Effects of seasonal vitamin D deficiency and respiratory acidosis on bone metabolism markers in submarine crewmembers during prolonged patrols

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Holy X, Collombet JM, Labarthe F, Granger-Veyron N, Bégot L. Effects of seasonal vitamin D deficiency and respiratory acidosis on bone metabolism markers in submarine crewmembers during prolonged patrols. J Appl Physiol 112: 587–596, 2012. First published December 1, 2011; doi:10.1152/japplphysiol.00608.2011.—The aim of the study was to determine the seasonal influence of vitamin D status on bone metabolism in French submariners over a 2-mo patrol. Blood samples were collected as follows: prepatrol and patrol days 20, 41, and 58 on crewmembers from both a winter (WP; n = 20) and a summer patrol (SP; n = 20), respectively. Vitamin D status was evaluated for WP and SP. Moreover, extended parameters for acid-base balance (PCO₂, pH, and bicarbonate), bone metabolism (bone alkaline phosphatase and COOH-terminal telopeptide of type I collagen), and mineral homeostasis (parathyroid hormone, ionized calcium and phosphorus) were scrutinized. As expected, SP vitamin D status was higher than WP vitamin D status, regardless of the considered experimental time. A mild chronic respiratory acidosis (CRA) was identified in both SP and WP submariners, up to patrol experimental time. A mild chronic respiratory acidosis (CRA) was identified in both SP and WP submariners, up to patrol day 41. Such an occurrence paired up with an altered bone remodeling coupling (decreased bone alkaline phosphatase-to-COOH-terminal telopeptide of type I collagen ratio). At the end of the patrol (day 58), a partial compensation of CRA episode, combined with a recovered normal bone remodeling coupling, was observed in SP, not, however, in WP submariners. The mild CRA episode displayed over the initial 41-day submersion period was mainly induced by a hypercapnia resulting from the submarine-enriched CO₂ level. The correlated impaired bone remodeling may imply a physiological attempt to compensate this acidosis via bone buffering. On patrol day 58, the discrepancy observed in terms of CRA compensation between SP and WP may result from the seasonal influence on vitamin D status.

hypercapnia; hypovitaminosis D; submariners; chronic obstructive pulmonary disease

Vitamin D is available to humans through UV-directed photo-synthesis in the skin (providing 90% vitamin D body stock) and through ingested vitamin D-rich food, such as oily fish and eggs (10% vitamin D body stock) (38). The main clinical indicator used to evaluate the vitamin D status for a given individual relies upon the measurement of serum 25 hydroxyvitamin D [25(OH)D] concentrations (17). The 25(OH)D active form is 1,25 dihydroxyvitamin D [1,25(OH)₂D], which plays an essential role in bone mineral homeostasis. It is commonly acknowledged that vitamin D deficiency in human subjects mainly results from a lack of sunlight exposure, owing to a wide array of contributing factors, namely, time of day, season, geographic localization, and atmospheric conditions (15, 23). Several distinctive studies have underscored vitamin D deficiency at the end of the winter season in free-living healthy young adults residing above and beyond the 37th parallel in the northern and southern hemispheres, respectively (37, 39). It is well documented that serum 25(OH)D levels are lower in winter than in summer since cutaneous vitamin D production is strongly reduced in winter due to sunlight deprivation (18, 23). In adults, vitamin D deficiency leads to secondary hyperparathyroidism, increases bone turnover, leading to progressive bone loss, and further heightens the risk for osteoporosis (16). Moreover, a recent cross-sectional study performed on a group of Northern Irishmen exhibiting low-trauma forearm fractures showed that vitamin D deficiency may possibly account for mineral density lessening [bone mineral density (BMD) measurements], increasing thereby conditions for further broken bone (42).

To date, few studies have targeted the evolution of vitamin D status in submariner populations during submersion (7, 11, 14, 25, 30). So far, related authors have reached the following consensus: serum 25(OH)D level significantly decreased in crewmembers during patrols. It is noteworthy, however, that none of these investigations have addressed, as yet, the issue of seasonal side-effect on submariner vitamin D status. Indeed, it can be assumed that submariners assigned to winter patrols (WP) are particularly prone to vitamin D deficiency. Insofar as the winter period fosters hypovitaminosis D (37, 39), we may legitimately hypothesize, therefore, that any additional sun deprivation due to long-term submersion is likely to further heighten vitamin D synthesis impairments. Hence, any sizeable vitamin D deficiency is likely to affect submariners’ bone remodeling and cause reduction of BMD. In a sophisticated study, Luria et al. (25) demonstrated that a 30-day submersion period induced a decrease in bone strength, as assessed by quantitative bone speed of sound (SOS) and bone metabolism markers. However, it would be worthwhile to further extend this investigation in terms of patrol duration in the context of
ballistic missile submarines (i.e., 2-mo continuous submersion) and in terms of seasonal vitamin D status influence over bone metabolism regulation.

