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Bone formation and resorption biological markers in cosmonauts during and after a 180-day space flight (Euromir 95)

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Long-term spaceflights induce bone loss as a result of profound modifications of bone remodeling, the modalities of which remain unknown in humans. We measured intact parathyroid hormone (PTH) and serum calcium; for bone formation, serum concentrations of bone alkaline phosphatase (BAP), intact osteocalcin (iBGP), and type 1 procollagen propeptide (PICP); for resorption, urinary concentrations (normalized by creatinine) of procollagen C-telopeptide (CTX), free and bound deoxypyridinoline (F and B D-Pyr), and Pyr in a 36-year-old cosmonaut (RTO), before (days −180, −60, and −15), during (from days 10 to 178, n = 12), and after (days +7, +15, +25, and +90) a 180-day spaceflight, in another cosmonaut (ASW) before and after the flight. Flight PTH tended to decrease by 48% and postflight PTH increased by 98%. During the flight, BAP, iBGP, and PICP decreased by 27%, 38%, and 28% respectively in CM1, and increased by 54%, 35%, and 78% after the flight. F D-Pyr and CTX increased by 54% and 78% during the flight and decreased by 29% and 40% after the flight, respectively. We showed for the first time in humans that microgravity induced an uncoupling of bone remodeling between formation and resorption that could account for bone loss.

It is now clearly established that the skeleton adapts its stucture to changes in mechanical load, leading to an increase or a decrease in bone mass and profound changes of bone architecture *[1, 2]*. This adaptation is regulated via changes in bone remodeling mediated by two cell types, osteoblasts and osteoclasts. Osteoblastic bone formation and osteoclastic bone resorption are balanced under physiological conditions. Spaceflight represents the ultimate challenge to demonstrate the role of gravity and mechanical forces in bone biology *[3]*. We *[4, 5]* and others *[6, 7]* reported that bone mass and bone formation were decreased in rat tibiae by the end of the first week of spaceflight, whereas resorption increased *[4, 5]* during the second week. No histologic data are available in humans, but the negative calcium balance observed in astronauts *[8]* suggests an increased bone resorption, and we observed a reduction of up to 25% in bone mineral densities (BMD) in cosmonauts after a 6-month spaceflight *[9]*.[3] Reliable biochemical evaluation of this phenomenon therefore requires specific and sensitive markers of bone remodeling for which assay methods have been recently developed.

Bone formation can be evaluated by various serum assays *[10]*. Osteocalcin (or BGP for bone gla protein) is a noncollagenous protein *[11]* synthesized by mature osteoblasts. Low concentrations are found in platelets and megakaryocytes *[12]*. One fraction of BGP (about 15%) is released into the systemic circulation in either intact or fragmented forms that can be measured separately *[13]*. Bone alkaline phosphatase (BAP), a tetrameric glycoprotein, is an enzyme involved in bone mineralization. It is released into the circulation as a dimer after cleavage by a phospholipase *[14]*. The carboxyl-terminal propeptide of human type 1 procollagen (PICP) splits off from the procollagen in a 1:1 molar ratio *[15]* and reflects osteoblastic differentiation.

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[3] Nonstandard abbreviations: BMD, bone mineral density; (i)BGP, (intact) bone gla protein; BAP, bone alkaline phosphatase; PICP, carboxyl-terminal propeptide of type 1 procollagen; Pyr, pyridinoline; (F) D-Pyr, (free) deoxypyridinoline; CTX, C-telopeptide of the α1 chain of type 1 collagen; PTH, parathyroid hormone; and IGF-1, somatomedin.

Bone resorption can be evaluated by measuring the urinary excretion of free and total pyridinoline (Pyr) cross-links *[16]* and type I collagen C-telopeptide (CTX) (Crosslaps™), which contains Pyr cross-links *[17]*. Pyr and deoxypyridinoline (D-Pyr) cross-links are trivalent molecules that stabilize the mature collagen molecule. These molecules are degraded by the lysosomal enzymes of osteoclasts during bone resorption *[18]*. After circulating in the blood, they are excreted in urine in a free form or bound to peptidic fragments (60%) *[19–21]*. CTX is an eight-amino-acid small peptide of known sequence. It is released when bone collagen is degraded and then excreted in the urine *[22]*.

