was conducted according to the ESA plan for standardization of bed rest studies [24], and was organized in 3 successive experimental campaigns. Each campaign consisted of 5 days of baseline data collection (BDC) during which subjects were ambulant, 21 intervention days (normoxic bed rest, hypoxic bed rest, or hypoxic ambulation), and 5 days of follow-up. For the latter, operational days are designated by 'R + i', meaning i days after re-ambulation. For BDC, operational days are indicated by 'BDC-n', meaning n days before the onset of bed rest. Prior to commencement, the study had received approval by the National Committee for Medical Ethics at the Ministry of Health of the Republic of Slovenia. In addition to the present sub-study, other sub-studies focused upon cardiorespiratory, muscular, immunological and thermoregulatory effects.

2.2. Test subjects

Inclusion and exclusion criteria for the study complied with the European Space Agency's standardization plan for bed rest studies [24] and aimed at selecting subjects who could safely undergo bed rest (*e.g.* exclusion of people with osteoporosis, with blood clotting disorders, history of deep vein embolism, lower back pain, and respiratory disorders). In addition, subjects were also excluded from this study if they had been exposed to altitudes >2000 m, two months prior to study commencement. Fourteen young healthy men participated in this study, with a mean age of 26.4 (SE 1.4) years, a height of 179.6 (SE 1.4) cm, a mass of 75.9 (SE 2.8) kg and a body mass index of 23.5 (SE 0.8) kg/m^2.

2.3. Bed rest and environmental protocol

Environmental conditions were controlled (ambient temperature = 24.4 ± 0.7 °C, relative humidity = 53.5 ± 5.4%) or assessed (ambient pressure 91.2 ± 5.3 kPa) throughout all campaigns. Capillary oxyhemoglobin saturation (SpO_2) was measured daily at 7:00 with a 3100 WristOx device (Nonin Medicals, Minnesota, USA) and also as part of a sleep polysomnographic study. To the latter purpose, full ambulatory polysomnography (Nicolet One, Viasys, Healthcare, Neurocare, Madison, WI, USA) was performed using standard setups [25] after subjects had spent at least two nights in the facility; during each campaign, measurements were conducted on three occasions: 1) baseline data collection day 3, 2) night 1 (N1), and 3) night 21 (N21) of the intervention. The light:dark cycle was set to 16:8 h, with bed time between 23:00 and 7:00.

During the bed rest phase of NBR and HBR campaigns subjects were confined to bed in the horizontal position for 24 h/day, and all activities of daily living took place in bed. One pillow was allowed for head support. Showers were taken on a specific gurney, and hospital equipment was used for bowel movements and urine collection. Compliance with the bed rest protocol was ascertained by supervision through members of staff and through closed-circuit television. No physical activity was allowed during NBR and HBR campaigns, except for changing position between supine, prone and lateral.

During HAmb, subjects were confined to the hypoxic area, but remained ambulatory and out of bed during the day. In order to replicate their habitual bone loading during the confinement periods, subjects performed low-level physical activity in two 30-minute bouts per day. This came as stepping, cycling and dancing exercise. Telemetric heart rate monitoring was used to achieve the targeted heart rate (123 ± 4 beats min^{-1}) during the exercises, which was set to the heart rate observed at 50% peak power output assessed at the onset of the intervention.

During HBR and HAmb campaigns, normobaric hypoxia was generated within the hypoxic area by a vacuum pressure swing adsorption system (b-Cat, Tiel, the Netherlands). Regulation of O_2 concentration was actuated within each room at 15-minute intervals. For safety reasons, subjects wore portable O_2 sensors (Rae PGM-1100, California, USA) at all times.

2.4. Diet

As reported earlier [26], the subjects were provided with an individually tailored, standardized and controlled diet throughout each intervention. Energy requirements were assessed with the Harris-Benedict method [27], and correction factors of 1.4 and 1.2 were used to account for activity levels in the ambulatory phases and the bed rest phases, respectively. In addition to setting the intake of fat to 30% of energy and the intake of protein to 1.2 g per kg body mass, sodium intake was set at <3500 mg per day. Subjects were supplemented with 1000 IU vitamin D3 per day. Fluid intake was *ad libitum*, but subjects were encouraged to drink at least 28.5 mL per kg per day. Importantly, menu plans

at were re-used for each subject across the 3 conditions, adjusting the quantity according to activity factors.

2.5. Peripheral quantitative computed tomography

pQCT scans were obtained from the shank and from the thigh on the 6th day of baseline data collection (BDC-6), on the 2nd, the 10th and the 21st intervention day (D02, D10 and D21, respectively), and at 14 days of follow-up (R + 14). Scans were obtained as previously described [28,29] by scout-viewing the tibio-talar joint and the knee joint in the frontal plane, to identify the distal and proximal tibia joint surfaces, and the distal femur surfaces as landmarks. Horizontal scans were then taken at 4%, 14%, 38%, and 66% of the tibia (assessed from its distal end, and at 4% and 33% of the femur.

