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Our cover: Transfection of human osteoblasts with the miR-DIAN microRNA Mimic with Dy547 at 100 nM. The nuclei are DAPI stained. 20x magnification with the Leica DM IL LED inverted microscope.


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Vitamin D and muscle function

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In 1922, at Johns Hopkins University in Baltimore, Professor McCollum discovered a factor, which has since been referred to as vitamin D, following the alphabetical order of the other vitamins identified up to that time. It is capable of curing rickets in children and osteomalacia in adults. Diseases in which, as we know from the first scientific descriptions published in London in the mid-seventeenth century, muscle involvement consisting of weakness and generalized hypotonia is associated with bone involvement, its main characteristic. Therefore, since the discovery of vitamin D, it has been associated not only with bone health but also with muscle health.

Paradoxically, at present, there is no consensus on the potential beneficial effects of vitamin D supplementation on muscle function, balance and risk of falls, a situation highlighted in the last meta-analysis published by Bolland et al. who review in 81 randomized clinical trials (RCTs) that include 53,537 participants the effect of vitamin D on fractures and falls as a primary outcome. The pooled analyses showed that vitamin D supplementation had no effect on falls (37 RCTs, n=34,144, RR=0.97, 95% confidence interval 0.93 to 1.02), what the authors concluded that "vitamin D supplementation does not exert significant effects in falls", affirming that "potential future trials will probably not alter those conclusions, and that, therefore, there is little justification for the use of vitamin D supplements, to maintain or improve musculoskeletal health, indicating that clinical guidelines should reflect these findings". From this publication, many physicians and patients could mistakenly conclude that they can stop prescribing or taking vitamin D supplements, which is a potentially dangerous message, given the high prevalence of vitamin D deficiency in Spain.

Loss of muscle strength and/or function, severe invalidating myopathy predominantly proximal with diffuse muscular or skeletal pain in adults, generalized muscle atrophy and electromyographic abnormalities, such as polyphasic motor unit, potentials with shortened duration and decreased range, involvement of Type II muscle fiber atrophy (of rapid contraction) and marked fatty infiltration are findings in severe and sustained vitamin D deficiency, in severe renal insufficiency, or in the congenital absence of the CYP27B1 gene due to inability to adequately synthesize 1,25 dihydroxyvitamin D (1,25 DHC), hormonally active metabolite of the endocrine system of vitamin D, with rapid improvement of muscle function after vitamin D or 1,25 DHC supplementation in these patients. More subtle changes in muscle function can be observed in subjects with less severe and perhaps less chronic vitamin D deficiency.

In our current issue, Gómez Alonso et al., present an article in which they observe that in patients of both sexes of the cohort EVOS (European Study of Vertebral Osteoporosis) that maintain serum levels of calcium higher than 20 ng/mL present greater grip strength in the hands, maintenance of daily activities and lower losses of bone mineral density in the hip, measured by densitometry in the proximal extremity of the femur, proven beneficial effects that constitute the novelty of this study. The mechanisms of action of vitamin D in muscle biology and the impact of its deficiency show that a possible link between muscle and vitamin D is plausible.

In fact, observational studies show a correlation between poor vitamin D status and frailty, muscle weakness or fatigue and falls. While a meta-analysis of 15 intervention studies performed on a total of 2,866 participants did not reveal a significant improvement in hand grip strength or walking tests, other meta-analyses showed a discrete beneficial effect on muscle strength and the balance, or only showed benefits in people with the levels of 25 hydroxyvitamin D (25OHD) lower (<10 ng/mL). Beaudart et al. they also found no effect on muscle mass, but observed a small positive effect on muscle strength in patients older than 65 years with vitamin D deficiency (<12 ng/mL). These data are supported by studies in patients with severe vitamin D deficiency in which the administration of vitamin D improves the symptoms of fatigue, muscle weakness, and energy recovery after physical exercise demonstrated in vivo by resonance spectrometry techniques. Nuclear magnetic. Some intervention studies show that administration of 800-1,000 IU of vitamin D3 per day, or slightly more than its weekly equivalent, improves strength and balance in the elderly with vitamin D deficiency.