Apart from mechanical forces, bone remodeling is regulated by systemic hormones, including parathyroid hormone (PTH) and 1,25-dihydroxycholecalciferol (calcitriol). To date, only slight and transient changes in these hormones have been observed in humans after spaceflight *[23]*. Other factors are therefore thought to be involved in the changes observed in bone remodeling: variations in stress-related hormones [cortisol, somatotropin (GH), somatomedin (IGF-1), or prolactin] or a direct effect of weightlessness on bone cells.

The aim of our study was to measure the variations of the specific bone markers of formation and resorption as well as calciotropic systemic hormones during a spaceflight. We also tried to determine the most specific and most sensitive markers of bone remodeling that can be proposed for medical follow-up of cosmonauts.

Subjects And Methods

During the Euromir 95 space mission (European crew embarked on the Mir station from September 1995 to March 1996, i.e., 180 days), four male cosmonauts (ASW, FCH, MGH, RTO), ages 40, 39, 46, and 38 years, respectively, underwent blood and urine sampling for biochemical evaluation of bone formation and resorption. Two cosmonauts (ASW and RTO) flew and FCH and MGH were their backup crew. Sampling was carried out 180, 60, and 15 days before launch in the four cosmonauts and 7, 30, 60, and 90 days after landing in the two cosmonauts who flew. During spaceflight, variables were analyzed 12 times in one subject (RTO) at days 10, 20, 31, 42, 55, 72, 86 104, 125, 301, 167, and 178.

During the flight, daily calcium intake was 800–1200 mg.

Vitamin K was given from days 86 to 130.

These procedures were carried out according to the current revision of the Helsinki Declaration.

SAMPLING

Before launch. Bood samples from fasting subjects were collected into dry glass tubes at 0900, centrifuged for 30 min, aliquoted within 30 min, and kept frozen at −80 °C after landing until assay.

Urine was sampled after discarding urine from the first micturition and ingestion of 200 mL of water, collected after 2 h and then frozen.

During the flight. Bood samples were collected at 0900, centrifuged for 30 min, aliquoted within 30 min, and kept frozen at −20 °C during the flight, and then at −80 °C until assay.

Urine sampling modalities were similar to those before launch.

After the flight. Bood samples were drawn between 0830 and 1000 and centrifuged, aliquoted, and frozen within 30 min.

Urine sampling modalities were similar to those before launch.

ASSAY METHODS

All samples were measured in duplicate during the same assay sequence.

Normal ranges were provided by manufacturers, except for cortisol, which was assayed in our laboratory.

Intact PTH assays were performed with the Allegro IRMA diagnostic kits (Nichols Institute Diagnostics). The cortisol RIA is a competitive binding assay (Immunotech International). The IRMA of IGF-I is a sandwich-type assay (Immunotech International).

Serum calcium was measured with a colorimetric assay (BioMérieux) that does not include deproteinization with *o*-cresolphthaleine. Creatinine was measured according to Jaffe's method without deproteinization (Merck Diagnostica).

Expected values: PTH, 10–65 ng/L; cortisol at 0800, 263–724 nmol/L (established in our laboratory on 65 healthy subjects); IGF-1, 107–410 μg/L; serum calcium, 80–102 mg/L; creatinine, 53–97 μmol/L.

Markers of bone formation. BAP was measured with the IRMA Tandem-R Ostase (Immunotech International), a two-site IRMA. The intra- and interassay CVs were <7% and <9%, respectively *[24]*. Expected values: 8–16.6 μg/L.

BGP assays: Elsa Ost-Nat IRMA (CIS-bio International) is a two-site IRMA for determination of the intact BGP (iBGP) concentration. The intra- and interassay CVs were <5% *[25]*. Expected values: men 31–51 years, 4.8–15.6 μg/L.

Elsa-Osteo IRMA (CIS-bio International) is a two-site IRMA for the determination of intact and fragmented BGP with a solid phase *[26]*. The intra- and interassay CVs were <4% and <6%, respectively. Expected values: men 31–50 years, 13.2–35.5 μg/L.

PICP (Orion Diagnostica): The PICP concentration was measured by a RIA. The intra- and interassay CVs were <3% and <5%, respectively *[15]*. Expected values: 38–202 μg/L.

Markers of bone resorption. All urine samples were run in duplicate in the same assay and were corrected for creatinine excretion.

