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# Bone metabolism, bone mass and structural integrity profile in professional male football players.

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***We thank the reviewers for their positive comments. We improved the manuscript according to the they suggestions. All the changes are underline with the yellow colour in the manuscript.***

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Commento 1

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General comment (originality, scientific accuracy, strengths and/or weaknesses): Originality: the aim is not new; good scientific accuracy. Strengths: attention to supplementation of athletes. Weaknesses: CUS in the evaluation of bone quality, and in the same time is useless in soccer players for at least two reasons: first they are very young (<24 yrs old) and second is well known that soccer is a protective factors for bone quality.

Major corrections (main criticisms): No major criticism

Minor corrections (page, paragraph, line where the author must make the corrections): Minor: please discuss the role of soccer in preventing bone quality and geometric properties.

***ANSWER**: Thanks to the reviewer for this positive comment. As suggested, we added the role of soccer in preventing bone quality and geometric properties (page 3, lines 14-20).*

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Commento 2

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General comment (originality, scientific accuracy, strengths and/or weaknesses): Overall this is a straightforward and well carried out pilot study of bone biochemical measures (i.e., serum parathyroid hormone, urinary calcium, serum Vitamin D etc.) in relation to joint, musculoskeletal injuries in 16 young adult male professional football players from October -May. Samples were obtained at the beginning (Oct) and end (May) of the season.

Major corrections (main criticisms): the researchers indicate the participants had less than 10 hours/week of sunlight. This is not verified nor are the methods to determine sunlight exposure given (i.e., self-report, direct observation). Please provide these details in the manuscript methodology section.

***ANSWER**: The sunlight exposure has been evaluated by using a self-report questionnaire (Hanwell HE, et al. J Steroid Biochem Mol Biol 2010;121:334-7). See new reference #37, page 5, line 8.*

One accepted measure of sunlight exposure is standard erythema doses (SEDs) and takes into account intensity and duration of exposure. Please provide the SEDs for the participants.

***ANSWER*: We agree with the referee that the standard erythema dose (SED)is a good and accepted methods for the sunlight exposure, however it has not been considered in this study.**

I also found it difficult to have confidence that the soccer players had less than 1 hour of exposure of sunlight per day from October-May. How was this determined and verified? Fuller methodological description is necessary here.

***ANSWER*: Valle d'Aosta is a mountain region sited in the Italian Alps. Weather is cold, rainy and snowy, especially in autumn and winter. The football players trained indoors and the estimated sun exposure for outdoors training is less than 10 hrs a week. We added this information also in the text (page 5, lines 10-12).**

The sample size is small (n=16) and non-parametric statistics are appropriate (i.e., Mann-Whitney U test).A short statement about why non-parametric tests were used (i.e, small sample, distribution free) would be useful for the reader as would a rationale for selecting the Mann-Whitney U test (as opposed to another independent sample ordinal test such as the Kolmogorov-Smirnov two sample test).

***ANSWER*: we better explain the rationale for selecting the Mann-Whitney U test (page 7, lines 11-13).**

Table 1: the standard deviations are quite large for a number of the biochemical parameters: serum alkaline phosphatase, serum parathyroid hormone, urinary calcium and urinary phosphorus. The researchers need to explain why there was so much variability in these measures. There is no indication of when (time) these measures were taken, which could contribute to the variability and lack of significant findings.

***ANSWER*: venous blood samples were obtained From 08:00 to 08:30 am after 12 hours fast. We added this information in the revised manuscript (page 6, line 8-10).**

Given the small sample size, the researchers should provide a power calculation to ensure the sample size was indeed large enough to see significant effects.

***<u>ANSWER</u>: The sample size was not provided because it is a pilot study performed on a small series of subjects who were all member of the soccer team of Valle d'Aosta.***

Minor corrections (page, paragraph, line where the author must make the corrections): There are some minor spelling and grammatical mistakes throughout the manuscript.

page 1, line 41. "Wit this pilot study...." should be "With this pilot study"

***<u>ANSWER</u>: We correct as suggested (page 2, line 19).***

page7, line 45 "confirming that vitamin D contributes with the correct function of musculoskeletal system." should be "...contributes to the correction function of...."

***<u>ANSWER</u>: We correct as suggested, also according to the next comment (page 9, lines 12).***

page 7, lines 41-45. This statement is too strong. A correlation is not causation. Please tone down.

***<u>ANSWER:</u> We reformulate this sentence as follow: "indicating that vitamin D might contribute to the correction function of musculoskeletal system" (page 9, lines 12).***



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Commento 3

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General comment (originality, scientific accuracy, strengths and/or weaknesses): The study has some interesting prospects and provides information which may be helpful to coaches and performance trainers.

Major corrections (main criticisms): There are no real major criticisms.

***<u>ANSWER</u>: We are grateful to the reviewer #3 for his positive comment.***

Minor corrections (page, paragraph, line where the author must make the corrections): Page Line Comment

2 41 "Wit" should be with".

***<u>ANSWER</u>: We correct as suggested (page 2, line 19).***

3 14-15 There doesn't seem to be much transition from the injury paragraph to the BMD paragraph. Plus, the 2nd paragraph doesn't seem to flow well.

***<u>ANSWER</u>: We decided to not separate the part of injury and BMD in two paragraphs, so we were***

***able to better connect these two (page 3, lines 7-8), and we described better the part of BMD (page 3, lines 14-20), adding also new references (#5, 11-17).***

3 33 Same problem; there doesn't seem to be a logic to transition to this paragraph. In addition, what is the relevance of list illnesses related to Vit D deficiency that is not relevant to your topic of BMD?

***ANSWER:* we reformulated the introduction of this paragraph related to vitamin D (page 3, lines 21-23).**

3 51 How does Vit D prevent falls {"decline in falls"]?

***ANSWER: we changed "decline" with "prevent". The role of vitamin D in preventing falls is explained at page 4, lines 1-4. We underlined that adding a sentence (page 4, line 3).***

4 2 This reference is in adolescent girls. Are there any studies relative to male athletes in various sports?

***ANSWER: we added Koundourakis NE et al, PLoS One 2014 (reference #30, page 4, line 4).***

4 17 "However, in the Italian population…."

***ANSWER: we changed it as suggested (page 4, line 11).***

4 26 Once more, the transition to the purpose does not seem to be there. Since there is only one study in Italian football players, how does that promote your study?

***ANSWER: as suggested, we better explain the conclusion and the bias of this Italian study (page 4, lines 13-17). In this way, the differences between our study and this previous study are better underlined.***

5 52 How long had they been fasting before the blood sample.