Prolonged exposure to elevated CO₂ levels has been found to result in respiratory acidosis, either in animal models (2, 35) or in human subjects (8). In all such investigations, respiratory acidosis induced modifications in the serum and/or urine calcium homeostasis. The involvement of respiratory acidosis on bone metabolism was more controversial. Indeed, a 14-day exposure of rats to 10% CO₂ failed to modify endosteal bone cell activity (2). Contrariwise, guinea pigs exposed to 1% CO₂ for up to 8 wk exhibited phasic changes in both bone calcium and bicarbonate (HCO₃⁻), suggesting thereby a bone deminer-alization process (35). More recently, by comparing the effect of 0.7% and 1.2% CO₂ environment in humans isolated for a 25-day period in a deep diving isolation chamber, Drummer et al. (8) demonstrated that elevated CO₂ atmosphere reduced bone formation and slightly increased bone resorption.

In French submarines, CO₂ concentration is between 0.6 and 1.0%, compared with the 0.03% CO₂ level found in atmospheric air. Therefore, respiratory acidosis and subsequent alterations of both mineral homeostasis and bone metabolism balances should be expected in submariners during submersion. By comparing several submarine patrols, crewmembers, acid-base balance data exhibited cyclic changes, alternating between metabolic and respiratory acidosis in ~20-day periods (27, 33, 34). Metabolic acidosis is characterized by a blood pH decrease induced by a reduction in serum HCO₃⁻ concentration, whereas, in respiratory acidosis, pH reduction is fostered by an increase in blood Pco₂ (9). To date, few investigations have focused on submariner mineral metabolism (e.g., calcium and phosphorus, mainly) over extended patrols (7, 12, 25, 27, 33, 34). However, the evolution of calcium and phosphorus levels during submarine patrols remained subject for debate. The discrepancy between these results may stem from the influence of the vitamin D status of submariners, depending on the boarding season. Such a point stands out as a critical issue that deserves further scrutiny. Hence, the goal of the present study was to determine the seasonal influence of vitamin D status on extended bone metabolism and mineral homeostasis parameters in submariners. To such an end, blood samples were collected from crewmembers during two distinctive patrols: 1) a winter patrol (WP; expected low vitamin D status); and 2) a summer patrol (SP; expected high vitamin D status). Analyses and comparison of bone metabolism and mineral homeostasis parameters obtained from submariners taking part in both patrols ought to shed light on the outstanding issue, namely, whether or not vitamin D supplementation should be administered to French crewmembers to counteract potential alteration of bone metabolism.

**MATERIAL AND METHODS**

**Subjects and patrol specifications.** Forty Caucasian men from two French ballistic missile submarines (20 crewmembers per submarine) were volunteers and gave their informed consent to take part in this study. All subjects were in excellent health, had never had major diseases nor any injury, and never received vitamin D supplementation during the last 4 mo before the submersion and during the experiment. To ensure a maximal discrepancy in vitamin D status between both patrols, submariners boarded either at the end of the summer (September) or in midwinter (February). The duration of both patrols amounted to 60 days. An identical atmospheric pressure was measured in both submarines with 999 ± 15 and 1,026 ± 11 mbar for SP and WP, respectively (pressure values are mean ± standard error of daily pressure records). Average CO₂ levels in submarines reached 0.72 ± 0.04% for SP and 0.69 ± 0.06% for WP (these values are means ± SE of daily recorded %CO₂ corrected for variation in submarine pressure). The corrected CO₂ levels measured by the day of sample collections are indicated in Table 1. All of these CO₂ levels are similar and range from 0.70 to 0.75%. The protocol for this study has been registered at the French Ministry of Health. It received official approval from the Ethical Committee known as “le Comité Consultatif de la Protection des Personnes dans la Recherche Biomédicale” in Brest (France), in keeping with the ethical standards laid down in the 1964 Declaration of Helsinki and the 2001 US Code of Federal Regulations related to the Protection of Human Subjects. The average age of submariners was 31 ± 2 yr for WP and 29 ± 1 yr for SP.

**Study design and sample collections.** Body weight was measured, and fasting venous blood samples were collected from subjects using Vaccumap tubes (within the first hour following awakening; without tourniquet to avoid local ischemia) before boarding (prepatrol control values) and 20, 41, and 58 days after the beginning of submersion. In the shore and submarine sick bays, sera were obtained from blood samples after centrifugation at 2,000 g for 10 min in anaerobic condition. Serum aliquots (500 μl) were immediately stored at −20°C for further biochemical analysis. However, measurements of ionized calcium (Ca²⁺), pH, Pco₂, and HCO₃⁻ were immediately carried out in anaerobic conditions before serum aliquot freezing, using a Stat Profile pHOx Plus analyzer (Nova Biomedical, 91965, Les Ulis, France).

**Endocrine regulators (vitamin D and parathyroid hormone).** Radioimmunoassay 25(OH)D 125I RIA and 1,25(OH)₂D 125I RIA kits (DiaSorin) were used to determine serum 25(OH)D and 1,25(OH)₂D concentrations, respectively. Preliminary extractions were necessary before the incubation step with specific 125I-labeled antibodies directed against 25(OH)D or 1,25(OH)₂D. In keeping with the supplier’s instructions, acetonitrile extraction was used for both 25(OH)D and 1,25(OH)₂D. An additional C₁₈ OH “extra clean” cartridge purification was performed solely for 1,25(OH)₂D. Concentrations of metabolites were expressed in nanograms per milliliter for 25(OH)D and in picograms per milliliter for 1,25(OH)₂D. The within- and between-run coefficients of variation (CV) amounted to 8.6 and 9.1% for 25(OH)D kit and 7.7 and 11.1% for 1,25(OH)₂D kit, respectively.