CTX (CrossLaps) is a competitive immunoassay on a microtiter plate (CrossLaps Elisa, Osteometer). The Crosslaps antigen coated on the microwell is a synthetic peptide with an amino acid sequence specific for a part of the C-telopeptide of the α1 chain of type 1 collagen (Glu-Lys-Ala-His-Asp-Gly-Gly-Arg: Crosslaps antigen) *[17]*. The intra- and interassay CVs were <6% and <8%, respectively, and the detection limit was 0.2 g/L.

Urinary free D-Pyr (F D-Pyr) was measured with a competitive RIA involving a monoclonal anti-D-Pyr antibody coated onto the inner surface of a polystyrene tube (Pyrilinks-D RIA, Metra biosystems). The intra- and interassay CVs were <5% and <7%, respectively, and the detection limit was 2 nmol/L. Expected values for men 25–55 years: 2.3–5.4 mmol/L per mmol/L creatinine.

The Pyrilinks® assay measures free pyridinium crosslinks, Pyr, and D-Pyr in urine (Pyrilinks, Metra biosystems). It is a competitive enzyme immunoassay in a microtiter plate. The interassay CV was <10% *[20]*. The detection limit was 7.5 nmol/L. Expected values: 14–23 nmol/mmol. Good correlations have been reported between immunologic assays and HPLC assays of Pyr *[20]*. Expected values: 99–293 μg per mmol/L.

Total D-Pyr was determined by hydrolyzing urine before analysis. Each sample was hydrolyzed by boiling in 6 mol/L HCl and heating at 110 °C for ≥18 h in a screw-capped glass tube. Urine was neutralized with 1 mol/L NaOH. Total D-Pyr determined by Pyrilinks-D correlates with both total D-Pyr measured by HPLC and F D-Pyr in nonhydrolyzed samples by Pyrilinks-D (respectively $r = 0.98$ and $r = 0.90$).

Data and statistical analyses. In the Figures, data are expressed in Z-scores calculated with the individual mean value and standard deviation of the preflight data of RTO. In RTO, the percentage variations given in the results during flight were calculated in relation to the mean preflight concentrations, and the percentage variations after flight were calculated in relation to the mean flight values of RTO. We did not perform any statistical analysis on the mean preflight, flight, and posflight values because of the time factor.

Results

Stress-related hormones. Data are summarized in Fig. 1. Serum concentrations of IGF-1 and cortisol remained within the normal range before, during, and after the flight in all cosmonauts except ASW, who exhibited increased concentrations of cortisol before launch.

Calcium and PTH concentrations. Data are sumarized in Fig. 2. In RTO, PTH decreased by 48% during the flight, but remained within the normal range. Serum PTH increased transiently above normal concentrations in both ASW and RTO at day 7 postflight after landing (i.e., + 200% between the last assay in space and day 7 for RTO) and subsequently returned to normal. Serum calcium concentrations remained within the normal range throughout the biochemical follow-up and exhibited no major variations.