***ANSWER: a 12-hrs fasting has been observed before blood sample. We added this information in the revised manuscript. (page 6, line 9).***

6 32 How was the bone quality index (BQI) calculated?

***ANSWER: BQI is calculated directly by the device software and is calculated from the***

***quantitative ultrasound index (QUI). We rewrote the part on "Calcaneal quantitative ultrasound measurement and radiographic calcaneal examination" and better explained how BQI, as well as the other ultrasound parameters, were calculated (page 6, line 21-24 and page 7, lines 1-8).***

6 41 Should "Ligue" be "League". It appears that these to re used interchangeably throughout. It would be better to use League since that is the English version.

***ANSWER**: as suggested, we changed "ligue" with "League" in the entire manuscript.*

6 45 It might be better to rearrange this sentence rather than start it with a lower-case letter, p.

***ANSWER**: we rearranged it as "Values of p…" (page 7, lines 14-15).*

7 6 Should "pf" be "of"?

***ANSWER**: we corrected it (page 7, line 20)*

7 28 "There was a correlation …." Should also give the correlation and express its significance level?

7 32-35 Again, should also give the correlations and express their significance level?

7 39 What does it mean to say that the correlations did not "persist" until the end of League play? How much did they drop off; was it a significant decline?

***ANSWER**: we reformulated the last part of the results according to reviewer comments (page 8, lines 6-16). In the revised version of the manuscript, we added the "r" of Pearson correlation (if significant) and the R and p value from the linear regression. Moreover, we also added the fold change between the beginning and the end of the League for the more significant parameters (see also page 7, line 23 and page 8, lines 1-2). Except for vitamin D levels, we did not observe significant differences of the different parameters between the beginning and the end of the League. However, phosphaturia, which is the parameters that changed more at the end of the League, was also the parameter with more significant correlation. We supposed that, this changing in phosphaturia, even if is not significant, could be the reason why we did not observe the correlations found at the beginning of the League also at its end. We added this comment also in the discussion (page 10, lines 6-11).*

7 52 Should there be a comma after "women's football"?

***ANSWER**: we corrected it (page 8, line 20)*

8 13 Again, the transition to this paragraph from the one talking about accidents, mistakes, and injuries seems t be lacking to immediately change tact to talking about Vit D,

***ANSWER:** we improved this part underlying the association between the injuries and vitamin D (page 9, lines 1-2).*

8 15 "it" should be "it's".

***ANSWER**: we changed the entire sentence (page 9, lines 1-2).*

8 41 "foud" should be "found"

***ANSWER**: we corrected it (page 9, line 14).*

8 45 Should be "with" be "to"?

***ANSWER**: we changed the entire sentence (page 9, line 16).*

8 49 "showed" should be "shown".

***ANSWER**: we corrected it (page 9, line 18).*

9 6 Eliminate the "i" between "which" and "remains".

***ANSWER**: we corrected it (page 9, line 22).*

9 19 "Moreover, QUS allows…."

***ANSWER**: we corrected it (page 10, line 4).*

9 19 "…the bone strength independent of BMD"

***ANSWER**: we corrected it (page 10, line 4).*

9 24-28 Where the correlations mentioned here positive or negative?

***ANSWER**: we better explained the observed correlations (page 10, lines 6-11).*

9 34 Eliminate "belonging"

***<u>ANSWER</u>*: we deleted it in two point of the manuscript (page 4, line 19 and page 10, line 14).**

10 2 "….and for improving athletic performance."

***<u>ANSWER</u>*: we corrected it (page 10, line 23).**

14 12 Should height and weight be included in this table?

***<u>ANSWER</u>*: we added this information in Table 1.**

**Bone metabolism, bone mass and structural integrity profile in professional male football players.**

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**Short Title:** Bone metabolism and hypovitaminosis D in football players.

**Conflict of interest:** the authors declare that they have no conflicts of interest.

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## Abstract

**BACKGROUND:** Physical exercise plays an important role in bone mineralization as well as factors involved in bone metabolism influence the athletic performance. In European countries, soccer is the most popular sport. The aim of the study was to investigate bone metabolism, bone mass and structural integrity profile in professional male adult football players.

**METHODS:** Sixteen professional male football players from a single team of the 2nd division Italian League (mean age 22.4±0.7 years) were enrolled. Bone biochemical parameters, including serum calcium, phosphorus, albumin, creatinine, alkaline phosphatase, intact plasma PTH, 25-hydroxy-vitamin D (25-OHD), 24-h urinary calcium and phosphorus, and calcaneal quantitative ultrasound (QUS), were evaluated at the beginning (October 2012) and at the end of the League (May 2013).

**RESULTS:** 25-OHD levels were significantly lower at the end of the League compared to the beginning (27.1 ± 5.9 *vs* 36.6 ± 9.5 ng/ml, fold change (FC)=0.25, *p=0.008*), and the prevalence of 25-OHD deficiency increased from 25 to 73%. Moreover, higher rate of previous bone, cartilage or ligament injuries correlated with 25-OHD deficiencies (*p=0.014*). T-score and Z-score were at the upper limits of the normality ranges, without significant difference between the beginning and end of the League. Phosphaturia was slightly decreased at the end of the League [(691.0 ± 364.5 *vs* 934.0 ± 274.3 mg/24h, FC=0.26, *p=0.06*)]. A significant correlation was found between phosphaturia and BQI (R square=0.28, *p=0.03*), and both T-s and Z-s (R square=0.28, *p=0.03*) at the beginning of the League.

**CONCLUSIONS:** With this pilot study, we demonstrated that vitamin D status significantly worsened at the end of the League. Therefore, vitamin D supplementation might be suggested in adult football players in order to prevent vitamin D deficiency and improve the athletic performance.


**Key Words:** Bone turnover; Bone Mineral Density; vitamin D; football players.

**Introduction**

Soccer is the worldwide most popular sport, with about 200 million players, both professionals and amateurs [1]. Several risk factors for soccer injuries have been described [2], including 1) the level of play, the risk appears to be higher in professional than amateur players; 2) the exercise load; and 3) the standard of training [3]. The main types of injury are traumatic, such as fracture, contusion or hematoma, laceration, muscle or tendon strain and joint or ligament sprains and/or overuse injuries [4]. To the other side, sports with high impact or odd-impact loading, including soccer, are associated with higher bone mineral composition and bone mineral density (BMD) [5]. Many studies reported that the increased BMD in athletes was mainly due to the strain resulting from impact bone loading, rather than, and independently from, muscle strength increase [6]. However, most studies have been conducted in girls and only occasionally in men [7, 8]. Exercise seems to have beneficial effects on bone tissue by stimulating the osteogenic responses [9, 10]. Soccer is characterized by various types of running with rapid changes of direction, starts, stops, jumping, and kicking resulting in large ground reaction forces at the skeleton, and thus can be classified as an impact loading sport [6]. Several studies have demonstrated that soccer have a positive effects on bone mass and those effects are greatest during growth [5, 11, 12]. However, this effect on bone health could be explained not only to an improvement of BMD, but also to an improvement of bone geometry, evaluated by with peripheral quantitative computed tomography or hip structural analysis [13, 14]. Different studies demonstrated that young adult soccer players had better bone geometry, including cortical area, periosteal circumference and volumetric bone mineral density, than matched controls in both genders [15-17].