Intact serum parathyroid hormone (PTH) level (in pg/ml) was measured by radioimmunometric assay with 125I-labeled antibody directed against human PTH (N-tact PTH SP IRMA kit, DiaSorin). The within- and between-run CVs were 3.6 and 3.4%, respectively.

**Biochemical assays.** Measurements of serum Ca²⁺, inorganic phosphorus (Pᵢ), pH, Pco₂, and HCO₃⁻ were performed on a Stat Profile pHOx Plus analyzer (Nova Biomedical, 91965, Les Ulis, France). The same apparatus was used for both patrol experiments in the shore (prepatrol control values) and submarine (patrol days 20, 41, Table 1. CO₂ levels in submarines by the day of sample collections

<table>
<thead>
<tr>
<th>Patrol Day</th>
<th>Summer patrol</th>
<th>Winter patrol</th>
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<tbody>
<tr>
<td>20</td>
<td>0.75</td>
<td>0.70</td>
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<tr>
<td>41</td>
<td>0.75</td>
<td>0.70</td>
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<tr>
<td>58</td>
<td>0.72</td>
<td>0.70</td>
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Values are the percentage of CO₂ levels recorded by the day of sample collections (patrol days 20, 41, and 58) in submarines from summer or winter patrols. These CO₂ percentages are corrected for variation in submarine atmospheric pressure using the following formula: corrected %CO₂ = daily recorded %CO₂ × (1,013/daily recorded pressure in mbar), in which 1,013 mbar is the mean atmospheric pressure at the mean sea level.
and 58) sick bays. Before measurement implementation, the apparatus was calibrated according to the supplier’s instructions, and three-level quality control (acidosis, normal, and alkalosis conditions) was assessed using Nova Biomedical ampoule control kits. Measured ion and HCO$_3^-$ concentrations were expressed in millimoles per liter, and PCO$_2$ in Torr.

**Markers of bone metabolism.** Bone-specific alkaline phosphatase (BAP) and COOH-terminal telopeptide of type I collagen (ICTP) were considered as specific serum markers for bone formation and bone resorption, respectively (5).

Serum BAP was quantified by ELISA immunoassay using monoclonal anti-BAP antibody (Alkphase-B kit from Metra Biosystems). The enzyme activity of the ELISA-captured BAP was determined with the p-nitrophenylphosphate substrate and expressed in international units per liter. Values are means ± SE. Statistical analyses were performed using a two-factor ANOVA with one between-groups factor (winter vs. summer submariners) and one repeated-measures factor (time). The interactions between both parameters (season × time) were also tested. Whenever ANOVA yielded any significant difference, post hoc comparisons were performed by least significant difference Fisher tests. For all statistical tests, data were analyzed with Statistica 7.1 software (StatSoft France), and significance level was set at $P < 0.05$.

**RESULTS**

**General parameters.** ANOVA revealed any effect neither of season ($P = 0.899$), nor of submersion time ($P = 0.646$) on body weight without any significant season × submersion time interaction ($P = 0.848$) (data not shown).

Concerning acid-base balance regulation, serum PCO$_2$, HCO$_3^-$, and pH parameters are presented in Fig. 1.

ANOVA exhibited a significant effect of both season ($P < 0.001$) and submersion time ($P < 0.01$) on PCO$_2$ without any significant season × submersion time interaction ($P = 0.102$). As shown in Fig. 1A, throughout the submersion period, serum PCO$_2$ increased compared with control value recorded either in WP ($\sim 22\%$ increase; $P < 0.001$) or SP groups ($\sim 21\%$ increase; $P < 0.001$). The PCO$_2$ level for WP was higher than for SP throughout the patrol.

ANOVA demonstrated a significant season × submersion time interaction for blood HCO$_3^-$ values ($P < 0.05$). In SP, a 16% increase was evidenced over the first 20 days of submersion ($P < 0.001$), and this value remained even, up to the end of the patrol (Fig. 1B). In WP, a first 10% rise was displayed on day 20 ($P < 0.01$), followed by a second 9% increase in HCO$_3^-$ values on day 58 ($P < 0.001$). Analyses of HCO$_3^-$ profile evolution between both patrols exhibited a significant difference on day 58 ($P < 0.05$). At this experimental time, WP HCO$_3^-$ quantity exceeded the SP value by 6%. ANOVA showed a significant effect of both season ($P < 0.05$) and submersion time ($P < 0.001$) on pH, without any significant season × submersion time interaction ($P = 0.310$). In both patrols, a significant pH decrease ($P < 0.05$) was evidenced over the first 41 days of submersion, followed by a compensation at a later experimental time, resulting in a return to control pH values at the end of the patrol (Fig. 1C). The pH level for WP was lower than for SP throughout the patrol.