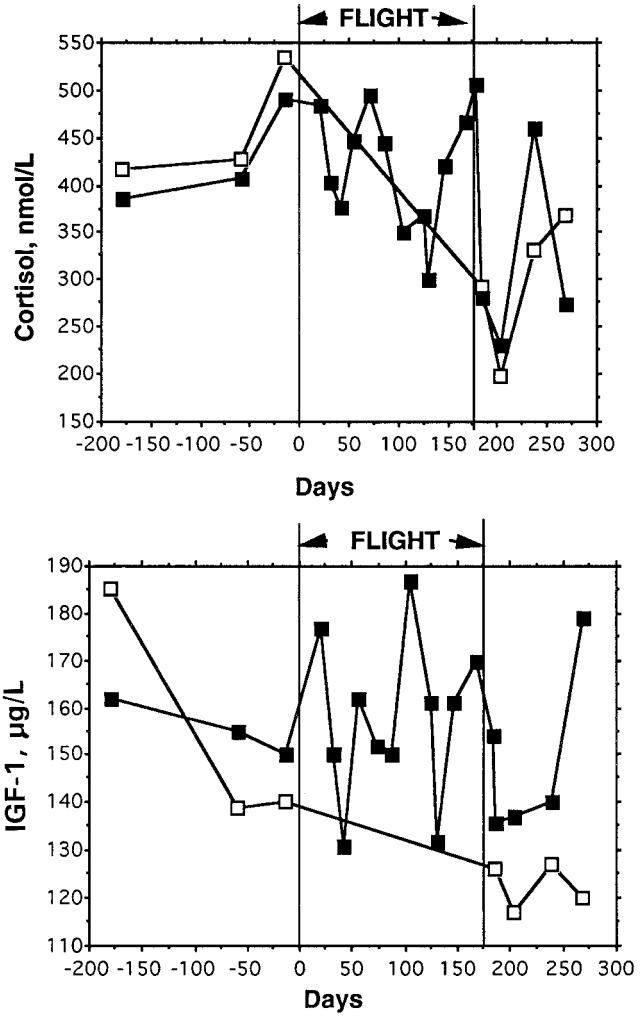

Fig. 1. Variations of cortisol and IGF-1 in the two cosmonauts ASW (□) and RTO (■).

Bone formation variables. Preflight values are summarized in Table 1. During the preflight period, bone formation variables remained within the normal range in the four cosmonauts. Z-scores for iBGP, BGP and fragments, BAP, and PICP of the two cosmonauts RTO and ASW are illustrated in Fig. 3. In RTO no overlapping between preflight and spaceflight concentrations was observed except for BGP. Values remained within the normal range except for one assay: postflight PICP of RTO. BGP, PICP, and BAP decreased (by 27%, 38%, and 28%, respectively) during the flight as compared with preflight concentrations, and these variables increased (by 42%, 132%, and 54%, respectively) after the flight as compared with flight concentrations.

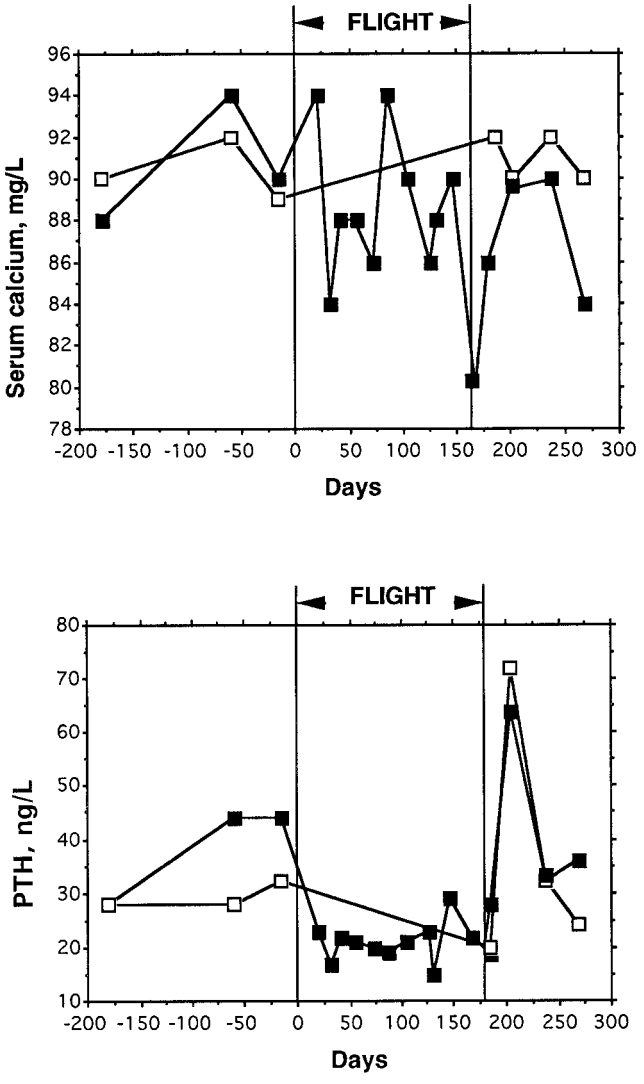

Fig. 2. Variations of serum calcium and PTH in the two cosmonauts ASW (□) and RTO (■).

Bone resorption variables. Preflight values are summarized in Table 1. During the preflight period, bone resorption variables remained within the normal range in the four cosmonauts. Fig. 4 illustrates changes in Z-scores of bone resorption variables for the two cosmonauts RTO and ASW. It is noteworthy that an increase in bone resorption markers, except for Pyr, was observed by the 20th day of flight. Free and total D-Pyr and CTX increased (54%, 35%, and 78% respectively) during the flight compared with preflight concentrations. CTX values returned to preflight values 30 days after landing, whereas F D-Pyr took longer to return to normal in ASW and remained increased in RTO. The preflight percentage of F/total D-Pyr was between 31% and 46% (Fig. 5). This ratio increased by 21% during the flight in RTO and increased further after landing by 15% compared with flight concentrations. No major variation in Pyr was observed during the flight. In ASW, a dramatic and unexplained increase in Pyr concentrations was observed during the postflight period.