A crucial role in bone health and mineralization is played by Vitamin D, due its role in the regulation of calcium and phosphorus metabolism [18]. Vitamin D deficiency is a worldwide health problem [19-23] and it is associated with impaired neuromuscular function, bone loss and fractures [24-26]. More specifically, it has been shown that vitamin D levels correlate with grip and quadriceps strength, physical fitness, and prevent falls and bone fractures [27]. Particularly, vitamin D deficiency predominantly affects the weight-bearing antigravity muscles of the lower limbs, which are necessary for walking and postural balance [27, 28], explained its role in preventing falls. Furthermore, vitamin D supplementation seems to boost muscular strength and restores balance [28-30]. Previous studies, on different small sized football players' populations from different European Countries, have been published regarding possible relationship between seasonal variation of serum vitamin D levels and bone turnover markers (BTMs), body mass, body composition, total body water, fat-free mass, muscle mass, exercise performance such as squat jump, countermovement jump, 10 and 20 meters sprint performance, maximal oxygen consumption ($VO_2max$), anthropometry, lower limb isokinetic function, race, fracture history, and the ability to obtain a contract position [30-35]. However, in the Italian population, only a single study on twenty-three football players evaluated the possible correlation between seasonal vitamin D levels and dual x-ray absorptiometry (DXA)-measured bone mineral content (BMC) [36]. The authors found that both vitamin D levels and BMC significantly decreased from autumn to winter, indicating that supplementation of vitamin D might be necessary during the winter period [36]. However, the authors did not evaluate other parameters involved in bone metabolism, including phosphorus, albumin, creatinine, alkaline phosphatase, parathyroid hormone (PTH), as well as they did not evaluate the bone structural integrity profile.

The aim of our study was to investigate the correlation between bone metabolism markers with bone mass and structural integrity profile in a professional male adult football players from a single team. Therefore, we evaluated also whether the rate of previous bone, cartilage or ligament injuries could be correlate with vitamin D deficiencies.

## Patients and Methods

### *Patients*

Sixteen professional male football players of the S.C. Vallée d'Aoste, a 2nd division Italian club (mean age 22.4±0.7 years, range 19-28 years), were enrolled and evaluated at the beginning (October 2012) and at the end of the league (May 2013). The study protocol was approved by the local ethics committee institutional board and was carried out in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki as revised in 2000. Informed consent was obtained from all the subjects included in this study. All subjects were placed on a standardized diet with greater than 1000 mg elemental calcium per day starting 2 months before the beginning of the study. None of the participants had been taking vitamin D supplements or drugs known to interfere with bone or mineral metabolism for the last 12 months. The sun exposure, evaluated by using a self-report questionnaire [37], was less than 10 hours/week for all participants. This could be explained by the fact that Valle d'Aosta is a mountain region sited in the Italian Alps where weather is generally cold, rainy and snowy, especially in autumn and winter, and that football players trained mostly indoors. Exclusion criteria were acute or chronic conditions that affect mineral metabolism or complete immobilization.

### *Clinical characteristics assessment*

Height, weight, body mass index (BMI), systolic blood pressure (SBP) and diastolic blood pressure (DBP) were evaluated by standard methods. Exercise duration was also assessed. BMI was measured as the ratio between the weight and the square of the height. A BMI between 25 and 30 Kg/m$^2$ indicated a condition of over-weight, whereas BMI greater than 30 Kg/m$^2$ indicated a condition of obesity [38]. Waist circumference (WC) was measured to the nearest 0.1 cm at the end of a normal expiration by measuring from the narrowest point between the lower borders of the rib cage and the iliac crest. The measurement were performed with the patients in standing position with relaxed abdomen, arms at sides, and joined feet [39]. Blood pressure was measured in the right arm, with the subjects in a relaxed sitting position. The average of 6 measurements (3 taken by each of 2 examiners) with a mercury sphygmomanometer was used. Arterial blood hypertension was diagnosed when DBP values were ≥ 85 mmHg and SBP values were ≥ 130 mmHg in line with Adult Treatment Panel III [40].

***Biochemical assessment***

From 08:00 to 08:30 am, venous blood samples were obtained following a ten-minute period of rest in a lying position, after 12 hours-fasting, in order to determine the concentration of all biochemical parameters. Serum concentrations of creatinine, calcium, phosphorus, albumin, total alkaline phosphatase (ALP) were determined at fasting by automated techniques (Roche Modular System). Serum concentrations of intact parathyroid hormone (iPTH) were measured by electrochemiluminescence immunoassay concentration. Serum concentrations of 25-hydroxyvitamin D (25-OHD) were measured with direct radioimmunoassay. According to the Endocrine Society guidelines [41], a condition of vitamin D insufficiency was defined by a serum concentration of 25-OHD between 20-30 ng/ml, while a condition of vitamin D deficiency was defined by serum concentrations of 25-OHD below 20 ng/ml. 24-h urine samples were analyzed for urinary calcium and phosphorus measurements. Creatinine clearance was also determined.

***Calcaneal quantitative ultrasound measurement and radiographic calcaneal examination***

Quantitative ultrasound (QUS) of the calcaneus was performed using the Sahara clinical bone sonometer (Hologic INC, USA). The calcaneus of participants was placed between two ultrasound transducers that are positioned on opposite sides to each other. This device provides measures of the velocity and frequency attenuation of the sound wave propagation through bone by the "speed of sound" (SOS; m/s) and the "broadband ultrasound attenuation" (BUA; dB/MHz) of an ultrasound beam passed through the calcaneus between the two transducers, and the "quantitative ultrasound index" (QUI), which is a combination between SOS and BUA. These two parameters reflect different bone properties, including density, elasticity and microarchitecture [42]. Bone mineral density (BMD), T-score (T-s) and Z-score (Z-s) were derived from the manufacturer reference database and provided by the software device. The QUI is converted directly by Sahara software in "bone quality index" (BQI), an assessment of calcaneus BMD ($g/cm^2$). BQI is easy to interpret and indicates bone composition and structure. Its normal range is between 0 to 150.