In addition to function, we have multiple observational studies that relate vitamin D deficiency with frailty and the incidence of falls. Thus, an analysis of 18 studies revealed an odds ratio (OR) of falls significantly greater than 1.23-1.44 for subjects with 25OHD concentrations below 10-20 ng/mL.
Muscle strength (especially proximal) can be modestly improved with vitamin D supplementation in the elderly with serum levels of 25OHD <12 ng/mL. According to this concept, supplementation for 9 months with 1,000 IU of vitamin D3 daily significantly decreased the first falls and the total of them in more than 50% of patients.

Several trials have examined the effect of vitamin D supplementation on fall rates. A meta-analysis of 9 RCTs showed that daily supplementation of less than 600 IU of vitamin D was not effective, while administration of between 700 and 1,000 IU significantly decreased the risk of falls.

In a Cochrane review, vitamin D supplementation reportedly reduced the risk of falls in institutionalized patients (RR=0.63, 95% CI: 0.46-0.85). In outpatients, supplementation with vitamin D did not reduce the risk of falls in a meta-analysis of all RCTs combined (RR=0.57, 95% CI 0.37-0.89), but it reduced the risk of falls in four studies that selected patients with lower levels of vitamin D (all four studies had cutoffs of <12, <20, <24 and <31 ng/mL, risk index=0.70, 95% CI: 0.56 to 0.87). The 30% reduction in the risk of falls in these studies (risk index=0.70, 95% CI 0.56 to 0.87) was significantly lower than in the other 9 studies evaluated in the meta-analysis that did not select participants according to vitamin D status (risk index=1.00, 95% CI 0.93-1.07, interaction p<0.01).

Supporting these data, a more recent meta-analysis of RCTs found that supplementation with vitamin D reduced the rate of fall only in subjects with an initial serum concentration of 25OHD below 20 ng/mL.

Megadoses employ intermittent supplementation regimens with long and variable dosing intervals of 100,000 IU of colecalciferol orally, every four months, or a month intramuscularly once a year, or 500,000 IU per year, of which its absence of effects or, even, the negative effects are known, increasing the risk of fractures and falls. They are, therefore, not recommended in guidelines or in usual practice because they are associated with oscillations in serum 25OHD concentrations (which means that serum concentrations do not remain above the normal threshold throughout the treatment period), and they have become obsolete and ineffective or harmful. Therefore, these designs with this posology should not be included in the meta-analyses and, however and surprisingly, have a weight of 50% of the meta-analysis proposed by Bolland et al.

In a study of elderly women with baseline vitamin D deficiency, falls occurred in 48% of the group treated with 24,000 IU of vitamin D3, in 67% of the group treated with 60,000 IU of vitamin D3, and in 66% of the group treated with vitamin D3 group that received 24,000 IU of vitamin D3 or more than 300 μg of calcifediol; the authors concluded from a post hoc analysis that 25OHD concentrations greater than 45 ng/mL may be associated with an increased risk of falls. Along the same lines, Smith et al. in a study conducted in women with vitamin D deficiency (<15 ng/dL) treated with a full range of daily doses of vitamin D3 (400-4,800 IU) vs. placebo for 1 year, they found a U-shaped association in falls, whose nadir occurred in the dose range of 1,600 to 3,200 IU per day; a greater number of falls were observed in the patients who received the highest doses of vitamin D.

Thus, in our usual practice we must be clear that the available evidence consistently indicates that vitamin D has important physiological effects on skeletal and cardiac muscle, that these effects are observed consistently when patients included in the studies have 25OHD levels with cut-off points at least below 30 ng/mL. That the administration between 800 and 1,000 IU daily of vitamin D3 are recommended, except in obese patients or in treatments that increase the catabolism of vitamin D, to obtain the proposed benefits; that higher doses may be harmful and that massive doses that become ineffective or harmful should not be administered, increasing the risk of falls and, potentially, the rate of fractures. So, maintaining adequate levels of 25OHD in patients should be a constant public health aim.

To obtain results, treatment must be maintained long-term both individually and in the design of clinical trials. Administration to patients with normal 25OHD serum levels will not help the patient, will not improve muscle health, nor will it prevent falls and, probably, not achieve other health objectives.