**Endocrine regulators (vitamin D status and PTH level).** ANOVA exhibited a significant season × submersion time interaction ($P < 0.001$) for serum 25(OH)D. As shown in Fig. 2A for prepatrol control values, serum 25(OH)D level in summer was roughly twice as high as the level measured in winter. In summer, a 31% decrease in 25(OH)D quantity was evidenced...
ANOVA revealed a significant effect of both season \((P < 0.05)\) and submersion time \((P < 0.01)\) on 25(OH)D levels, without any significant season \(\times\) submersion time interaction \((P = 0.784)\). Under submersion conditions, a 15\% reduction in 25(OH)D amounts was evidenced at the end of the patrol \((day\ 58)\) for both WP and SP \((Fig.\ 2B)\). The 1,25(OH)D level for WP was lower than for SP throughout the patrol.

Concerning PTH \((Fig.\ 2C)\), ANOVA showed a significant season \(\times\) submersion time interaction \((P < 0.01)\). Serum WP PTH levels consistently exceeded SP PTH values (between 58 and 91\% higher) in prepatrol conditions and at submersion days 20 and 41. A continuous reduction of PTH levels was observed for both WP (10\% decrease; \(P < 0.05\)) and SP (26\% reduction; \(P < 0.01\)) up to patrol day 41. At the end of the patrol \((day\ 58)\), WP PTH level was subsequently reduced by ~15\% \((P < 0.05)\), whereas SP PTH level returned to SP prepatrol value (29\% increase; \(P < 0.05\)).

**Bone metabolism markers.** Serum BAP and ICTP measurements are considered as valuable markers to estimate bone formation or resorption, respectively \((Fig.\ 3)\). ANOVA showed a significant season \(\times\) submersion time interaction \((P < 0.001)\) for serum BAP. Similar BAP levels were found in both WP and SP prepatrol conditions. In WP, a 13\% decrease in BAP was measured as early as patrol day 20 \((P < 0.01)\), compared with prepatrol level \((Fig.\ 3A)\). This low quantity of BAP remained consistent over the rest of the submersion period. A similar pattern was reached for SP conditions (16\% reduction in BAP on patrol day 20 compared with control data; \(P < 0.001\)), except that BAP was increased on patrol day 58, even exceeding prepatrol SP levels \((P < 0.001)\). When comparing both patrols, WP BAP was 38\% lower than SP BAP on patrol day 58 only \((P < 0.05)\).

ANOVA demonstrated a significant season \(\times\) submersion time interaction for serum ICTP \((P < 0.05)\). Serum ICTP levels exhibited no difference in prepatrol SP and WP \((Fig.\ 3B)\). ICTP levels rose by 22\% on patrol day 20 for SP \((P < 0.001)\) and by 10\% on patrol day 41 for WP \((P < 0.05)\). This bone resorption marker remained elevated until the end of both patrols, although there was no significant difference compared with prepatrol values at the end of the WP submersion \((day\ 58)\).

In addition, we used BAP-to-ICTP level ratios \((BAP/ICTP)\) as an index of bone remodeling coupling \((Fig.\ 3C)\). ANOVA revealed a significant season \(\times\) submersion time interaction for BAP/ICTP \((P < 0.001)\). Similar control BAP/ICTP were found in both WP and SP prepatrol conditions. Submersion proved detrimental to bone remodeling balance, since a persistent reduction in BAP/ICTP was measured in both SP \((\sim\ 28\%\ decrease; \(P < 0.001)\) and WP \((\sim\ 18\%\ reduction; \(P < 0.001)\) populations, up to 41 days of submersion. At the end of the patrol \((day\ 58)\), a complete recovery of control BAP/ICTP value was evidenced for SP submariners \((P < 0.001)\), but not for WP submarine crews. Indeed, WP BAP/ICTP remained low, thus indicating that the altered bone remodeling coupling lasted until the end of the winter assignment.

**Blood mineral homeostasis.** ANOVA showed a significant season \(\times\) submersion time interaction \((P < 0.001)\) for serum Ca\(^{2+}\). Serum Ca\(^{2+}\) levels exhibited no difference in prepatrol SP and WP \((Fig.\ 4A)\). For SP, a significant 1.1\% rise of Ca\(^{2+}\) level was evidenced as early as patrol day 20 \((P < 0.05)\) and was further followed by a reduction of this parameter up to the
ANOVA exhibited a significant effect of both season ($P < 0.001$) and submersion time ($P < 0.001$) on Pi levels without any significant season $\times$ submersion time interaction ($P = 0.959$). As shown in Fig. 4B, SP serum phosphorus concentration was significantly higher than in WP (between 14 and 19% higher), regardless of the experiment time. Compared with prepatrol control values, a similar increase in serum Pi content was demonstrated on patrol day 20 in both SP ($+15\%; P < 0.001$) and WP ($+18\%; P < 0.001$), reaching a plateau extending to the end of both patrols.