**Statistical Analysis**

Numerical data are expressed as mean ± standard deviation (SD). Due the small sample size and the non-normally-distributed data, differences between the experimental periods in the measured parameters were analyzed by the non-parametric Mann-Whitney U test. A logistic regression analysis and a linear correlation analysis by calculating the Pearson's coefficient were performed. Values of $p$ <0.05 were considered statistically significant. Statistical analysis was performed with SPSS statistical software package (version 20.0, SPSS; Chicago, IL).



**Results**

Clinical, biochemical and BMD parameters both at the beginning and at the end of the League were summarized in Table 1. At the beginning of the League all evaluated parameter were within the normal range (Table 1).

Phosphaturia was slightly but not significantly decreased at the end compared to the beginning of the League [(691.0 ± 364.5 *vs* 934.0 ± 274.3 mg/24h, fold change (FC)=0.26, *p=0.06*)], while serum calcium, phosphorus and iPTH as well as calciuria were unchanged (Table 1). 25-OHD serum levels were significantly reduced at the end of the League in comparison to the beginning (27.1 ± 5.9 *vs* 36.6 ± 9.5 ng/ml, FC=0.25, $p=0.008$). In parallel, the prevalence of 25-OHD deficiency increased from 25 to 73% at the end of the League. The rate of previous bone, cartilage or ligament injuries correlated with the 25-OHD deficiencies ($p=0.014$) (Figure 1). Particularly, subjects with lower vitamin D levels had a history of higher number of injuries.

No significant differences between the beginning and the end of the League were observed for BQI (138.5 ± 17.7 *vs* 133.7 ± 15.3, FC=0.03, $p=0.45$), T-s and Z-s (in both cases, 1.7 ± 1.1 *vs* 1.6 ± 1.0, FC=0.05, $p=0.48$), which were at the upper limits of normal range. BQI significantly correlate with both T-s and Z-s (r=1, R square=0.99, $p<0.001$) at the two time-points of the study. Interestingly, a significant positive correlation was found between phosphaturia and bone parameters, including BQI (R square=0.28, $p=0.03$), and both T-s and Z-s (R square=0.28, $p=0.03$, for both) at the beginning of the League. Moreover, phosphaturia also significantly correlated with the duration of soccer activity (R square=0.68, $p=0.0005$). A trend of positive correlation was also demonstrated between both T-s and Z-s and the duration of soccer activity (R square=0.23, $p=0.09$) and calciuria (R square=0.24, $p=0.052$). However, these trends were observed only at the beginning of the League, whereas were not found after its end.



**Discussion and conclusions**

The greater frequency of football accidents in recent years across all sectors, both professional and amateur [43, 44], youth football [45] and women's football [46], has been the subject of several studies and publications. Many accidents are unforeseeable as they are the natural consequences of sport in which speed, strength and explosive action are accompanied by physical contact, tackling and collisions with an opponent. Many others, however, could be avoided because they stem from mistakes, underestimation of the risk factors, or insufficient consideration of the ways in which they can be prevented. One risk factor could be represented by vitamin D deficiency, which impairs bone loss, neuromuscular function and postural balance, increasing the risk of fractures [24-28]. Morton et al. [47] demonstrated a significant reduction in serum levels of 25-OHD in a group of professional soccer players of the English Premier League at the latitude of 53˚ N between the summer and the winter period (in August and December). Similarly, a study in Spanish professional soccer players who trained at the latitude of 37˚ N showed a statistically significant reduction in serum levels of 25-OHD between these two time periods (October and February)[31]. Moreover, several studies have shown a considerable correlation of vitamin D levels with muscle structure and strength [31, 48, 49]. Visser et al. [50] found that a 25-OHD level of 30 ng/ml guarantees optimal muscle function. Close et al [51] suggest that decreased vitamin D levels are one of the important causes of reduced exercise capabilities in athletes.

In accordance with previous studies [31, 47], we observed that 25-OHD serum levels were significantly reduced at the end of the League as compared to the beginning, as well as the prevalence of 25-OHD deficiency according to the Endocrine Society guidelines [41] increased from 25 to 73%. We also found that the rate of previous bone, cartilage or ligament injuries correlated with the 25-OHD deficiencies, indicating that vitamin D might contribute to the correction function of musculoskeletal system.

Conflicting results on the impact of physical activity on serum of PTH are reported in the literature. Particularly, it has been shown that in athletes pursuing performance disciplines, serum levels of PTH were lower than in physically inactive subjects [52]. In our study we did not observed any differences in PTH levels during the beginning and the end of the League. Only phosphaturia was slightly but not significantly decreased at these two time points.

Bone mass status was evaluated using the QUS method. Different from the DXA, which remains the gold standard technique for measuring the BMD, QUS is an attractive alternative method of bone assessment because it is a non-invasive technique, radiation-free, low-cost, simple, and is portable.

In recent years, QUS has been applied in the prediction of osteoporotic fractures, in monitoring therapies, and in the investigation of secondary osteoporosis [53]. Several studies showed the positive correlation between calcaneal QUS and BMD of the spine or the proximal femur assessed by DXA [52, 54]. Moreover, QUS allows us to measure the BQI that describes the bone strength independent of BMD [55]. In our cohort, we did not find any difference between BQI at the beginning and at the end of the study. As expected, BQI significantly correlated with both T-s and Z-s, confirming that BQI could be use as parameter to evaluate bone status. We observed that phosphaturia positively correlated with BQI, T-s, Z-s and duration of soccer activity. However, this correlation was found only at the beginning of the League. This could be explained by the fact that phosphaturia decreased at the end of the League (FC=0.26) and, even though this decreased was not significant, it appeared to be enough to change its correlations with other parameters at the end of the League.

The strength of the study is its prospective nature. Moreover, we will able to evaluate a complete profile of markers of bone metabolism and correlate them with bone mass and structural integrity profile in adult football players from a single team. The limit of the study is the small number of included subjects and our results should be confirmed in larger series of football players. However, this would represent a pilot study for future larger studies on such a category of athletes to adequately investigate complete bone metabolism and bone mass and structural integrity profiles by a non-invasive and reliable approach, in order to identify, together with other parameters, such as the ones recognized in the FRAX assessment [56], athletes at risk for bone fragility and, consequently, reduced physical performances.

Due to our findings, we concluded that vitamin D supplementation might be suggested in adult football players during the League, in order to prevent vitamin D deficiency with consequence mineralization defects and muscle weakness, and for improving the athletic performance.