2.6. Biological sample collections

Twenty-four hour urine collections were obtained on days BDC-2, BDC-1, D01, D02, D03, D04, D08, D10, D12, D14, D16, D18, D21, R + 2, R + 3 and R + 4. 24-hour urine collection started at 07:00 h. Urine voids were kept until final pooling in the 24-hour urine pool. Aliquots were stored at −20 °C, with calcium and phosphorous aliquots being acidified before freezing.

Fasting blood samples were collected from an antecubital vein at 07:00 h on days BDC-2, BDC-1, D02, D05, D10, D14 and D21. Whole blood was centrifuged (3000 rpm, 4 °C, 10 min) after coagulation, and serum was distributed in 1.5 mL Eppendorf tubes and immediately frozen at −20 °C (PINP, FGF and Klot) and −80 °C (bAP, Scle, Ocal, PTH and 25-OH-D) until analysis.

2.7. Biochemical analysis

Serum levels of bone specific alkaline phosphatase (bAP), parathyroid hormone (PTH) and 25-Hydroxyvitamin D (25-OH-D) were assessed by using commercially available radioimmunoassays (RIA); (bAP, PTH: Immunotech, Prague, Czech Republic; 25-OH-D: DiaSorin, Saluggia, Italy). Serum levels of Procollagen-I-N-terminal propeptide (P1NP) were assessed by using RIA assays (Orion Diagnostica, Espoo, Finland). Regulators of bone metabolism, namely Dickkopf-related protein 1 (DKK1) and Sclerostin (SOST) were analyzed by enzyme-linked immunosorbent assay (ELISA) (DKK1: Biomedica, Vienna, Austria, SOST: Quidel/TECOmedical, San Diego, USA). Inter-assay CV and intra-assay CV were assessed as follows: bAP (4.1%, 5.5%), PTH (5.85%, 7.95%), 25-OH-D (N/A*, 12.5%), P1NP (2.45%, 2.05%), DKK1 (6.3%, 5.4%).

Serum and urinary calcium and phosphate were analyzed by an automated analyzer for clinical chemistry by spectrophotometric enzymatic colorimetric tests (COBAS INTEGRA 400 plus, Roche Diagnostics, Mannheim, Germany), at wavelengths of 340/378 nm for calcium and at 700/340 nm for phosphorus. N-terminal telopeptide (UNTX) as a distinct marker for bone resorption was detected *via* ELISA measurement in urine samples (Immunodiagnostic Systems Ltd., Bolden, UK). Inter-assay CV and intra-assay CV for UNTX were 5.75% and 3.55%, respectively.

2.8. Respiratory gas measurements

End-tidal carbon dioxide concentration was measured on day BDC-5 while exposed to a normoxic environment and on day D17 of each intervention, breathing the prevailing gas mixture, which was either normoxic (NBR) or hypoxic (HBR, HAmb). Measurements were performed with the subject resting supine and breathing *via* an oronasal mask and a two-way respiratory valve that was connected to a capnograph (Quark CPET, Cosmed, Rome, Italy). Breath-by-breath values of end-tidal partial pressure of CO_2 ($P_{ET}CO_2$) were averaged during the last min of a 15-minute period of motionless rest. Prior to each experiment, the capnograph was calibrated using two different gas mixtures.

2.9. Data processing

The volumes of the 24 h urine samples were multiplied by the urine concentrations of NTX, Ca, and P to yield the 24 h urinary excretion of these compounds. pQCT image analyses were performed with the integrated XCT software (version 6.20B). Regions of interest (RoIs) around the tibia and fibula in shank scans, and around the femur in the thigh scans, were outlined by a rater blinded to the nature of the data. In the epiphyseal/metaphyseal scans, very narrow RoIs were defined, as these sites require relatively low segmentation thresholds, in order to suppress soft tissue contributions to bone data. The segmentation thresholds were individualized so that the highest threshold was used that was still able to achieve a clean segmentation. For each individual and site, the same threshold was used for image analysis at the different time points. For the diaphyseal RoIs, simple rectangular RoIs were used and the segmentation threshold was set to 650 mg/cm^3 [30,31]. All RoIs were checked by one of the authors as second expert (UM), and images were then automatically processed with the XCT software by using the 'loop' and 'automatic analysis' tools. From the resulting database, we extracted the bone mineral content (BMC) as TOT_CNT.

2.10. Statistical analyses

Statistical analyses were carried out using the R-environment (version 3.1.1 for the 64-bit Windows platform, www.r-project.org). Data were analyzed following the *intent-to-treat principle* and are given as best linear unbiased predictors (BLUPs) and their SE, expressed as percent of BDC values of NBR and HAmb campaigns in the text and in Table 2, and as means and standard errors (SE) in the figures. The level for statistical significance was set to 0.05.