**DISCUSSION**

Seasonal influence on vitamin D status in submariner populations. In currently available research publications, it is widely acknowledged that UV-depending vitamin D production in the skin is higher in summer than in winter for people living above and beyond the 37th parallel within the northern and southern hemispheres, respectively (15, 23, 41). Even if there are slight discrepancies regarding the threshold level for vitamin D status from country to country, it has been suggested that a level of $25(OH)D > 30$ ng/ml ($>75$ nmol/l) should be reached. However, in the present study, the vitamin D status of both SP and WP groups was below the recommended level in winter, suggesting a seasonal hypovitaminosis D. This finding is consistent with previous reports showing that vitamin D status is lower in winter than in summer (15, 23, 41). The lack of significant difference between the two groups in summer could be attributed to the higher UV exposure during this season. In conclusion, the present study highlights the importance of vitamin D supplementation during winter months to maintain adequate vitamin D status in submariners.
maintained throughout the year to be healthy (17). A vitamin D insufficiency status is characterized by a 25(OH)D concentration < 30 ng/ml (<75 nmol/l) in human blood serum; furthermore, a vitamin D deficiency status is considered when the serum 25(OH)D level is < 20 ng/ml (<50 nmol/l) (18). At last, a severe vitamin D deficiency is defined by a 25(OH)D level < 15 ng/ml (<37.5 nmol/l) (21).

As expected, a marked difference in vitamin D status was evidenced between our two submariner populations, depending on the boarding season. Indeed, serum 25(OH)D levels were ~36 and 17 ng/ml for summer and winter boarding, respectively. This indicates that, even before the beginning of submarine patrols, winter crewmembers already suffered from vitamin D deficiency, whereas summer submariners exhibited a vitamin D sufficiency status. In summer, a rapid decrease in 25(OH)D was observed as early as the first 20 days of patrol (25 ng/ml), resulting in a vitamin D insufficiency. During the rest of SP, the reduction in 25(OH)D level in crewmembers’ serum endured to a lesser extent, however, thus reaching a concentration of 21 ng/ml on day 58. Such a finding implies, therefore, that, at the end of the SP, crewmember 25(OH)D levels were close to the vitamin D deficiency borderline, whereas, in winter, vitamin D deficiency is notably aggravated by the end of the SP or WP.

Similarly to 25(OH)D, however, to a lesser extent, a difference in 1,25(OH)2D was observed between SP and WP submariners, regardless of the experimental time (prepatrol control, patrol days 20, 41, and 58). Indeed, SP 1,25(OH)2D level consistently exceeded the WP level. For each patrol, a decrease in 1,25(OH)2D concentrations was evidenced at the end of the assignment (patrol day 58). Due to the hydroxylation of 25(OH)D into its 1,25(OH)2D active form by 1α-hydroxylase, the origin of low 1,25(OH)2D could be explained by substrate 25(OH)D deficiency. It is, nevertheless, noteworthy that all measured 1,25(OH)2D quantities rested within the range of physiological standard concentrations (20–50 pg/ml).

Our findings are congruent with earlier publications regarding vitamin D status following submarine patrols (4, 7, 11, 14, 25, 30). In all of these studies, a drop of serum 25(OH)D level was observed during patrols lasting from 30 to 90 days. Hence, the intensity of vitamin D decline seemed to be related to the length of the submarine assignment. Additionally, prepatrol vitamin D concentrations found in two distinct experiments were correlated with the boarding season, as demonstrated in our present study. Indeed, 25(OH)D levels were 13.7 (30) or 31.0 ng/ml (7) for winter or summer boarding, respectively. In terms of serum 1,25(OH)2D concentration, Dlugos and coauthors (7) observed that this parameter did not change after a 68-day submersion period, a finding that differs therefore from our data collected herein.

To conclude, we demonstrated a physiological difference in 25(OH)D status and a moderate variation of the biologically active vitamin D form between crewmembers involved either in SP or WP. Thus such parameter differences substantiate the valuable contribution from our experiment aimed at assessing seasonal influence on bone metabolism in long-term confined submariner populations.

**Ballistic missile submarine confinement induces a chronic respiratory acidosis.** Submariners are also subjected to prolonged exposure to elevated CO2 concentrations (ranging between 0.7 and 1.0%) compared with atmospheric air (0.03% CO2). It is common knowledge that breathing of CO2 excess induces hypercapnia and further deregulation of acid/base homeostasis (3, 8, 25, 35, 42). According to the Henderson-Hasselbalch equation, a close relationship exists between blood pH, PCO2, and HCO3 level. We, therefore, measured these parameters on board within the serum of our ballistic missile submarine crewmembers. By and large, our data revealed a noticeable increase in both PCO2 (about +7 Torr) and HCO3 (about +4 mmol/l) concentration associated with a slight blood pH acidification (~0.02 pH unit) during the first 41 days of submersion compared with prepatrol values, regardless of the boarding season. Such a pattern is commonly encountered during respiratory acidosis episodes (9). Indeed, in an atmosphere enriched in CO2, breathing usually leads to the accumulation of CO2 in serum (PCO2 level increase). Subsequently, this CO2 excess reacts with H2O molecules to release HCO3 (HCO3 level increase) and H+ (pH decrease). Protons generated in this way must be excreted via the kidneys. If insufficient H+ is eliminated, a respiratory acidosis results. Furthermore, according to Davenport’s diagram (graphic representation of the Henderson-Hasselbalch’s equation) generally used for the diagnosis of acid-base disorders, our submariners would experience mild chronic respiratory acidosis (CRA) episodes, independently from the patrol season. Additionally, a recent reevaluation of acid-base prediction rules has been performed in patients with stable CRA (26). In such patients suffering from chronic obstructive pulmonary disease (COPD), the deregulation of acid-base balance parameters (pH, PCO2, HCO3 values) roughly coincides with the results we obtained in our submarine crewmember populations during the first 41 days of submersion. Nevertheless, it is noteworthy that our crewmembers are healthy people exposed to specific confinement conditions involving abnormal CO2 levels, while COPD patients exhibit respiratory symptoms (chronic cough, wheezing, expectoration, and dyspnea during physical activities). Therefore, it would be inappropriate to classify the moderate acidosis exhibited by our submariners as CRA in the strict sense of the word. Hence, we prefer to use the CRA-“like” terminology.