**Figure Legend:**

**Figure 1. Correlation between 25-OHD serum levels and rate of bone, cartilage or ligament injures**.

We observed a negative correlation between vitamin D levels and numbers of bone, cartilage or ligament injures (*p=0.014*). Thus, subjects with lower vitamin D levels had a history of higher number of injuries.



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**Table 1. Clinical, biochemical and densitometric parameters in 16 professional male football players at the beginning and the end of league.**

| Parameters | Mean±SD | | Normal range | *p* |
|---|---|---|---|---|
| | Beginning | End | | |
| SBP (mmHg) | 111.8 ± 10.8 | 11.8 ± 10.8 | 100-130 | ns |
| DBP (mmHg) | 76.8 ± 9.5 | 75.0 ± 9.2 | 60-90 | ns |
| Hight (cm) | 176.2 ± 7.6 | - | - | - |
| Weight (kg) | 69.0 ± 8.2 | 69.2 ± 7.4 | - | ns |
| BMI (kg/m$^2$) | 22.1 ± 1.3 | 22.2 ± 1.1 | 19-24 | ns |
| WC (cm) | 81.9 ± 4.4 | 81.8 ± 4.4 | 75-90 | ns |
| Serum albumin (g/L) | 4.5 ± 0.5 | 4.3 ± 0.3 | 4-5 | ns |
| Serum creatinine (mg/dl) | 1.0 ± 0.1 | 1.0 ± 0.1 | 0.9-1.3 | ns |
| Creatinine clearance (ml/min) | 88.3 ± 11.8 | 88.1 ± 12.3 | 67-107 | ns |
| Serum 25-OHD (ng/ml) | 36.6 ± 9.5 | 27.1 ± 5.9 | 30-100 | *0.008* |
| Serum ALP (U/L) | 109.5 ± 20.3 | 111.1 ± 37.7 | 71-140 | ns |
| Serum iPTH (pg/ml) | 18.6 ± 5.5 | 21.4 ± 24.4 | 11-31 | ns |
| Serum calcium (mg/dl) | 9.9 ± 0.4 | 9.9 ± 0.5 | 9.2-10.5 | ns |
| Serum phosphorus (mg/dl) | 4.0 ± 0.5 | 4.0 ± 0.4 | 3.1-4.9 | ns |
| Urinary calcium (mg/24h) | 151.6 ± 59.4 | 168.5 ± 92.1 | 51-260 | ns |
| Urinary phosphorus (mg/24h) | 934.0 ± 274.3 | 691.0 ± 364.5 | 350-1575 | ns |
| Calcaneal ultrasonometer T-s | 1.7 ± 1.1 | 1.6 ± 1.0 | 0.2-3.4 | ns |
| Calcaneal ultrasonometer Z-s | 1.7 ± 1.1 | 1.6 ±1.0 | 0.2-3.4 | ns |
| BQI (right feet) | 138.5 ± 17.7 | 133.7 ± 15.3 | 0-150 | ns |

Abbreviation: 25-OHD: 25-hydroxyvitamin D; ALP: total alkaline phosphatase; BMI: body mass index; BQI: bone quality index; DBP: diastolic blood pressure; iPTH: parathyroid hormone; ns: *p* not statistically significant; SBP: systolic blood pressure; T-s: T-score of the calcaneus; WC: waist circumference; Z-s: Z-score of the calcaneus; -: not applicable.

**Bone metabolism, bone mass and structural integrity profile in professional male football players.**

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**Short Title:** Bone metabolism and hypovitaminosis D in football players.

**Conflict of interest:** the authors declare that they have no conflicts of interest.

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## Abstract

**BACKGROUND:** Physical exercise plays an important role in bone mineralization as well as factors involved in bone metabolism influence the athletic performance. In European countries, soccer is the most popular sport. The aim of the study was to investigate bone metabolism, bone mass and structural integrity profile in professional male adult football players.

**METHODS:** Sixteen professional male football players from a single team of the 2nd division Italian League (mean age 22.4±0.7 years) were enrolled. Bone biochemical parameters, including serum calcium, phosphorus, albumin, creatinine, alkaline phosphatase, intact plasma PTH, 25-hydroxy-vitamin D (25-OHD), 24-h urinary calcium and phosphorus, and calcaneal quantitative ultrasound (QUS), were evaluated at the beginning (October 2012) and at the end of the League (May 2013).

**RESULTS:** 25-OHD levels were significantly lower at the end of the League compared to the beginning (27.1 ± 5.9 *vs* 36.6 ± 9.5 ng/ml, fold change (FC)=0.25, $p=0.008$), and the prevalence of 25-OHD deficiency increased from 25 to 73%. Moreover, higher rate of previous bone, cartilage or ligament injuries correlated with 25-OHD deficiencies ($p=0.014$). T-score and Z-score were at the upper limits of the normality ranges, without significant difference between the beginning and end of the League. Phosphaturia was slightly decreased at the end of the League [(691.0 ± 364.5 *vs* 934.0 ± 274.3 mg/24h, FC=0.26, $p=0.06$)]. A significant correlation was found between phosphaturia and BQI (R square=0.28, $p=0.03$), and both T-s and Z-s (R square=0.28, $p=0.03$) at the beginning of the League.

**CONCLUSIONS:** With this pilot study, we demonstrated that vitamin D status significantly worsened at the end of the League. Therefore, vitamin D supplementation might be suggested in adult football players in order to prevent vitamin D deficiency and improve the athletic performance.

**Key Words:** Bone turnover; Bone Mineral Density; vitamin D; football players.

## Introduction

Soccer is the worldwide most popular sport, with about 200 million players, both professionals and amateurs [1]. Several risk factors for soccer injuries have been described [2], including 1) the level of play, the risk appears to be higher in professional than amateur players; 2) the exercise load; and 3) the standard of training [3]. The main types of injury are traumatic, such as fracture, contusion or hematoma, laceration, muscle or tendon strain and joint or ligament sprains and/or overuse injuries [4]. To the other side, sports with high impact or odd-impact loading, including soccer, are associated with higher bone mineral composition and bone mineral density (BMD) [5]. Many studies reported that the increased BMD in athletes was mainly due to the strain resulting from impact bone loading, rather than, and independently from, muscle strength increase [6]. However, most studies have been conducted in girls and only occasionally in men [7, 8]. Exercise seems to have beneficial effects on bone tissue by stimulating the osteogenic responses [9, 10]. Soccer is characterized by various types of running with rapid changes of direction, starts, stops, jumping, and kicking resulting in large ground reaction forces at the skeleton, and thus can be classified as an impact loading sport [6]. Several studies have demonstrated that soccer have a positive effects on bone mass and those effects are greatest during growth [5, 11, 12]. However, this effect on bone health could be explained not only to an improvement of BMD, but also to an improvement of bone geometry, evaluated by with peripheral quantitative computed tomography or hip structural analysis [13, 14]. Different studies demonstrated that young adult soccer players had better bone geometry, including cortical area, periosteal circumference and volumetric bone mineral density, than matched controls in both genders [15-17].