Discussion

In the present study, we report, for the first time, the variations of new markers of bone remodeling as well as stress-related and calcitropic hormones before, during, and after a 6-month spaceflight in humans. The absence of change in cortisol and IGF-1 concentrations in the two cosmonauts who flew suggested that stress-related hormones *[27]* were not involved in long-term bone cell response to spaceflight or landing. We showed that PTH decreased during spaceflight, though remaining within the normal range. Morey-Holton et al. *[28]* reported no change in biologically active PTH measured with a receptor assay in four astronauts undergoing an 8-day spaceflight. A decrease in PTH could be explained either by an increase in serum calcium or an increase in calcitriol, which directly inhibits transcription of preproPTH mRNA. We did not measure calcitriol concentrations in our study, but Morey-Holton et al. *[28]* showed a transient rise in serum concentrations of 1,25-$(OH)_2$ vitamin D only after the first 18 h of spaceflight in two astronauts, followed by a rapid return to preflight concentrations. Spaceflight has previously been reported to induce hypercalciuria *[8]* that could be related to increased bone resorption. Our data confirmed that increased bone resorption occurred during spaceflight. The decrease in PTH observed during the flight could therefore be the consequence of a relative increase in serum calcium concentrations due to this increased bone resorption. Seven days after landing, PTH increased above normal concentrations in both cosmonauts (64 in RTO and 72 ng/L in ASW), whereas serum calcium concentrations remained within the normal range. This combination suggests the development of transient secondary hyperparathyroidism. This could be related to relative hypocalcemia due to the intense water intake used to correct postflight-induced hypovolemia. The second possible explanation could be that postflight bone hyperremodeling, suggested by our results, induced a great calcium influx towards the skeleton that might transiently decrease serum calcium concentrations and induce secondary hyperparathyroidism. In conclusion, changes in systemic hormones regulating calcium metabolism seem to be a consequence rather than a cause of changes in bone remodeling, in the absence of any significant alteration in stress-related hormones, suggesting a direct effect of long-term microgravity on bone cells.

Our present study confirmed that microgravity strongly depresses bone formation in humans as consistently reported by ourselves *[4, 5]* and others *[6, 7]* in bone histomorphometric studies of rat tibiae after spaceflights of various durations.

The four bone formation markers reflects the three different steps of osteoblastic differentiation, as suggested by in vitro data *[29]*: type I collagen synthesis (PICP), bone

Table 1. Preflight mean values of biochemical markers of bone remodeling in four cosmonauts.

Marker, normal range	RTO	ASW	FCH	MGM
Bone formation				
BGP 4.8–15.6 μg/L	15.3 ± 2.5	16.0 ± 2.7	15.7 ± 2.3	6.4 ± 2.9
iBGP + fragments 13.2–35.5 μg/L	28.7 ± 5.1	31.3 ± 2.5	32.0 ± 2.7	13.0 ± 2.7
Type 1 procollagen propeptide 38–202 μg/L	164.7 ± 33.0	147.0 ± 9.0	115.3 ± 6.3	92.0 ± 10.5
BAP 8–16.6 μg/L	11.0 ± 0.3	8.4 ± 1.3	6.7 ± 0.4	9.8 ± 0.2
Bone resorption				
FD-Pyr 2.3–5.4 nmol/mmol	4.8 ± 0.6	4.9 ± 0.6	3.9 ± 0.5	2.2 ± 1.4
Total D-Pyr nmol/mmol	9.4 ± 1.1	10.1 ± 1.1	8.0 ± 1.5	3.0 ± 0.3
Pyr 12.8–25.6 nmol/mmol	21.9 ± 3.2	19.4 ± 2.4	21.1 ± 3.2	12.4 ± 8.6
CTX 99–293 μg/mmol	189.5 ± 36.9	216.1 ± 38.3	218.5 ± 27.8	122.7 ± 49.5