To address the primary goal of the study, namely to test for hypoxia effects during bed rest, linear mixed effect (LME) models were constructed with time and condition (either NBR or HBR) as fixed effects and subject as random effect, and with the HAmb data excluded. The HAmb data were then used to address secondary goal of the study, *via* LMEs with time as fixed effect and subject as random effect. LME models were optimized according to Akaike's information criterion (see p. 353 and p. 652 in [32]). Data were box-cox transformed when non-linear quantile-quantile plots or heteroscedacity was found. Data from the baseline data collection (BDC) phase were lumped together. Models for statistical testing of the primary hypothesis were simplified in a step-wise manner. Firstly, the time * condition interaction term was discarded where justified by non-significance and Akaike's criterion, and the condition term was discarded in the next step. Any significant effects were followed up with treatment contrasts, using BDC and NBR as reference.

3. Results

3.1. Study conduct and subjects

Subjects generally tolerated the interventions well. However, one subject showed signs of acute mountain sickness at the onset of HBR and was moved to a room in which the hypoxia was equivalent to approximately 3000 m altitude. Thereafter, the simulated altitude was increased by 500 m per day for this subject, until attainment of the requisite altitude of 4000 m. For this subject, the same protocol was used during the subsequent HAmb campaign. Two subjects did not return for the 3rd campaign, and one subject had to be withdrawn during the 3rd campaign because of health problems unrelated to the study.

Average day-time SpO_2 amounted to 97.1 (SE 0.1) % during NBR, and was reduced to 88.2 (SE 0.2) % in HBR and to 87.9 (SE 0.2) % in HAmb (both $P < 0.001$). These day-time SpO_2 values tended to recover during the experimental phases of both the HAmb and HBR trials ($P < 0.05$). Night-time SpO_2 amounted to 94.3 (SE 0.4) during the first night of NBR, which was lowered to 83.2 (SE 0.5) % and 81.6 (SE 0.5) % during

the first nights of HBR and HAmb, respectively ($P < 0.001$). Similar to day-time SpO_2 values, night time values tended to recover for HBR and HAmb over time ($P < 0.001$). Statistical analysis yielded a time * condition interaction for PCO_2 (see Table 1), indicating lowered $PETCO_2$ and thus hyperventilation during intervention phases of HBR and HAmb. Serum levels of 25-OHD during BDC of the different campaigns were 14.2 (SE 1.3) $ng \cdot mL^{-1}$ for NBR, 16.2 (SE 0.6) $ng \cdot mL^{-1}$ for HBR and 13.8 (SD 2.2) $ng \cdot mL^{-1}$ for HAmb. 25-OHD levels were comparable across conditions ($P = 0.25$) and ranged between 7.0 and 21.3 ng/mL.

3.2. Bone mineral content

NBR and HBR data revealed the expected reductions in BMC, which were observed on day R + 14 at all, but the 33% femur site (see Table 2). Additionally, the 4% femur site depicted bone loss also on day BR21 ($P = 0.020$). Conversely, and counter-intuitively, statistical analysis indicated increases over baseline BMC values at the 66% tibia site on D10 and D21 ($P = 0.0058$ and $P = 0.0030$, respectively).

With regards to hypoxia effects, no time * hypoxia interaction was observed in NBR and HBR data for the tibia and for the 33% femur site ($P = 0.058$ for the 4% femur site, and $P > 0.5$ for all other sites, see Table 2). HAmb data yielded small but significant increases in BMC at the 66% tibia site and at the 33% femur site, which, however, was limited to the 10th day of HAmb only (see Table 2).

3.3. General effects upon bone metabolism

NBR and HBR data likewise yielded expected bed rest effects for all measures of bone metabolism ($P = 0.004$ for P1NP and all other $P < 0.001$, see Table 3). There were generally no main effects of condition in these bed rest data, except for PTH ($P = 0.045$) and DKK1 ($P = 0.0052$). However, these latter effects were insubstantial, so that baseline conditions were comparable between campaigns.

With regards to hypoxia, NBR and HBR data revealed time * hypoxia interactions only for serum levels of PTH ($P < 0.001$), for urinary excretion of calcium ($P = 0.029$) and of phosphate ($P = 0.0010$). Conversely, HAmb data depicted significant effects of time for most variables, except for serum levels of P1NP and NTX excretion. Taken together, therefore, hypoxia had clear effects upon bone metabolism in HAmb, and much less during BR.

3.4. Urinary excretion

3.4.1. NTX excretion

The expected increase during bed rest (see Fig. 1) was significant for all days from D04 to R + 3 ($P = 0.047$ for D04 and $P < 0.01$ for all other days), amounting to up to 79% (SE 11.2%). In addition, we unexpectedly observed a decrease at D01 by 32.3% ($P = 0.001$), and an increase by the same amount at D02 ($P = 0.0065$). Notably, there was no time * hypoxia interaction ($P = 0.69$) in NBR and HBR data, but a trend for time in HAmb data ($P = 0.072$).

3.4.2. Calcium excretion

Increases were observed during NBR from day D08 until R + 3 (all $P \le 0.01$, see Fig. 1) by up to 69% (SE 12%). A time * hypoxia interaction indicated attenuation of increased calcium excretion by hypoxia on days D08 and D10 of HBR ($P < 0.001$ and $P = 0.034$, respectively), but not during the second half of HBR (all $P \ge 0.18$). Calcium excretion was decreased between D04 and D14 of HAmb (all $P < 0.017$), with the decrements ranging between 28% and 37% (SE 13%).