Interestingly, a partial compensation of CRA-like episode was only evidenced at the end of the SP (day 58). This physiological attempt to compensate the acidosis is characterized by a stabilization of both HCO3 and PCO2 levels associated with a pH rise, leading to a recovery of prepatrol pH value. Contrariwise, this partial CRA compensation is not observed in the WP. The difference of CRA compensation between both patrols could stem from the discrepancy of vitamin D status, depending on the boarding season. Indeed, new insights on vitamin D and respiratory health further substantiated the hypothesis for a potential beneficial effect of vitamin D over the function of airway epithelial cells (19). Therefore, we may argue that elevated vitamin D status (above a threshold that remains to be determined) is likely to improve the expulsion of CO2 accumulated in blood, fostering, thereby, CRA compensation. The bone’s ability to buffer the phenomenon of blood acidification may further be involved in the partial CRA-like episode compensation, evidenced in our present experiment. The latter feature stands as the main issue for further discussion hereunder.

Few publications catered to acid-base balance regulation in the context of submarine confinement have been released as yet.
A mild respiratory acidosis resulting from a decrease in pH, combined with a significant increase in both PCO2 and HCO3− plasma levels, was identified in submarine patrols lasting either 35 (29) or 56 days (27). In addition, Messier and coworkers (27) noticed that the acidosis appeared to be compensated by the end of the patrol. Such findings are entirely congruent with our present data. However, in a report involving 13 Polaris submarine patrols, Schaefer (33) concluded that a cyclic acid-base balance modification was observed throughout the submersion period. The time course of plasma pH, HCO3−, and PCO2 showed alternations between a metabolic acidosis phase during the first 15–20 days, followed by a respiratory acidosis phase (20–40 days) and a subsequent metabolic acidosis phase during the period extending between 40 and 60 days. The only difference with our present data analysis involved the first 15–20 days of submarine patrols, since the second episode of metabolic acidosis described by Schaefer (patrol days 40–60) could also be interpreted as a respiratory acidosis compensation (Ref. 27 and our data). Due to the lack of details on conditions for blood sample collection and subsequent analysis in Schaefer’s report, the discrepancy between his data and our results may not lend themselves to further investigation. Nevertheless, the fact remains that such a discrepancy (Ref. 29 and our data) cannot be explained by the variation of ambient CO2 levels measured in submarines (ranging between 0.7 and 1.5%). Indeed, Schaefer (34) provided conclusive evidence regarding the existence of two different physiological responses, depending on the ambient CO2 level. Renal regulation (HCO3− reabsorption) is fully active during exposure to high CO2 concentrations (>3%), but becomes less effective during exposure to low CO2 quantity (<3%). Under the latter conditions, bone buffering with its slow time constant emerges as a salient factor. According to Schaefer’s theory, in all submarine experiments described above, bone buffering should be the predominant physiological response to counterbalanced blood acidosis.

The effects of season and hypercapnia conditions on bone metabolism. Since vitamin D deficiency and physiological response to compensating mild hypercapnia-related acidosis (bone buffering) could have valuable repercussions on bone integrity, we evaluated the impact of both seasonal and moderate hypercapnia (CO2 level < 3%) on bone metabolism markers in our submariner populations. To our knowledge, our study provides the first approach associating seasonal variation of vitamin D with bone remodeling parameters.

In terms of bone formation, we showed that submersion induced a decrease in BAP for both SP and WP, up to patrol day 41. This reduction in bone formation was sustained for WP until the end of the patrol (day 58), whereas a complete recovery of this parameter was observed for SP even exceeding prepark control value. Concerning bone resorption, measurements of serum ICTP marker indicated an increase in bone degradation significantly initiated on patrol day 20 for SP and later for WP (patrol day 41). Additionally, for both patrols at each experimental time, we used BAP/ICTP as an indicator of bone remodeling coupling. The resulting BAP/ICTP graph exhibited a thoroughly identical pattern with the pH graph for SP during prepark and all submersion period. For WP, patterns of BAP/ICTP and pH graphs were similar for prepark, only during the first 41 days of submersion, but not on patrol day 58. Therefore, we may legitimately hypothesize that bone metabolism balance is predominantly influenced by hypercapnia during the first 41 days of submersion, to exert a bone-buffering role to compensate for the CRA-like episode. At the end of submersion (patrol day 58), it is most likely that the vitamin D status related to the seasonal changes superimposed the hypercapnia influence to regulate bone metabolism. Indeed, we may infer, therefore, that the higher vitamin D status in SP fosters the swift recovery of normal coupling between bone formation and resorption after the CRA-like episode compensation process. Contrariwise, the very low vitamin D status in WP (close to a severe deficiency status) would prevent such bone remodeling coupling recovery.