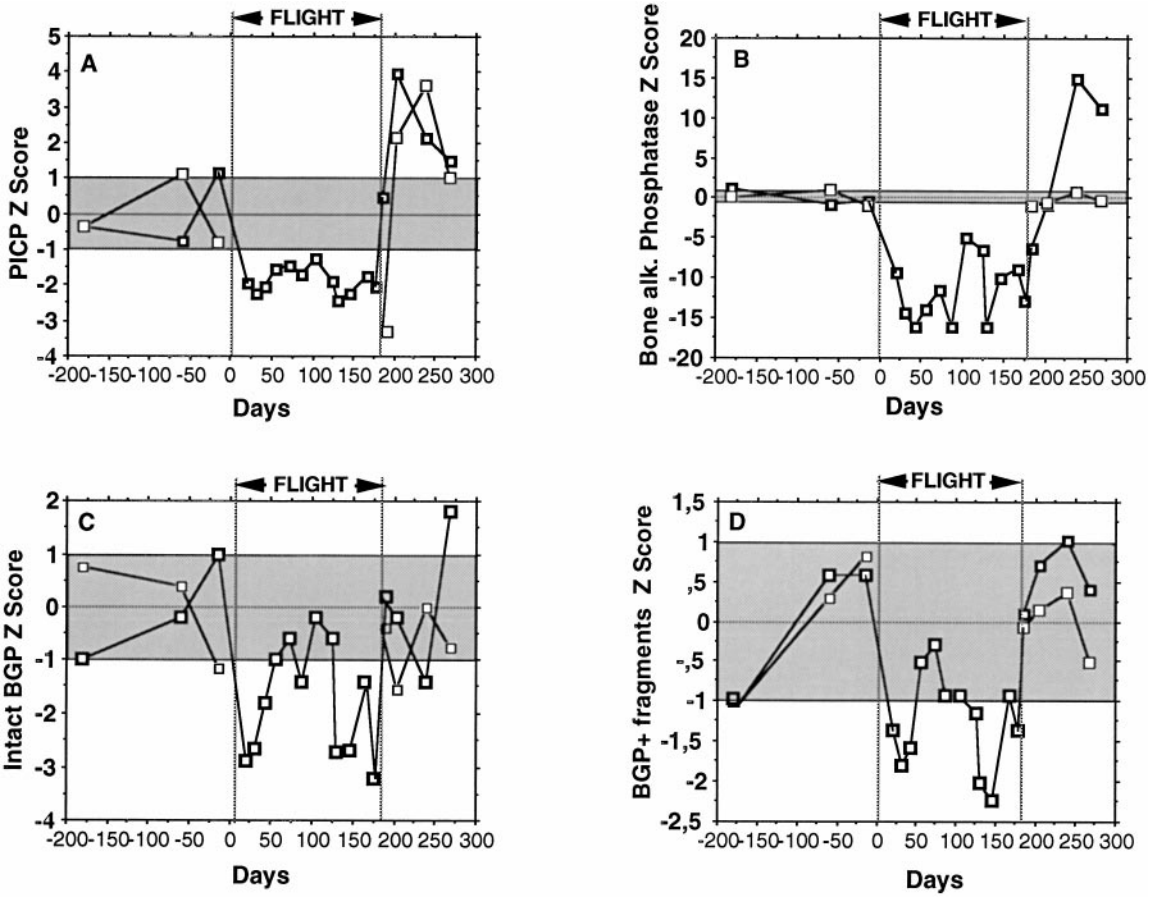

Fig. 3. Evolution of *Z*-scores of bone formation markers in the two cosmonauts RTO (*bold squares*) and ASW (*thin squares*).
A, PICP; *B*, BAP; *C*, iBGP; *D*, BGP and fragments.