3.4.3. Phosphate excretion

NBR data showed increases in phosphate excretion by 30%, by 26% and by 31% (SE 13%) on days D02, D04 and D08 of bed rest, respectively (all $P < 0.04$). Strong opposing time * hypoxia interaction effects were found on days D01 and D02, and a trend on day D04 ($P = 0.0059$, $P = 0.0036$, and $P = 0.09$, respectively), which implied decreases in phosphate excretion by −47%, by −51% and by −28%, respectively. HAmb data yielded decreased phosphate excretion on days D01, D02, D03, D04 and D10 of HAmb (all $P \le 0.030$) by down to −38%, but increases of comparable magnitude on days R + 2 and R + 3 after HAmb (both $P = 0.002$).

3.5. Serum biochemistry

3.5.1. P1NP levels

Decreases on days D10, D14 and D21 of NBR ($P = 0.003$, $P = 0.021$ and $P = 0.043$, respectively) amounted to −10.6%, −8.3% and −7.3% (SE 3.8%). Neither was a time * hypoxia interaction observed in NBR and HBR data ($P = 0.88$), nor a time effect in HAmb data ($P = 0.97$).

3.5.2. bAP levels

Contrasting with P1NP levels, there were increases in bAP levels by 10.0% to 13.0% (SE 3.5%) between day D02 and day D14 of NBR (all $P < 0.005$). No time * hypoxia effect was found in NBR and HBR data ($P = 0.97$), and increases by 8.6% to 12.0% were found on D02, D05 and D14 of HAmb (all $P \le 0.05$).

3.5.3. Calcium levels

A main effect of time in NBR and HBR data indicated increases on all days from D02 to D21 (all $P < 0.001$) by 0.059 to 0.091 mM (SE 0.009 mM). There was no time * hypoxia interaction ($P = 0.83$). HAmb data revealed a quantitatively similar effect of time as HBR data, with increases on all but the 14th day of HAmb (all $P \le 0.022$).

3.5.4. Phosphate levels

Increases on days D05, D10, D14 and D21 (all $P < 0.001$) by 0.081 mM to 0.16 mM (SE 0.02 mM) were observed in NBR and HBR data, but no time * hypoxia interaction ($P = 0.73$). Conversely, HAmb data yielded significant decreases on D02, D05, and D21 days of HAmb ($P < 0.05$) by 0.064 mM to 0.224 mM (SE 0.032 mM).

3.5.5. PTH levels

NBR and HBR data yielded the expected decrease on all days (all $P < 0.001$) by down to −31% (SE 3%) on D21. Trends for time * hypoxia interactions indicated mitigation of the PTH decrease on days D10 and D14 ($P = 0.069$ and $P = 0.077$, respectively), and a significant time * interaction effect was found on day D21 ($P < 0.001$). During HAmb, PTH levels were increased on D14 ($P = 0.0043$) by 13%, and a trend indicated a decrease on D05 ($P = 0.06$).

3.5.6. Sclerostin levels

NBR and HBR data yielded increases for all days from D02 to D21 (all $P < 0.001$) by 9.9% to 25.9% (SE 2.6%). No time * hypoxia effect was found ($P = 0.61$). Notably, sclerostin was also increased on D02, D10 and D14 of HAmb (all $P < 0.01$) by 8.5% to 11.7% (SE 3.2%).

3.5.7. DKK1 levels

Increases were observed during NBR and HBR on days D05, D10 and D14 (all $P < 0.02$) by 18.3 to 40.5% (SE 7.8%), with a trend towards an increase also on day D21 ($P = 0.053$). No time * hypoxia interaction was

Table 1
End-tidal CO_2 partial pressure ($PetCO_2$) at rest, assessed at BDC and D17 in each condition.

Day	NBR	HBR	HAmb
BDC	33.7 (2.5)	34.2 (1.9)	34.4 (2.2)
D17	33.4 (1.8)	26.9*** (1.5)	27.3*** (1.9)

*** Denotes significant time * condition interaction with $P < 0.001$.

Table 2
Changes in BMC as obtained by pQCT for the 4 tibial and the 2 femural measurement sites. The presented values are best linear unbiased predictors (BLUPs) and their standard errors, as obtained by mixed effect analysis, expressed in percent of the BDC values in NBR and HAmb campaigns, respectively.