To our knowledge, a single study recently focused on bone structural modification in the context of submarine confinement (25), as assessed by quantitative ultrasound (QUS) measurements of tibial SOS. QUS measurements of tibial SOS evolved for the diagnosis of osteoporosis are an accepted indirect method for bone strength determination in which QUS is significantly correlated to BMD (31). Luria and coauthors (25) evidenced a decrease in SOS following a 30-day submersion period, and submariners regained baseline SOS levels only 6 mo after disembarkation. Furthermore, determination of serum bone metabolism marker concentrations exhibited a significant reduction in both bone formation and resorption, indicating thereby a decreased bone turnover after 30 days of submersion (25). These results are consistent with our present data, except for bone resorption, which was transiently, yet markedly, highlighted by long-term submersion in either our WP or SP submariner populations.

Similar bone metabolism alterations have been observed in COPD patients, a population we previously paralleled with our submariner populations in terms of respiratory acidosis. Indeed, COPD patients had significantly lower total trabecular BMD and cortical BMD in the distal forearm bone compared with control subjects, using peripheral quantitative computerized tomography technique (6). Moreover, authors demonstrated that the serum bone resorption marker was heightened in association with a decrease in bone formation marker among COPD patients compared with control subjects. These bone metabolism results are consistent with our present data, emphasizing the potential close relationship of CRA episodes induced in either COPD patients or submariner population during patrols.

Influence of seasonal and hypercapnia on serum ionic homeostasis in submariners. During the CRA-like episode identified in our SP and WP submariners (patrol days 20 and 41), only a transient increase in SP serum Ca2+ levels was detected at day 20. On patrol day 58, a significant decrease was obtained in SP Ca2+ concentration compared with prepark control level, while the opposite situation was evidenced in WP (increase in serum Ca2+ compared with prepark control level). For serum Pi level, a rapid increase in this parameter was observed on patrol day 20 and was maintained until the end of the patrols. In related research publications, highly heterogeneous data were found with regards to the evolution of serum calcium and Pi concentrations during submarine patrols extending to ~60 days (7, 12, 25, 27, 33, 34). Three different time course evolutions have been highlighted. In the first profile, both serum calcium and Pi time course complied with the cyclic changes of metabolic and respiratory acidosis in ~20-day periods (33, 34). Metabolic acidosis was associated
with a reduction of both calcium and Pi concentrations, whereas respiratory acidosis was correlated with an increase in both ion concentrations. In the second profile, plasma calcium decreased, while phosphorus remained unmodified, during the submersion period (27). In the third profile, both calcium (12, 7, 25) and Pi (12, 7) levels rose during the submersion. This third profile is globally consistent with our present data, at least for WP patrol. We suggest that, during the submersion period, the increase in serum Pi could be due to the bone buffering process underscored above (heightened bone resorption would result in a release of Pi in blood).

The main significant variations in serum Ca\(^{2+}\) concentrations were observed on day 58 in both SP and WP, compared with their respective prepatrol control values. Ca\(^{2+}\) level decreased in SP, whereas this level increased in WP. We previously hypothesized that vitamin D status was to be held accountable for the presence (SP) or lack (WP) of an acidosis compensation at the end of patrols (day 58). Thus this different response to patrol in terms of serum Ca\(^{2+}\) may result from the vitamin D status. Since a fine regulation between vitamin D status and PTH has been demonstrated (32), PTH level could also be involved in the balance of mineral homeostasis in submariners (1). Few studies to date have focused on the PTH activity could be regulated by the submarine-induced CRA episode (22). These factors could also foster this impairment of normal bone remodeling coupling at the end of the patrol time. Therefore, a vitamin D supplementation could be provided to crewmembers assigned to WP so as to counteract the detrimental effects of hypovitaminosis D on bone metabolism. The issue of vitamin D intake over the health of submariners must not be neglected. However, the conditions set for intake remain to be determined in terms of vitamin D dose, supplementation duration, and onset of supplementation (i.e., before or during the submersion period). Duplessis et al. (10) have already assessed the efficacy of a daily 400 IU vitamin D supplementation in underway submariners. Unfortunately, the latter study revealed that this supplementation was insufficient to gain sufficient vitamin D status. This could be due to an inappropriate low dose of delivered vitamin D. According to Holick (14), the vitamin D requirement in submariners subjected to a 3-mo patrol is close to 600 IU per day. Indeed, submariners who took daily multivitamin pills containing 600 IU vitamin D exhibited a restoration of control 25(OH)\(D\) blood concentrations at the end of the patrol, despite a transient vitamin D insufficiency observed after 1.5 mo of submersion. Based on a extensive data analysis study, Vieth (40) estimated that vitamin D supplementation in submariners should be higher than 600 IU per day and suggested a daily dose of 2,000 IU.