**Conflict of interests:** The authors declare no conflict of interest.
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Calcidiol levels and muscle function maintenance, functional capacity and bone mineral bone density in non-selected Spanish population

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Summary
Introduction: Vitamin D offers beneficial effects that reportedly help maintain musculoskeletal function.
Aim: To analyze the effect of calcidiol levels on muscle function in both hands, on activities of daily life and on changes in bone mineral density (BMD) in an unselected population.
Material and methods: The EVOS study cohort was used, which carried out, among others, measures of muscular strength of grip in both hands, questions related to difficulty in performing daily activities, densitometric study in the lumbar and hip spine, and biochemistry to determine the levels of calcidiol.
Results: Calcidiol values ≥20 ng/mL were associated with greater grip strength in both hands. After adjusting for age, sex, BMI and seasonality, calcidiol levels <20 ng/mL were independently associated with lower grip strength only in the left hand (OR=2.35; 95% CI: 1.03‐5.38). Likewise, the inability or difficulty to “pick up a book or object from a high shelf” and “get up from the bed” were significantly associated with calcidiol levels <20 ng/mL. Levels of calcidiol <20 ng/mL were associated with greater BMD losses in the femoral neck and total hip. These associations were maintained in the multivariate analysis.
Conclusions: Maintaining levels of calcidiol ≥20 ng/mL was associated with greater muscular strength of grip in the hands, maintenance of daily activities and lower BMD losses in the hip. This study corroborates the utility of maintaining adequate levels of vitamin D to maintain musculoskeletal function.
Key words: calcidiol, muscle strength, functional capacity, bone mineral density.

INTRODUCTION
The aging process is associated with a loss of muscle mass and strength, as well as a decrease in bone mineral density (BMD), which can lead to reduced mobility, greater risk of falls and the appearance of fractures2,4. In recent years, special emphasis has been placed on maintaining an adequate vitamin D status to optimize muscle strength and BMD in order to reduce falls and fractures1,5. Although a recent meta-analysis questions the usefulness of vitamin D supplements to reduce the risk of falls, BMD decrease and fractures6, there are sufficient arguments that demonstrate the importance of vitamin D on muscle and bone health. Vitamin D stimulates the absorption of calcium from the intestine and maintains the serum calcium levels that are required for normal bone mineralization and for the maintenance of muscle function7. Several in vivo studies suggest vitamin D’s role in regulating muscle mass and its function. Observational studies show that vitamin D deficiency in the elderly is associated with reduced muscle mass and strength8,10, lower physical performance6,11, and increased risk of falls12. In addition, a meta-analysis of 17 clinical trials showed that supplementation with vitamin D in subjects with basal calcidiol levels below 10 ng/mL had a positive effect on hip muscle strength13. These studies suggest that vitamin D can affect muscle mass and function. However, it is not clear whether vitamin D plays a direct or indirect role. In recent years, the local conversion of calcidiol to calcitriol, the most active vitamin D metabolite, which is synthesized mainly in the kidney through its precursor calcidiol, has been increasingly important14. This local synthesis has been reported in several other cell types, such as in osteo-
Calcidiol levels and muscle function maintenance, functional capacity and bone mineral bone density in non-selected Spanish population

MATERIAL AND METHODS
The initial study protocol was designed to ascertain the prevalence of vertebral fracture. To do this, 624 men and women over 50 years of age were randomly selected from the municipal registry of Oviedo, Spain. The protocol consisted of all subjects completing a questionnaire on risk factors related to osteoporosis. This questionnaire was designed for the EVOS study, translated into several languages, and had an adequate reproducibility index. Similarity, the entire cohort underwent two lateral radiographs (this radiographic study was not completed in only two cases), the collection of anthropometric measurements such as height and weight to determine the body mass index (BMI), and a densitometric study. All subjects had sufficient ambulatory capacity to climb two floors without a lift and 99% lived in their own home.

After the prevalence study, this cohort was followed prospectively for 4 years by means of 2 postal questionnaires to find out the incidence of non-vertebral osteoporotic fracture. In the fourth year of the follow-up period (between the second and the third postal questionnaire), participants who had answered at least one of the two previous questionnaires were invited to repeat the same tests performed in the prevalence study, to measure muscular strength of grip in both hands added to him with a dynamometer that owns a scale that goes from the minimum 0 to the maximum of 300 mm of Hg, a survey with 12 items on the difficulty or not to carry out daily life activities, as well as a biochemical study of general markers and bone and mineral metabolism. In this second cross-sectional study, 404 subjects participated (212 women and 192 men), of which 322 agreed to take part in the biochemical study. A total of 32 subjects (9.9%) were excluded from the analysis as they had undergone osteoporotic treatment, including treatment with vitamin D. From a total of 290 subjects, we had all the data in both cross sections.