A crucial role in bone health and mineralization is played by Vitamin D, due its role in the regulation of calcium and phosphorus metabolism [18]. Vitamin D deficiency is a worldwide health problem [19-23] and it is associated with impaired neuromuscular function, bone loss and fractures [24-26]. More specifically, it has been shown that vitamin D levels correlate with grip and quadriceps strength, physical fitness, and prevent falls and bone fractures [27]. Particularly, vitamin D deficiency predominantly affects the weight-bearing antigravity muscles of the lower limbs, which are necessary for walking and postural balance [27, 28], explained its role in preventing falls. Furthermore, vitamin D supplementation seems to boost muscular strength and restores balance [28-30]. Previous studies, on different small sized football players' populations from different European Countries, have been published regarding possible relationship between seasonal variation of serum vitamin D levels and bone turnover markers (BTMs), body mass, body composition, total body water, fat-free mass, muscle mass, exercise performance such as squat jump, countermovement jump, 10 and 20 meters sprint performance, maximal oxygen consumption ($VO_2max$), anthropometry, lower limb isokinetic function, race, fracture history, and the ability to obtain a contract position [30-35]. However, in the Italian population, only a single study on twenty-three football players evaluated the possible correlation between seasonal vitamin D levels and dual x-ray absorptiometry (DXA)-measured bone mineral content (BMC) [36]. The authors found that both vitamin D levels and BMC significantly decreased from autumn to winter, indicating that supplementation of vitamin D might be necessary during the winter period [36]. However, the authors did not evaluate other parameters involved in bone metabolism, including phosphorus, albumin, creatinine, alkaline phosphatase, parathyroid hormone (PTH), as well as they did not evaluate the bone structural integrity profile.

The aim of our study was to investigate the correlation between bone metabolism markers with bone mass and structural integrity profile in a professional male adult football players from a single team. Therefore, we evaluated also whether the rate of previous bone, cartilage or ligament injuries could be correlate with vitamin D deficiencies.

## Patients and Methods

### *Patients*

Sixteen professional male football players of the S.C. Vallée d'Aoste, a 2nd division Italian club (mean age 22.4±0.7 years, range 19-28 years), were enrolled and evaluated at the beginning (October 2012) and at the end of the league (May 2013). The study protocol was approved by the local ethics committee institutional board and was carried out in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki as revised in 2000. Informed consent was obtained from all the subjects included in this study. All subjects were placed on a standardized diet with greater than 1000 mg elemental calcium per day starting 2 months before the beginning of the study. None of the participants had been taking vitamin D supplements or drugs known to interfere with bone or mineral metabolism for the last 12 months. The sun exposure, evaluated by using a self-report questionnaire [37], was less than 10 hours/week for all participants. This could be explained by the fact that Valle d'Aosta is a mountain region sited in the Italian Alps where weather is generally cold, rainy and snowy, especially in autumn and winter, and that football players trained mostly indoors. Exclusion criteria were acute or chronic conditions that affect mineral metabolism or complete immobilization.

### *Clinical characteristics assessment*

Height, weight, body mass index (BMI), systolic blood pressure (SBP) and diastolic blood pressure (DBP) were evaluated by standard methods. Exercise duration was also assessed. BMI was measured as the ratio between the weight and the square of the height. A BMI between 25 and 30 $Kg/m^2$ indicated a condition of over-weight, whereas BMI greater than 30 $Kg/m^2$ indicated a condition of obesity [38]. Waist circumference (WC) was measured to the nearest 0.1 cm at the end of a normal expiration by measuring from the narrowest point between the lower borders of the rib cage and the iliac crest. The measurement were performed with the patients in standing position with relaxed abdomen, arms at sides, and joined feet [39]. Blood pressure was measured in the right arm, with the subjects in a relaxed sitting position. The average of 6 measurements (3 taken by each of 2 examiners) with a mercury sphygmomanometer was used. Arterial blood hypertension was diagnosed when DBP values were ≥ 85 mmHg and SBP values were ≥ 130 mmHg in line with Adult Treatment Panel III [40].

***Biochemical assessment***

From 08:00 to 08:30 am, venous blood samples were obtained following a ten-minute period of rest in a lying position, after 12 hours-fasting, in order to determine the concentration of all biochemical parameters. Serum concentrations of creatinine, calcium, phosphorus, albumin, total alkaline phosphatase (ALP) were determined at fasting by automated techniques (Roche Modular System). Serum concentrations of intact parathyroid hormone (iPTH) were measured by electrochemiluminescence immunoassay concentration. Serum concentrations of 25-hydroxyvitamin D (25-OHD) were measured with direct radioimmunoassay. According to the Endocrine Society guidelines [41], a condition of vitamin D insufficiency was defined by a serum concentration of 25-OHD between 20-30 ng/ml, while a condition of vitamin D deficiency was defined by serum concentrations of 25-OHD below 20 ng/ml. 24-h urine samples were analyzed for urinary calcium and phosphorus measurements. Creatinine clearance was also determined.

***Calcaneal quantitative ultrasound measurement and radiographic calcaneal examination***

Quantitative ultrasound (QUS) of the calcaneus was performed using the Sahara clinical bone sonometer (Hologic INC, USA). The calcaneus of participants was placed between two ultrasound transducers that are positioned on opposite sides to each other. This device provides measures of the velocity and frequency attenuation of the sound wave propagation through bone by the "speed of sound" (SOS; m/s) and the "broadband ultrasound attenuation" (BUA; dB/MHz) of an ultrasound beam passed through the calcaneus between the two transducers, and the "quantitative ultrasound index" (QUI), which is a combination between SOS and BUA. These two parameters reflect different bone properties, including density, elasticity and microarchitecture [42]. Bone mineral density (BMD), T-score (T-s) and Z-score (Z-s) were derived from the manufacturer reference database and provided by the software device. The QUI is converted directly by Sahara software in "bone quality index" (BQI), an assessment of calcaneus BMD (g/cm$^2$). BQI is easy to interpret and indicates bone composition and structure. Its normal range is between 0 to 150.