Moreover, it may prove relevant to address the issue of the onset of vitamin D supplementation for our WP crewmembers. To answer this issue, it is necessary to take into account the fact that hypercapnia has been shown to foster calcification in tubules of the renal cortex of guinea pigs exposed to 1.5% CO\(_2\) over 91 days (36). A similar mechanism of kidney calcification could be suspected in the hypercapnic submariner populations (4). This is conceivable with epidemiological studies showing that 40–60 per annum United States Navy submariners develop nephrolithiasis, even if Dlugos and colleagues (7) suggested that the submarine environment produces physiological changes that decrease the risk for renal stone formation. Furthermore, vitamin D supplementation is likely to increase the potential risk for stone formation because of the effects of vitamin D on the intestinal absorption and renal excretion of calcium. Taking into account all of these facts, the ideal setup for vitamin D supplementation would be a compromise between a condition preventing winter-vitamin D deficiency and a condition avoiding a heightened blood vitamin D status, which could trigger nephrolithiasis when associated with submarine hypercapnia. Hence, we suggest that vitamin D supplementation could be provided to submariners before winter boarding rather than during submersion. Indeed, a prolonged supplementation during submersion is not recommended so as to avoid the association of elevated blood vitamin D status with hypercapnia, which could potentially generate renal stone formation in submariners.

As an alternative hypothesis, PTH and 1\(\alpha\)-hydroxylase activity could be regulated by the submarine-induced CRA episodes. An experiment performed in rats subjected to severe hypercapnia is arguing in favor of this mechanism (2). These results showed a decrease in 1,25(OH)\(_2\)D level without any change in plasma concentration of PTH. The authors suggested that a reduction in the 1\(\alpha\)-hydroxylase activity during CRA episodes might account for the significant reduction in 1,25(OH)\(_2\)D level. A similar mechanism could be present in our submariner populations.

Is a vitamin D supplementation necessary to ensure proper health conditions for submariners? We suggested above that vitamin D deficiency detected in WP submariners could prevent the recovery of normal bone remodeling coupling at the end of the patrol time. Therefore, a vitamin D supplementation could be provided to crewmembers assigned to WP so as to counteract the detrimental effects of hypovitaminosis D on bone metabolism. The issue of vitamin D intake over the health of submariners must not be neglected. However, the conditions set for intake remain to be determined in terms of vitamin D dose, supplementation duration, and onset of supplementation (i.e., before or during the submersion period). Duplessis et al. (10) have already assessed the efficacy of a daily 400 IU vitamin D supplementation in underway submariners. Unfortunately, the latter study revealed that this supplementation was insufficient to gain sufficient vitamin D status. This could be due to an inappropriate low dose of delivered vitamin D. According to Holick (14), the vitamin D requirement in submariners subjected to a 3-mo patrol is close to 600 IU per day. Indeed, submariners who took daily multivitamin pills containing 600 IU vitamin D exhibited a restoration of control 25(OH)D blood concentrations at the end of the patrol, despite a transient vitamin D insufficiency observed after 1.5 mo of submersion. Based on a extensive data analysis study, Vieth (40) estimated that vitamin D supplementation in submariners should be higher than 600 IU per day and suggested a daily dose of 2,000 IU.

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**Conclusion.** To conclude, valuable changes in vitamin D status were demonstrated in our French submarine populations, depending on the boarding season. For prepatrol control conditions, SP submariners presented an adequate vitamin D status, while WP crewmembers suffered from vitamin D deficiency. A detrimental effect of submersion was evidenced at the end of the patrols (day 58) since the SP submariner population was close to the vitamin D deficiency limit, and WP crewmembers exhibited a severe vitamin D deprivation.

A CRA-like episode was evidenced in both SP and WP crewmembers, up to 41 days after submersion. This CRA-like episode is mainly induced by a hypercapnia, resulting from the
enriched CO₂ level encountered in submarines. In the meantime, measurements of bone metabolism markers demonstrated a bone remodeling impairment (reduced bone formation associated with a heightened bone resorption), which could constitute a physiological attempt to compensate this acidosis, via a bone-buffering regulation. However, an unbalanced bone remodeling coupling could induce osteopenia in submariner populations. At a later experimental time (patrol day 58), a partial acidosis compensation was detected in SP crewmembers, but was completely absent in WP submariners. Such a discrepancy could result from the seasonal influence on vitamin D status. Therefore, we suggest that a vitamin D supplementation could be provided to submariners assigned to WP to avoid alteration of bone metabolism at the end of their assignment. Thus this vitamin D intake would be a valuable preventive treatment against potential risks toward chronic osteopenia. In addition, we recommend delivering vitamin D intake before boarding rather than during submersion to minimize the potential occurrence of renal stone formation induced by the synergistic effect of both vitamin D excess and hypercapnia.

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No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

REFERENCES