Densitometric evaluation
The BMD was measured with a Hologic® QDR-1000 DXA densitometer (Hologic Inc., Waltham, Massachusetts, USA). In all cases, the anterior-posterior lumbar spine (L2-L4) and the density of the right femur were measured. For the evaluation of lumbar BMD, 4 subjects with marked degenerative osteoarthritis were excluded. The coefficients of variation (CV) were 1.2% and 1.9%, respectively. In the fourth year of the follow-up period, BMD was also determined in the same areas as those measured in the first cross-sectional study, using the rate of change in BMD between both cross-sectional studies as a method to evaluate BMD development over time.

Biochemical analysis
In the fourth year of follow-up and over 1 year, a sample of blood and urine was taken in fasting from each subject: 33% of the blood samples were taken in the spring, 12% in the summer; 32% in the autumn and 23% in winter. Once the serum was separated, it was stored frozen together with the urine at -80°C until the analyses were carried out. Serum levels of calcium, creatinine, total alkaline phosphatase and resistant tartrate acid phosphatase were determined using an autoanalyzer (Hitachi Mod. 717, Ratiogen, Germany). The serum levels of calcidiol (25(OH)D) were determined by previous extraction with acetonitrile (IDS, Ltd., Bolton, United Kingdom), whose intra- and interassay coefficients of variation (CV) were, respectively, 5.2% and 8.2% respectively.

Levels of 1,25-dihydroxyvitamin D were measured by radio-immunoassay (IDS, Ltd.); the intra- and interassay CVs were 6.5% and 9%, respectively. Intact levels of PTH were measured using radio-immunoassay methods (Nichols Institute, San Juan de Capistrano, California, USA); the intra- and interassay CV values were 2.6% and 5.8%, respectively.

All the studies carried out followed the principles set out in the Helsinki Declaration and were formally approved by the Clinical Trials Committee of the Principality of Asturias.

Statistic analysis
The analysis of the data was carried out using version 17.0 of SPSS for Windows. The quantitative variables were analyzed by Student’s t test. The qualitative variables were analyzed by chi square.

To analyze at multivariate level the effect of calcidiol levels on muscle strength, the muscular strength of grip in both hands was categorized as 0 for values equal to 300 mm Hg (maximum pressure of the dynamometer) and 1 for values <300 mm Hg. The logistic regression analysis was adjusted for age, sex, BMI and seasonality (season of the year in which blood extraction was carried out).

To study the association between the performance of daily life activities with serum levels of calcidiol, a logistic regression analysis was carried out after adjusting for age, sex, BMI and seasonality. When statistically significant associations were found between the levels of calcidiol and the rate of change in BMD in the univariate analysis, a linear regression was performed adjusted for age, sex, BMI and seasonality.

RESULTS
Table 1 shows sociodemographic, anthropometric, clinical variables and biochemical markers of the cohort analyzed as a function of serum levels of calcidiol. In those with calcidiol levels of ≥20 ng/mL, there was a predominance of men, younger age, higher BMD values in all the skeletal segments analyzed, lower frequency of previous fractures, higher levels of calciotriol and lower levels of PTH and total alkaline phosphatase.

Calcidiol values ≥20 ng/mL (28.6% of the cohort) were associated with greater grip strength in both hands compared to levels <20 ng/mL (Figure 1). After adjusting for age, sex, BMI and seasonality, calcidiol levels ≥20 ng/mL alone were associated independently with decreases in grip strength (OR=2.35; 95% CI: 1.03-5.38). On the other hand, that association was lost in the right hand (OR=1.91; 95% CI: 0.92-3.98).