**Statistical Analysis**

Numerical data are expressed as mean ± standard deviation (SD). Due the small sample size and the non-normally-distributed data, differences between the experimental periods in the measured parameters were analyzed by the non-parametric Mann-Whitney U test. A logistic regression analysis and a linear correlation analysis by calculating the Pearson's coefficient were performed. Values of $p$ <0.05 were considered statistically significant. Statistical analysis was performed with SPSS statistical software package (version 20.0, SPSS; Chicago, IL).

**Results**

Clinical, biochemical and BMD parameters both at the beginning and at the end of the League were summarized in Table 1. At the beginning of the League all evaluated parameter were within the normal range (Table 1).

Phosphaturia was slightly but not significantly decreased at the end compared to the beginning of the League [(691.0 ± 364.5 *vs* 934.0 ± 274.3 mg/24h, fold change (FC)=0.26, *p=0.06*)], while serum calcium, phosphorus and iPTH as well as calciuria were unchanged (Table 1). 25-OHD serum levels were significantly reduced at the end of the League in comparison to the beginning (27.1 ± 5.9 *vs* 36.6 ± 9.5 ng/ml, FC=0.25, *p=0.008*). In parallel, the prevalence of 25-OHD deficiency increased from 25 to 73% at the end of the League. The rate of previous bone, cartilage or ligament injuries correlated with the 25-OHD deficiencies (*p=0.014*) (Figure 1). Particularly, subjects with lower vitamin D levels had a history of higher number of injuries.

No significant differences between the beginning and the end of the League were observed for BQI (138.5 ± 17.7 *vs* 133.7 ± 15.3, FC=0.03, *p=0.45*), T-s and Z-s (in both cases, 1.7 ± 1.1 *vs* 1.6 ± 1.0, FC=0.05, *p=0.48*), which were at the upper limits of normal range. BQI significantly correlate with both T-s and Z-s (r=1, R square=0.99, *p<0.001*) at the two time-points of the study. Interestingly, a significant positive correlation was found between phosphaturia and bone parameters, including BQI (R square=0.28, *p=0.03*), and both T-s and Z-s (R square=0.28, *p=0.03*, for both) at the beginning of the League. Moreover, phosphaturia also significantly correlated with the duration of soccer activity (R square=0.68, *p=0.0005*). A trend of positive correlation was also demonstrated between both T-s and Z-s and the duration of soccer activity (R square=0.23, *p=0.09*) and calciuria (R square=0.24, *p=0.052*). However, these trends were observed only at the beginning of the League, whereas were not found after its end.



**Discussion and conclusions**

The greater frequency of football accidents in recent years across all sectors, both professional and amateur [43, 44], youth football [45] and women's football [46], has been the subject of several studies and publications. Many accidents are unforeseeable as they are the natural consequences of sport in which speed, strength and explosive action are accompanied by physical contact, tackling and collisions with an opponent. Many others, however, could be avoided because they stem from mistakes, underestimation of the risk factors, or insufficient consideration of the ways in which they can be prevented. One risk factor could be represented by vitamin D deficiency, which impairs bone loss, neuromuscular function and postural balance, increasing the risk of fractures [24-28]. Morton et al. [47] demonstrated a significant reduction in serum levels of 25-OHD in a group of professional soccer players of the English Premier League at the latitude of 53˚ N between the summer and the winter period (in August and December). Similarly, a study in Spanish professional soccer players who trained at the latitude of 37˚ N showed a statistically significant reduction in serum levels of 25-OHD between these two time periods (October and February)[31]. Moreover, several studies have shown a considerable correlation of vitamin D levels with muscle structure and strength [31, 48, 49]. Visser et al. [50] found that a 25-OHD level of 30 ng/ml guarantees optimal muscle function. Close et al [51] suggest that decreased vitamin D levels are one of the important causes of reduced exercise capabilities in athletes.

In accordance with previous studies [31, 47], we observed that 25-OHD serum levels were significantly reduced at the end of the League as compared to the beginning, as well as the prevalence of 25-OHD deficiency according to the Endocrine Society guidelines [41] increased from 25 to 73%. We also found that the rate of previous bone, cartilage or ligament injuries correlated with the 25-OHD deficiencies, indicating that vitamin D might contribute to the correction function of musculoskeletal system.

Conflicting results on the impact of physical activity on serum of PTH are reported in the literature. Particularly, it has been shown that in athletes pursuing performance disciplines, serum levels of PTH were lower than in physically inactive subjects [52]. In our study we did not observed any differences in PTH levels during the beginning and the end of the League. Only phosphaturia was slightly but not significantly decreased at these two time points.

Bone mass status was evaluated using the QUS method. Different from the DXA, which remains the gold standard technique for measuring the BMD, QUS is an attractive alternative method of bone assessment because it is a non-invasive technique, radiation-free, low-cost, simple, and is portable.

In recent years, QUS has been applied in the prediction of osteoporotic fractures, in monitoring therapies, and in the investigation of secondary osteoporosis [53]. Several studies showed the positive correlation between calcaneal QUS and BMD of the spine or the proximal femur assessed by DXA [52, 54]. Moreover, QUS allows us to measure the BQI that describes the bone strength independent of BMD [55]. In our cohort, we did not find any difference between BQI at the beginning and at the end of the study. As expected, BQI significantly correlated with both T-s and Z-s, confirming that BQI could be use as parameter to evaluate bone status. We observed that phosphaturia positively correlated with BQI, T-s, Z-s and duration of soccer activity. However, this correlation was found only at the beginning of the League. This could be explained by the fact that phosphaturia decreased at the end of the League (FC=0.26) and, even though this decreased was not significant, it appeared to be enough to change its correlations with other parameters at the end of the League.

The strength of the study is its prospective nature. Moreover, we will able to evaluate a complete profile of markers of bone metabolism and correlate them with bone mass and structural integrity profile in adult football players from a single team. The limit of the study is the small number of included subjects and our results should be confirmed in larger series of football players. However, this would represent a pilot study for future larger studies on such a category of athletes to adequately investigate complete bone metabolism and bone mass and structural integrity profiles by a non-invasive and reliable approach, in order to identify, together with other parameters, such as the ones recognized in the FRAX assessment [56], athletes at risk for bone fragility and, consequently, reduced physical performances.

Due to our findings, we concluded that vitamin D supplementation might be suggested in adult football players during the League, in order to prevent vitamin D deficiency with consequence mineralization defects and muscle weakness, and for improving the athletic performance.

**Figure Legend:**

**Figure 1. Correlation between 25-OHD serum levels and rate of bone, cartilage or ligament injures**.

We observed a negative correlation between vitamin D levels and numbers of bone, cartilage or ligament injures (*p=0.014*). Thus, subjects with lower vitamin D levels had a history of higher number of injuries.