	Bed rest		BR: Hypoxia interaction	Ambulatory hypoxia	
	Effect (SE)	*P*-value	*P*-value	Effect (SE)	*P*-value
Tibia 4%	R + 14: −1.37% (0.26%)	<0.001	0.66	–	0.16
Tibia 14%	R + 14: −0.30% (0.09%)	0.0044	0.85	–	0.41
Tibia 38%	R + 14: −0.38% (0.13%)	0.032	0.60	–	0.26
Tibia 66%	D10: +0.23% (0.08%) D21: +0.25% (0.08%) R + 14: −0.35% (0.08%)	<0.001	0.86	D10: +0.37% (0.11%)	0.0073
Femur 04%	D21: −0.39% (0.16%) R + 14: −0.55% (0.16%)	0.0031	0.058	–	0.38
Femur 33%	–	0.096	0.95	D10: +0.36% (0.12%)	0.024

found ($P = 0.94$). HAmb data yielded increases on D10 and D21 of HAmb (all $P < 0.05$) by 24.5% (SE 8.9%) and by 31.2% (SE 8.6%), respectively.

4. Discussion

This study has explored the effects of hypoxia upon bed rest-induced bone losses and calcium homeostasis during a 21-day bed rest paradigm in young healthy men at a simulated altitude of ~4000 m. The most important finding of this study is that time * hypoxia interactions during bed rest were non-significant for bone resorption (urinary NTX excretion), but highly significant for calcium homeostasis (serum PTH, urinary excretion of calcium and phosphate). To the best of our knowledge, the only preceding study was performed five decades ago by Lynch et al. [10]. That study used hypobaric (as opposed to normobaric) hypoxia equivalent to 10,000 and 12,000 ft (3048 m and 3658 m, respectively), but was unable at the time to analyse markers of bone turn-over, and was inconclusive regarding phosphorus metabolism. There was partial congruence regarding calcium excretion between studies, although the sparing of bed rest-induced calcium excretion seems more pronounced and longer-lasting in the study by Lynch et al.'s [10] study than in the present one.

4.1. Bed rest effects

The salient features of bed rest-related alterations of bone metabolism have all been replicated in this study, namely increased bone resorption and reduced bone formation [33], increased excretion of calcium and phosphate [34], loss of bone mineral from the lower extremities [2], decreased serum PTH [4], and increased serum levels of sclerostin and DKK1 [35]. Notably, vitamin D had been supplemented throughout the study. Accordingly, there is little reason to assume that D-deficiency, which is quite common nowadays in young people, had substantial influence on these results. This study has also replicated the finding that bone resorption, after an initial and moderate increase on day 2 or 3 of bed rest [36], is fully activated only around the 8th day (see Fig. 1 in [37], Fig. 2 in [38] and Fig. 1 in this publication) – a finding that, although previously published for experimental bed rest [38] and for spaceflight [37] has been mostly ignored. Likely explanations involve time-delays in cellular communication between osteoblasts and osteoclasts, in the activation and maturation within the monocytic-osteoclastic lineage, as well as in homing of osteoclastic cells in the bone matrix.

Table 3
Overview of statistical results for biological sample data. *P*-values for main effects of time and hypoxia, and for time * hypoxia interaction, listed for bed rest data (columns 2–4), and for hypoxic ambulatory data (HAmb column 5). Significant effects are set in bold face, and trends are marked with **(T)**.

	Bed rest			Ambulatory hypoxia
	Time	Hypoxia	Time * Hypoxia	Time
Serum levels				
P1NP	**0.004**	0.18	0.88	0.97
bAP	<**0.001**	0.43	0.97	**0.016**
Calcium	<**0.001**	0.20	0.83	<**0.001**
PTH	<**0.001**	**0.045**	<**0.001**	**0.0013**
P	<**0.001**	0.96	0.73	<**0.001**
Sclerostin	<**0.001**	0.73	0.61	**0.002**
DKK1	<**0.001**	**0.0052**	0.94	**0.0057**
Urinary excretion				
NTX excretion	<**0.001**	0.66	0.69	0.072 **(T)**
Calcium excretion	<**0.001**	0.067 **(T)**	**0.029**	<**0.001**
Phosphate excretion	<**0.001**	0.60	**0.0010**	<**0.001**

4.2. Responses to hypoxia

Overall, the present data do not suggest that normobaric hypoxia equivalent to 4000 m *per se* would exaggerate bone resorption in healthy humans. This is in stark contrast to the numerous *in-vitro* studies that demonstrate profound effects of hypoxia on osteoclasts, enhancing their differentiation from pre-cursor cells [20,39], modulating their binding to resorption sites [20], and affecting their resorption activity [39]. Moreover, expectations that hypoxia would hamper osteoclastic activity through reduced sclerostin release [19] are not supported by our results, as serum levels of sclerostin, as well as of DKK1 were found to be increased during ambulatory hypoxic conditions (see Fig. 3). Notably, increased sclerostin and DKK1 levels normally indicate enhanced bone resorption [35,40]. There is no doubt about the effectiveness of the hypoxic stimulus in this study (normobaric equivalent to 4000 m), as reflected in profound systemic hypoxemia (reduced SpO_2) noted in all participants throughout both hypoxic campaigns. The magnitude of the SpO_2 reduction is in line with previous studies investigating normobaric hypoxic exposures to the same simulated altitudes [41]. Patients with moderate to severe COPD, who lack the ability of hyperventilation, are supposed to receive long term oxygen therapy (LTOT) when their $SpO_2 < 88\%$ or their $PaO_2 < 7.3$ kPa [42]. Notably, SpO_2 was 88% in hypoxic conditions in this study. However, patients with severe COPD develop pathologic retention of CO_2 and concomitant blood acidification [13]. In contrast to that, subjects in this study even lowered their arterial CO_2 levels, which is the classic hyperventilation response to high altitude.