The daily life activities as a function of calcidiol levels are reflected in table 2. Of the 12 activities analyzed, the inability or difficulty to “lean to catch a soil object” was significantly associated with lower levels of calcidiol (p=0.009, table 2).
Likewise, the difficulty or inability to: “get out of bed” was associated with lower levels of calcidiol; “Pick up a book or object from a high shelf”; “Lean from a chair to take object from the floor”; “Remove the stockings or socks” and “run 100 meters without stopping” (Table 2). Only “bending down to get an object from the floor” and “getting up from the bed” were significantly associated with calcidiol levels after multivariate adjustment for age, sex, BMI and seasonality. Thus, increments of 10 ng/mL of calcidiol were associated with a decrease of 30% and 58%, respectively, in the difficulty or inability to “bend over to pick up an object from the floor” or to “get up from bed”.

The stratification of calcidiol levels showed that, in the multivariate adjustment, the presence of calcidiol deficiency (<10 ng/mL) not only significantly increased the inability or difficulty to “get out of bed” (OR=2.14; 95% CI: 1.21-3.77) but also to “take a book or object from a high shelf” (OR=2.02; 95% CI: 1.09-3.73), “lean from a chair to take object from the floor” (OR=1.78; 95% CI: 1.03-3.07) and “remain seated in a hard chair for 1 hour” (OR=1.78; 95% CI: 1.03-3.07).

The percentage of change in BMD at the level of the lumbar spine, femoral neck and total hip as a function of the serum levels of calcidiol is shown in table 3. The presence of calcidiol levels <20 ng/mL was associated with greater losses of BMD, both at the femoral neck and total hip level, with no significant differences at the lumbar level. After a multivariate analysis, changes in BMD at femoral neck and total hip level were independently associated with calcidiol levels <20 ng/mL (typified beta coefficient=0.130, p=0.041 and typified beta coefficient=0.142, p=0.033, respectively).

### DISCUSSION

In this study, low levels of calcidiol (<20 ng/mL) have been found to contribute to a lower muscular strength of grip in the hands, to more difficulties to perform certain activities of daily life, as well as to greater losses of BMD in the hip.

There are both basic and clinical evidences that support the participation of vitamin D in skeletal muscle function. In recent work in people with spinal cord injuries requiring rehabilitation, low levels of calcidiol were independent predictors of decreased physical function. The lower muscle strength of grip in relation to the low levels of calcidiol found in our study has also been reported by other authors. In a longitudinal study of Dutch adults, aged between 55 and 85 years, serum levels of calcidiol below 10 ng/mL were associated with a 40% loss in grip strength compared to baseline.

### Table 1. Demographic, anthropometric characteristics, clinical variables and biochemical markers as a function of serum levels of calcidiol

<table>
<thead>
<tr>
<th></th>
<th>Calcidiol &lt;20 ng/mL</th>
<th>Calcidiol ≥20 ng/mL</th>
<th>Value of p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex man (n)</td>
<td>95 (45.4%)</td>
<td>53 (63.9%)</td>
<td>0.005</td>
</tr>
<tr>
<td>Age (years)</td>
<td>69.0 ± 8.4 (n=207)</td>
<td>65.7 ± 8.2 (n=83)</td>
<td>0.002</td>
</tr>
<tr>
<td>BMI (kg/cm²)</td>
<td>28.3 ± 8.4 (n=207)</td>
<td>27.7 ± 3.3 (n=83)</td>
<td>0.249</td>
</tr>
<tr>
<td>BMD lumbar spine (g/cm²)</td>
<td>0.932 ± 0.179 (n=153)</td>
<td>1.003 ± 0.158 (n=61)</td>
<td>0.007</td>
</tr>
<tr>
<td>BMD femoral neck (g/cm²)</td>
<td>0.743 ± 0.129 (n=207)</td>
<td>0.788 ± 0.130 (n=83)</td>
<td>0.008</td>
</tr>
<tr>
<td>Total hip DMO (g/cm²)</td>
<td>0.858 ± 0.147 (n=207)</td>
<td>0.910 ± 0.149 (n=83)</td>
<td>0.007</td>
</tr>
<tr>
<td>Vertebral fracture according to Genant (n)</td>
<td>36 (17.4%)</td>
<td>11 (13.3%)</td>
<td>0.362</td>
</tr>
<tr>
<td>Previous fracture (n)</td>
<td>53 (25.6%)</td>
<td>11 (13.3%)</td>
<td>0.032</td>
</tr>
<tr>
<td>Falls (n)</td>
<td>49 (23.7%)</td>
<td>27 (20.5%)</td>
<td>0.532</td>
</tr>
<tr>
<td>Calcium (mg/dL)</td>
<td>9.4 ± 0.3 (n=207)</td>
<td>9.4 ± 0.4 (n=83)</td>
<td>0.547</td>
</tr>
<tr>
<td>Calcidiol (ng/mL)</td>
<td>11.7 ± 4.2 (n=207)</td>
<td>27.7 ± 7.4 (n=83)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Calcitriol (pg/mL)</td>
<td>38.7 ± 14.4 (n=207)</td>
<td>47.8 ± 18.2 (n=83)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>PTH (pg/mL)</td>
<td>55.4 ± 24.5 (n=207)</td>
<td>45.6 ± 18.7 (n=83)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Total alkaline phosphatase (UI/L)</td>
<td>183 ± 76 (n=207)</td>
<td>162 ± 54 (n=83)</td>
<td>0.025</td>
</tr>
<tr>
<td>Creatinine (mg/dL)</td>
<td>1.06 ± 0.16 (n=207)</td>
<td>1.01 ± 0.19 (n=83)</td>
<td>0.051</td>
</tr>
<tr>
<td>FATR (UI/L)</td>
<td>2.1 ± 0.7 (n=207)</td>
<td>2.0 ± 0.5 (n=83)</td>
<td>0.547</td>
</tr>
</tbody>
</table>
Figure 1. Grip strength measurements (mm Hg) in hand A) left; B) right according to the serum levels of calcidiol. *p<0.05 for calcidiol <10 ng/mL and calcidiol between 10-20 ng/mL