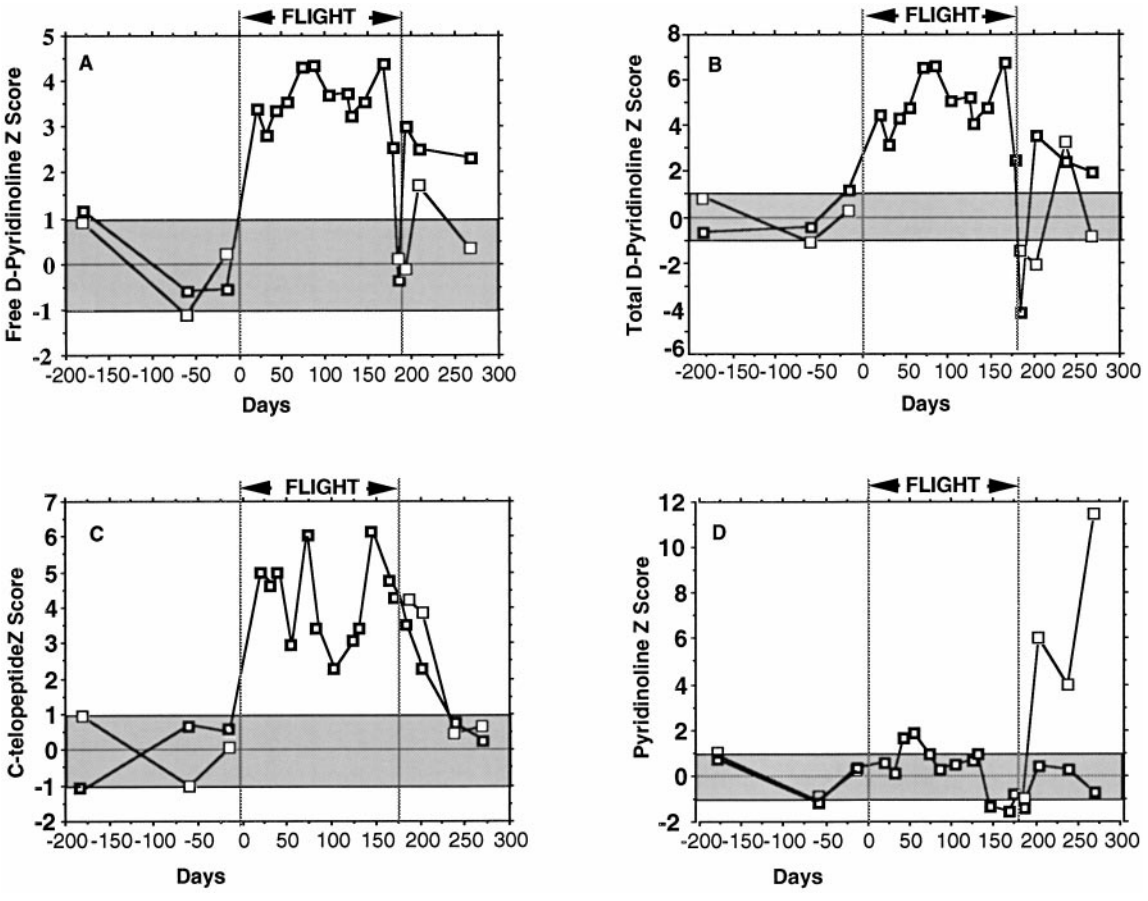

Fig. 4. Evolution of *Z*-scores of bone resorption markers in the two cosmonauts RTO (*bold squares*) and ASW (*thin squares*). *A*, F D-Pyr; *B*, total D-Pyr; *C*, CTX; *D*, Pyr.

matrix maturation (BAP), and matrix mineralization (BGP). During the flight, a decrease of the four markers was observed compared with preflight concentrations. This decrease occurred by the 20th day of flight. In a previous study, we reported trends towards a decrease in these variables during a 1-month flight (space mission Euromir 94), followed by a return to preflight concentrations 7 days after landing *[30]*. Therefore, although changes in bone formation concentrations appear to occur early, they still require a lag time of 20 to 30 days to be detected. This suggests that the adaptation to microgravity might be achieved by newborn osteoblasts, whereas osteoblasts born before launch kept their synthetic activities. Fig. 3 shows that the kinetics of both BGPs differed from PICP and BAP, with a transient return to preflight values between days 55 and 104 of the flight. RTO received vitamin K supplementation between days 86 and 130. However, although such treatment might alter the amount of carboxylation of BGP, it could not alter the total BGP concentration *[31]*. This biphasic response of BGP to flight might be comparable with that reported in rats flown for 14 days *[5, 32]*. Bone formation tended to rebound after an initial decrease observed after the first week of flight.

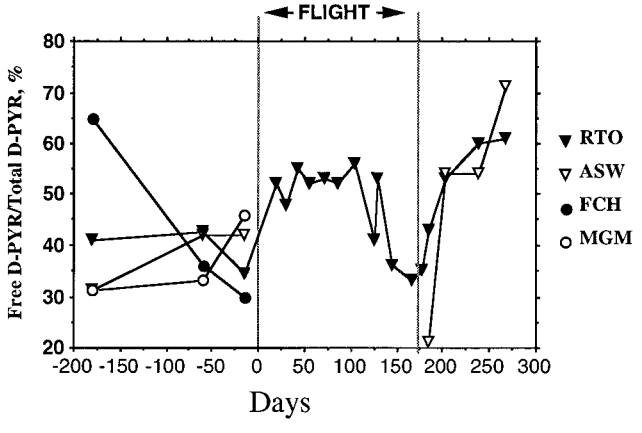

Fig. 5. Evolution of the F/bound D-Pyr ratio in the four cosmonauts.