Therefore, we discuss the possibility here that hypoxia-induced hypocapnia in this study can explain part of the results. Hyperventilation, *i.e.* lowering of P_{CO2} within the body through ventilatory dissipation of CO_2, causes alkalization. This, in turn, causes immediate reductions in serum concentrations of ionized, but not total calcium [43]. Within minutes, this leads to lowering of serum phosphate levels and moderate increases in serum calcium through intermediate effects in tissue metabolism [44]. Decreased phosphate levels in serum, and increased calcium levels, as observed during the onset of hypoxic ambulatory conditions (Fig. 2) in this study are therefore in keeping with these purported short-term hyperventilation effects. However, the question

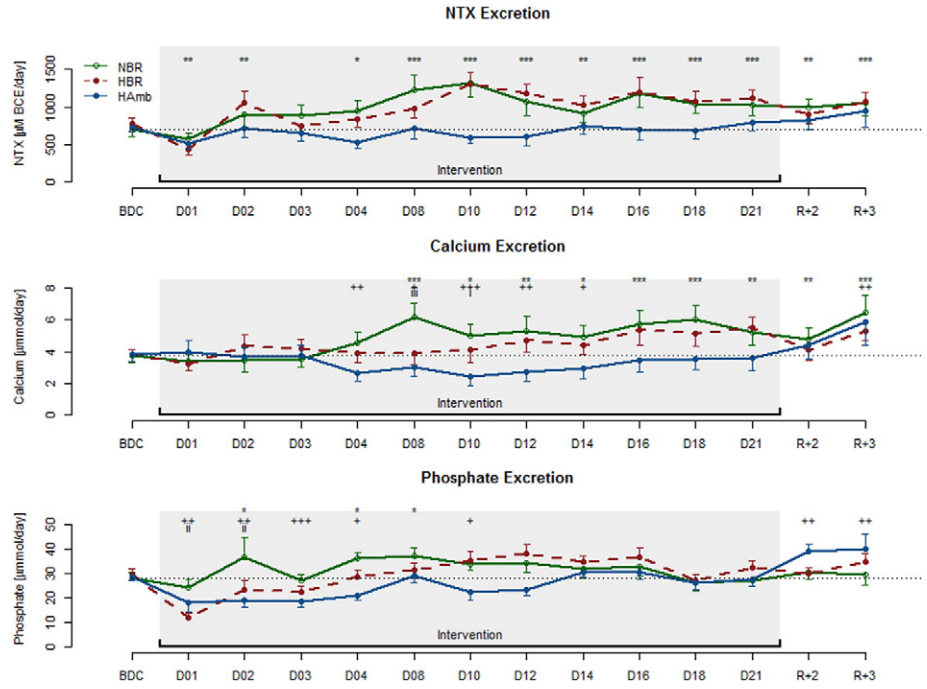

Fig. 1. Urinary excretion for normoxic and hypoxic bed rest (NBR and HBR, respectively) and for hypoxic ambulatory confinement (HAmb). Excretion of the bone resorption marker NTX, of calcium and of phosphate are plotted over time for baseline data collection (BDC), for intervention days (D, being either bed rest or ambulatory hypoxia) and for recovery days (R). Data are means and their standard errors. Main effects of bed rest (BR) are denoted as $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; BR*: Hypoxia interaction effects: $^{ii}P < 0.01$; $^{ii}P < 0.001$; time effects during HAmb: $^{+}P < 0.05$; $^{++}P < 0.01$; $^{+++}P < 0.001$.

arises what mechanisms lead to increased serum phosphate levels at the later stages (see Fig. 2). Whatever these mechanisms may be, it seems possible to explain the nonappearance of exaggerated bone resorption during hypoxic ambulatory conditions by compensatory effects of hyperventilation-related alkalization.

4.3. Alterations of bed rest responses by hypoxia

Interactions between bed rest and hypoxia were few and limited in time. Specifically we observed: i) reduced phosphate excretion on the 1st and 2nd days of bed rest (Fig. 1), reduced calcium excretion on the