Table 2. Levels of calcidiol (ng/mL) depending on the difficulty or not to perform certain activities of daily life

<table>
<thead>
<tr>
<th>Activity</th>
<th>Without difficulty</th>
<th>Unable to do it or with difficulties</th>
<th>Value of p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Take a book or object from a high shelf</td>
<td>16.9 ± 8.9 (n=232)</td>
<td>13.5 ± 8.4 (n=58)</td>
<td>0.009</td>
</tr>
<tr>
<td>Carry an object of 10 kg for 10 meters</td>
<td>16.8 ± 8.6 (n=169)</td>
<td>15.4 ± 9.4 (n=120)</td>
<td>0.186</td>
</tr>
<tr>
<td>Wash and dry yourself</td>
<td>16.3 ± 8.8 (n=257)</td>
<td>15.7 ± 1.0.4 (n=33)</td>
<td>0.718</td>
</tr>
<tr>
<td>Lean forward to pick up an object from the ground</td>
<td>17.4 ± 9.2 (n=174)</td>
<td>14.6 ± 8.3 (n=116)</td>
<td>0.009</td>
</tr>
<tr>
<td>Wash your hair in a sink</td>
<td>16.3 ± 8.8 (n=251)</td>
<td>15.8 ± 9.7 (n=39)</td>
<td>0.728</td>
</tr>
<tr>
<td>Sit one hour in a hard chair</td>
<td>16.7 ± 9.6 (n=203)</td>
<td>15.2 ± 9.7 (n=85)</td>
<td>0.179</td>
</tr>
<tr>
<td>Standing in a queue for 30 minutes</td>
<td>17.0 ± 9.2 (n=157)</td>
<td>15.4 ± 8.6 (n=133)</td>
<td>0.137</td>
</tr>
<tr>
<td>Get up in bed</td>
<td>17.4 ± 9.2 (n=214)</td>
<td>13.1 ± 7.2 (n=76)</td>
<td>0.000</td>
</tr>
<tr>
<td>Remove socks or similar clothes from the feet</td>
<td>17.0 ± 9.0 (n=193)</td>
<td>14.7 ± 8.7 (n=97)</td>
<td>0.040</td>
</tr>
<tr>
<td>Leaning from a chair to pick up an object from the floor</td>
<td>17.4 ± 9.0 (n=183)</td>
<td>14.4 ± 8.4 (n=107)</td>
<td>0.006</td>
</tr>
<tr>
<td>Raise a box of 6 full bottles and place them on a table</td>
<td>16.8 ± 8.5 (n=176)</td>
<td>15.5 ± 9.5 (n=114)</td>
<td>0.226</td>
</tr>
<tr>
<td>Running 100 meters without stopping</td>
<td>17.4 ± 9.2 (n=167)</td>
<td>14.8 ± 8.4 (n=123)</td>
<td>0.015</td>
</tr>
</tbody>
</table>