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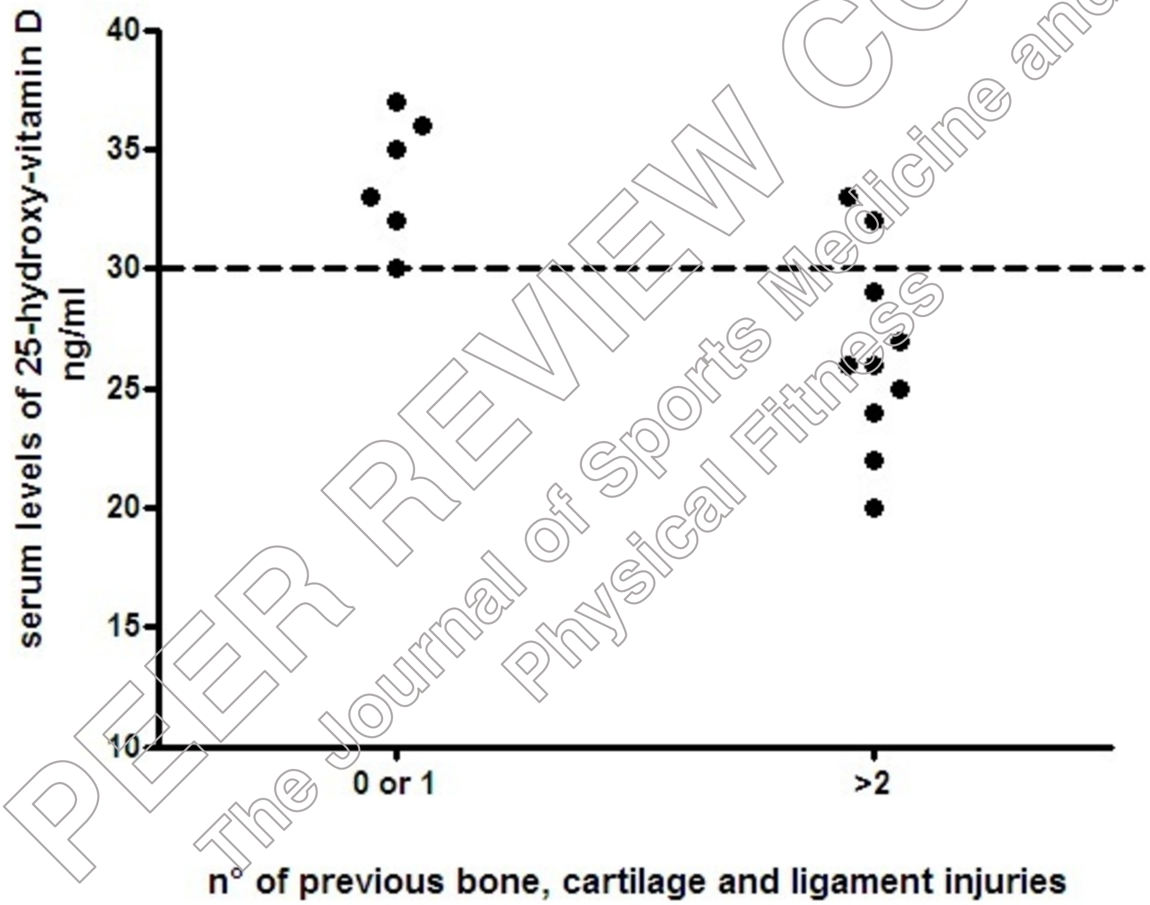
**Table 1. Clinical, biochemical and densitometric parameters in 16 professional male football players at the beginning and the end of league**.

| Parameters | Mean±SD | | Normal range | *p* |
|---|---|---|---|---|
| | Beginning | End | | |
| SBP (mmHg) | 111.8 ± 10.8 | 11.8 ± 10.8 | 100-130 | ns |
| DBP (mmHg) | 76.8 ± 9.5 | 75.0 ± 9.2 | 60-90 | ns |
| Hight (cm) | 176.2 ± 7.6 | - | - | - |
| Weight (kg) | 69.0 ± 8.2 | 69.2 ± 7.4 | - | ns |
| BMI (kg/m$^2$) | 22.1 ± 1.3 | 22.2 ± 1.1 | 19-24 | ns |
| WC (cm) | 81.9 ± 4.4 | 81.8 ± 4.4 | 75-90 | ns |
| Serum albumin (g/L) | 4.5 ± 0.5 | 4.3 ± 0.3 | 4-5 | ns |
| Serum creatinine (mg/dl) | 1.0 ± 0.1 | 1.0 ± 0.1 | 0.9-1.3 | ns |
| Creatinine clearance (ml/min) | 88.3 ± 11.8 | 88.1 ± 12.3 | 67-107 | ns |
| Serum 25-OHD (ng/ml) | 36.6 ± 9.5 | 27.1 ± 5.9 | 30-100 | *0.008* |
| Serum ALP (U/L) | 109.5 ± 20.3 | 111.1 ± 37.7 | 71-140 | ns |
| Serum iPTH (pg/ml) | 18.6 ± 5.5 | 21.4 ± 24.4 | 11-31 | ns |
| Serum calcium (mg/dl) | 9.9 ± 0.4 | 9.9 ± 0.5 | 9.2-10.5 | ns |
| Serum phosphorus (mg/dl) | 4.0 ± 0.5 | 4.0 ± 0.4 | 3.1-4.9 | ns |
| Urinary calcium (mg/24h) | 151.6 ± 59.4 | 168.5 ± 92.1 | 51-260 | ns |
| Urinary phosphorus (mg/24h) | 934.0 ± 274.3 | 691.0 ± 364.5 | 350-1575 | ns |
| Calcaneal ultrasonometer T-s | 1.7 ± 1.1 | 1.6 ± 1.0 | 0.2-3.4 | ns |
| Calcaneal ultrasonometer Z-s | 1.7 ± 1.1 | 1.6 ±1.0 | 0.2-3.4 | ns |
| BQI (right feet) | 138.5 ± 17.7 | 133.7 ± 15.3 | 0-150 | ns |

Abbreviation: 25-OHD: 25-hydroxyvitamin D; ALP: total alkaline phosphatase; BMI: body mass index; BQI: bone quality index; DBP: diastolic blood pressure; iPTH: parathyroid hormone; ns: *p* not statistically significant; SBP: systolic blood pressure; T-s: T-score of the calcaneus; WC: waist circumference; Z-s: Z-score of the calcaneus; -: not applicable.


serum levels of 25-hydroxy-vitamin D ng/ml
40
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n° of previous bone, cartilage and ligament injuries