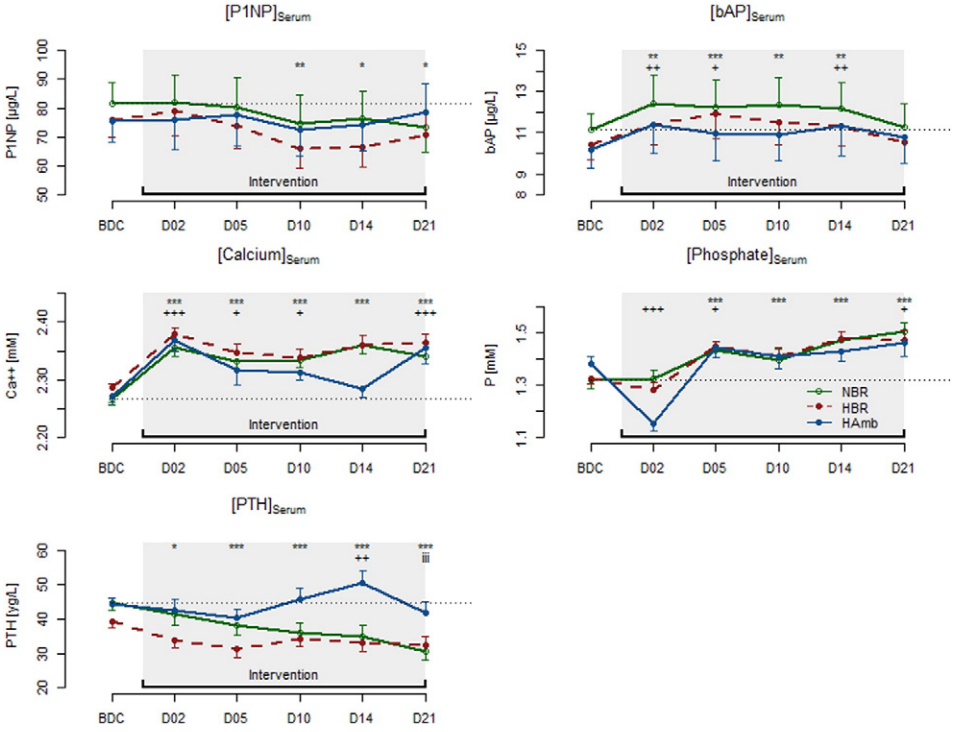

Fig. 2. Serum levels of bone formation marker P1NP, calcium, phosphate and PTH. Significant contrasts for time in the bed rest data are denoted as * ($P < 0.05$), ** ($P < 0.01$) and *** ($P < 0.001$); significant contrasts for time in HAmb data are denoted as + ($P < 0.05$), ++ ($P < 0.01$) and +++ ($P < 0.001$); a significant time * condition interaction effect for bed rest data with $P < 0.001$ is denoted as iii.

8th and 10th days (Fig. 1), and normalization of PTH levels that became significant on the 21st day of bed rest (Fig. 2). These three interaction effects can be interpreted from Figs. 1 and 2 as linear superposition of hypoxic effects onto bed rest effects (as evidenced *e.g.* by coinciding '+' and 'i' symbols in Figs. 1 and 2).

Both sclerostin and DKK1 levels were increased by bed rest as well as under hypoxic ambulatory conditions. While the former was expected [35,45], the latter is in some contrast to results from *in-vitro* studies [19]. Notably, the sclerostin elevations during hypoxic ambulatory conditions in this study were neither associated with decreased P1NP levels nor with increased NTX excretion (Figs. 2 and 1, respectively). Moreover, hypoxia added onto bed rest did not lead to further increases in sclerostin serum levels (lack of interaction effects in Fig. 3). The physiological implications of sclerostin in hypoxic bone responses therefore are currently not clear.

4.4. Clinical implications

Arterial hypoxemia has been postulated to cause systemic inflammation by activation of regulatory pathways and cytokines, thus causing bone loss and musculoskeletal dysfunction [11]. While COPD is associated with cachexia and musculoskeletal wasting [15], the present results suggest no effect of hypoxia on disuse-induced bone losses. In contrast to our healthy subjects who hyperventilated in the hypoxic conditions, and were consequently most likely hypocapnic compared to the normoxic condition, COPD patients are both hypoxic and hypercapnic in normoxic conditions [11]. Thus chronic acidification of blood pH, which supposedly could contribute to bone loss, is not seen in a healthy, hyperventilating person. To add further argument to the notion of respiratory effects on bone metabolism we refer to the sleep apnea syndrome. During the hypoxic trials, subjects experienced sleep apnea, which resulted in a hypercapnic and a greater hypoxic stimulus, albeit episodically. Notwithstanding, bone resorption is elevated in severe cases of sleep apnea syndrome, and continuous positive airway pressure therapy reverses this elevation [46].

4.5. Implications for space flight

There was no negative impact on bone metabolism by hypoxia in this study, and both the present study as well as the one by Lynch et al. [10] indicate potentially effects the risk of renal concrements in space [47]. This indicates that changing spacecraft atmospheres from sea level to 4000 m equivalents is unlikely to aggravate bone-related risks in long-term space sojourns. However, this conclusion should be taken with two important restrictions. Firstly, it should be considered that elevated CO_2 concentration, as typically present in spacecraft atmospheres [48] could counteract the positive hyperventilatory effects surmised above. Second, hypoxia interferes with the capacity to perform aerobic exercise, and it can hamper muscular benefits of resistive exercise [49]. Thus, because there was no exercise involved in the bed rest conditions of this study, one has to consider that hypoxia could negatively impact upon efficacy of exercise countermeasures that have proven successful under normoxic conditions.