Table 3. Percentage of change in BMD at the level of the lumbar spine, femoral neck and total hip between the two cross-sectional studies as a function of serum levels of calcidiol

<table>
<thead>
<tr>
<th></th>
<th>Calcidiol &lt;20/mL</th>
<th>Calcidiol ≥20 ng/mL</th>
<th>Valor de p</th>
</tr>
</thead>
<tbody>
<tr>
<td>% change in BMD at the lumbar level</td>
<td>-0.59 ± 4.14 (n=153)</td>
<td>0.39 ± 5.29 (n=61)</td>
<td>0.193</td>
</tr>
<tr>
<td>% change in BMD at femoral neck level</td>
<td>-1.49 ± 5.29 (n=160)</td>
<td>-0.10 ± 5.09 (n=66)</td>
<td>0.036</td>
</tr>
<tr>
<td>% change in BMD at the total hip level</td>
<td>-0.30 ± 3.61 (n=160)</td>
<td>1.08 ± 4.54 (n=66)</td>
<td>0.017</td>
</tr>
</tbody>
</table>
In our study, some activities of daily life were found to be compromised by the low levels of calcidiol, this effect being more marked in the presence of calcidiol deficiency (<10 ng/mL). The activities that were most affected were those that had more to do with the functional capacity of the organism than those dependent on greater muscular strength such as “transporting an object of 10 kg for 10 meters” or “lifting a box with 6 full bottles and place them on a table.” Other recent studies have also associated low levels of vitamin D with the greatest difficulty in performing activities of daily living. Thus, in a recent study by Arbex Borim et al., the reduction of muscle strength or dynapenia combined with low levels of calcidiol were found to be a risk factor that conditioned the development of daily life activities in a sample of 4,630 people over 50 free of disability at the outset of the study, followed for 2 years. Similarly, Wicherts et al., analyzing a study of men and women between 65 and 88 years old, found that those with calcidiol levels below 20 ng/mL, had a worse state and physical performance at both baseline and 3 years of follow-up compared to those with levels higher than 30 ng/mL. Another Dutch prospective study showed that vitamin D levels were associated with functional limitations in the age stratum between 55 to 65 years and in those over 65 years. However, others authors have not found any association between calcidiol levels below 10 ng/mL and lower hip flexion, knee extension force, grip strength, gait speed or disability in activities related to mobility of the upper extremities. This last study was carried out in 628 women over 65 years of age followed for 3 years and who presented a moderate to severe disability at the beginning of the study. The existence of very few participants with low calcidiol values may have limited the possibility of obtaining differences.

The association between levels of calcidiol and BMD revealed in a recent meta-analysis is more contradictory. Epidemiological evidence indicates that the highest levels of calcidiol are associated with higher BMD in both the young and aging population, maintaining a linear relationship to levels of 30 ng/mL, an association that does not seem so clear and solid in black populations, or Hispanics of North America. Our data indicate a direct association between calcidiol levels ≤20 ng/mL and BMD at femoral neck and total hip level. A 2014 meta-analysis concluded that there was very little evidence that vitamin D influenced BMD, since there was no consistent relationship between vitamin D supplementation and BMD in most of the anatomical sites analyzed (lumbar spine, total hip, trochanter, whole body or forearm), although a positive association was observed in the femoral neck, as was observed in our study. Similarly, a recent article shows that patients with hip fractures have lower levels of calcidiol, lower bone mass, decreased bone quality and an increased risk of fracture. It is important to highlight that in our study all subjects who were receiving treatment for osteoporosis, including supplements with vitamin D, were eliminated, which does not allow us to assess the possible effect of vitamin D supplementation on bone mass.

Our study has limitations, but also strengths. Regarding the former, the fact of having a single biochemical determination (after 4 years of follow-up) without knowing the values at the beginning of the study limits the associations found. On the other hand, the questionnaire on difficulties to carry out activities of daily life was not self-administered but administered by an interviewer, which could have biased the responses of the participants, especially in those questions that referred to personal hygiene difficulties. As strengths, the analyzed cohort participated in the EVOS-EPOS study, being one of the few groups that completed all the study guidelines. The participation percentages of more than 80% in the four postal follow-ups carried out during 8 years guarantee the representativeness of the sample analyzed. In addition to the articles published with data from the full cohort of the EVOS-EPOS study, the cohort of the city of Oviedo, which has been used for this study, has contributed individually to the publication of several original articles in high-impact journals.