We previously studied the variations of bone formation and resorption after a 7-day spaceflight *[33]* in two American astronauts (51 G mission). No significant variation in pre- and postflight BAP and BGP concentrations was observed. Our present study in a much longer flight showed a rebound in the four bone formation variables during the postflight period, with a return to preflight concentrations 90 days after landing except for BAP in

RTO. In conclusion, BGP measurements, combined with BAP and PICP, providing indications about various aspects of osteoblastic activities, might be necessary for reliable follow-up of bone formation in cosmonauts *[13, 15]*. Interestingly, according to Delmas *[34]*, measuring intact osteocalcin molecule + fragments reduces by 50% the long-term precision error when measurements are repeated over months in a single patient, and therefore seems more appropriate in the space field.

Type 1 collagen represents 90% of bone matrix *[16]* and measurement of its degradation reflects osteoclastic resorption *[35]*. Our results showed that all resorption variables except for Pyr increased during spaceflight (Table 3). This might reflect the lower specificity of Pyr for bone since it is also known to be present in nonbone collagen *[36]*.

Previous studies demontrated that Pyr and D-Pyr are excreted in urine in either the free form (40%) or the bound form (60%) *[20, 21]*. The total D-Pyr/F D-Pyr ratio is constant in healthy subjects free of bone disease, and increases in elderly men and women because of an increase in the amount of the free form *[20]*. This ratio also varies with bone diseases or various treatments *[16, 20, 21, 37]*. The preflight F/total D-Pyr ratio was between 30% and 46% in the four cosmonauts. It increased by 20.5% during the flight in RTO and increased by a further 15% after landing as compared with flight concentrations. A similar discrepancy was observed by Garnero et al. *[19]*, who reported that bisphosphonate therapy was able to decrease total D-Pyr with no change in F D-Pyr in osteoporotic patients and by Brazier et al. *[38]* in patients treated with vitamin D. These data suggested that bisphosphonate, vitamin D deficiency, and spaceflight might affect osteoclast breakdown of bone collagen in different ways compared with physiological conditions, suggesting different patterns of type 1 collagen proteolysis.

Seven days after landing, a dramatic decrease in pyridinium cross-links was observed, followed by a rebound. However, in contrast with formation markers, resorption markers did not return to preflight concentrations, except for CTX, which decreased by 40% during the postflight period. We previously reported *[30]*, after the 60-day Euromir 94 space mission, a tendency towards an increase in Pyr with no variation in D-Pyr. Although Pyr is considered to be an accurate marker of bone resorption in pre- and postmenopausal women *[22]*, it did not appear to be appropriate for follow-up of bone remodeling in space. In conclusion, CTX and D-Pyr assays could be proposed for follow-up of cosmonauts.

We showed that spaceflight induced changes in biochemical markers of bone remodeling measured in blood and urine. In animals, short spaceflights (2–6 weeks) affected primarily weight-bearing bones *[6]*. However, the fact that in the present study we observed such systemic variations indicated that either the amplitude of variation was large in weight-bearing bones or that non-weight-bearing bones were affected as well after a 6-month spaceflight. This latter hypothesis is corroborated by BMD measurements performed after a 6-month spaceflight in humans that showed a bone mass decrease in tibiae and in radii *[9]*.

In conclusion, we report for the first time the evolution of biological markers of bone remodeling during and after a 6-month spaceflight compared with preflight concentrations. We found that bone formation was reduced during spaceflight, whereas bone resorption was increased. The postflight period was characterized by a return to preflight concentrations of formation markers and CTX and by a biphasic response for pyridinium cross-links. Our findings support the hypothesis of a direct effect of microgravity on bone, independent of stress-related hormones. BAP, BGP, D-Pyr, and CTX, which are known to be specific markers of bone formation and resorption, seem to provide consistent information about bone remodeling. These findings need to be confirmed by further studies in a larger number of subjects.

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