4.6. Limitations

A few limitations of this study need to be considered. Firstly, its design is not full factorial, *i.e.* there was no specific normoxic ambulatory campaign. To organize another campaign would have raised financial, organizational and ethical issues. To recruit suitable subjects for a 3-campaign cross-over trial is by no means easy, and recruiting subjects for a four-campaign trial was not feasible at this stage. However, normoxic ambulatory conditions were present during the seven days of baseline collection in all three campaigns. Thus, some of the information that would emerge from a fourth, normobaric ambulatory campaign is contained in the changes from baseline in the hypoxic ambulatory campaign. Second, the sample size in this study was relatively small, and thus with limited statistical power. A larger study therefore might be able to find more significant effects. Third, subjects in this study were younger than typical astronauts. Recent results suggest that bone metabolic responses to bed rest are comparable between men in their 3rd and in their 6th decade of life (Buehlmeier et al.,

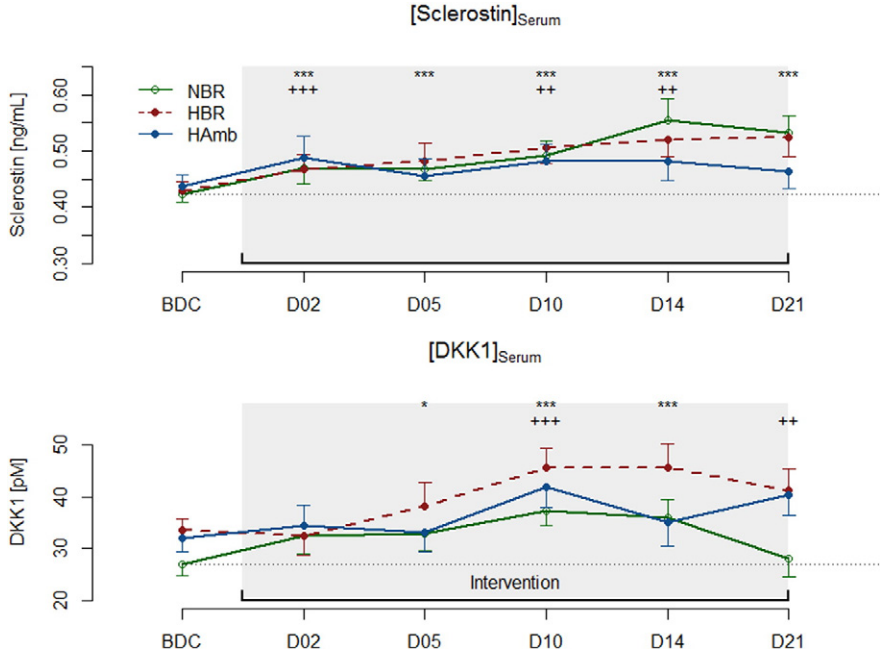

Fig. 3. Serum levels of Wnt-signaling molecules sclerostin and DKK1. Significant contrasts for time in the bed rest data are denoted as * ($P < 0.05$) and *** ($P < 0.001$); significant contrasts for time in HAmb data are denoted as ++ ($P < 0.01$) and +++ ($P < 0.001$).

unpublished results), but older individuals might be more vulnerable to hypoxic exposures or hypoxic disease states than younger individuals due to the blunted hypoxic ventilatory response [50]. While hypoxemic systemic inflammation in COPD patients appears to be driven by white adipose tissue [51], we do not suspect this mode of action in our young, healthy individuals. Pulmonary remodeling as a known consequence of hypoxia was not to be expected in our test population. It would therefore be highly interesting to repeat the experiment with subjects, who often depict moderate levels of arterial hypoxemia, *e.g.* with older people, in order to find out whether reduced ventilatory compensation of hypoxic challenges would cause more profound effects upon bone metabolism. Fourth, this study has used normobaric hypoxia, which may not fully represent the hypobaric hypoxia condition that is envisaged in future spacecrafts. Finally it needs to be mentioned that pQCT results in this study were not very conclusive. This has to be assigned to the relatively short duration (21 days), which turns out to be just long enough to assess effects of bed rest, but probably not long enough to decipher modulatory effects.

5. Conclusion

The present study refutes the idea of strong modulatory effects by hypoxia upon bed rest-induced alterations in bone metabolism, at least in young men. There was, however, a consistent and moderately strong effect upon calcium homeostasis, with reductions in urinary excretion of calcium and phosphate. As these two compounds play a major role in the formation of renal concrements, the present data could imply a moderate mitigation effect for the known risk of renal stone in bed rest [4] and spaceflight [47]. These benefits are, however, likely limited, as the effect was most pronounced in the initial half of the bed rest period and faded away thereafter. Finally, the study also demonstrates that hypoxia-induced perturbation of calcium metabolism leaves bone resorption responses unaffected. This adds further evidence to the understanding that BR-induced bone losses are mainly driven by the mechanical environment, and are quite independent of hormonal or endocrine changes.

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