To sum up, calcidiol levels above 20 ng/mL are associated with greater muscle grip strength in the hands, better performance in activities of daily life such as “bending over to pick up an object from the ground” and “Get up from the bed” and with a greater BMD in total hip and femoral neck, suggesting that maintaining calcidiol levels above 20 ng/mL would favor an adequate musculoskeletal function.

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Conflict of interests: The authors declare no conflict of interest.
Effects of mechanical stimulation on communication between bone cells

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Summary
Mechanical force is important for modeling, remodeling and bone regeneration. It stimulates the osteocytes, causing an alteration in the production and secretion of signaling molecules that regulate osteoblast and osteoclast activity. The present study aims to evaluate the effect of the conditioned medium of mechanically stimulated mouse osteocytic cells on the proliferative and migratory capacity of mesenchymal cells and bone cells. For this, the proliferation and migration of mouse pre-osteoblastic cells, human pre-adult mesenchymal cells and mouse macrophages in the presence of the conditioned medium of osteocytic cells were analyzed, after 6 and 24 hours after being subjected to a mechanical stress of 10 dynes/cm² by fluid flow (FF) for 10 minutes. The migration of pre-osteoblastic cells has been found to increase significantly in the presence of conditioned media of osteocytic cells compared to the static control group (SC) (SC=12.63±5.44, FF6h=23.03±11.57, FF24h=29.72±15.76, p<0.0001). In the same way, the pre-adipose cells also significantly increased their migration in the presence of this conditioned media (SC=11.48±4.75, FF6h=18.43±9.94, FF24h=18.80±10.03, p≤0.0007). However, macrophage migration decreased in the presence of the conditioned medium collected at 24 hours with respect to the static control group (SC=69±22.71, FF24h=26.57±5.47, p<0.0001). These effects were associated with decreased protein expression of certain chemokines, such as the monocyte chemotactic protein type I (SC=0.25±0.06, FF24h=0.09±0.005, p=0.0262), the protein of group I of high mobility (SC=0.25±0.04, FF24h=0.15±0.05, p=0.0159) and the regulatory protein of the activation of T lymphocytes and monocytes (SC=3.29±0.88, FF6h=1.33±1.09, FF24h=0.97±0.66, p≤0.0314), by the osteocytes in the presence of mechanical stimulation with respect to the static control group. In conclusion, this in vitro study demonstrates that osteocyte mechanotransduction enhances recruitment of osteoblasts and pre-adipose mesenchymal cells while reducing the migration of macrophages.

Key words: osteocytes, osteoblasts, macrophages, mesenchymal cells, mechanical stimulation, chemokines.

INTRODUCTION
Mechanical force is one of the most important stimuli that the bone receives to regulate bone mass, shape and microarchitecture. The endoskeleton reacts to an increase in load by forming more bone or decreasing its mass in the absence of mechanical stress1. This is because the stimulation triggers the mechanotransduction process in which osteocytes, considered bone’s key mechanosensory cells, when stimulated, send chemical signals that affect the paracrine regulation of osteoblast and osteoclast behavior2,3. It also has been found to have an anti-apoptotic effect on osteocytes4.

With mechanical loading, the expression of sclerostin, which is an inhibitor of the protein signaling pathway Wnt/β-catenin constitutively secreted by osteocytes, decreases thus causing an increase in osteoblastogenesis5,6. On the other hand, apoptotic osteocytes induce the secretion of the receptor activator for nuclear factor κ B ligand (RANKL), indirectly stimulating osteoclastogenesis7. In addition, some chemokines, a family of chemotactic cytokines, could be involved in bone remodeling when expressed by bone cells and provide key signals to recruit different cellular subpopulations8.

Recent studies indicate that the high mobility group box 1 protein (HMGB1), the regulated upon activation, normal T cell expressed, and secreted protein or chemokine (C-C motif) ligand 5 (RANTES or CCL5) and the monocyte chemoattractant protein 1 or chemokine (C-C motif) ligand 2 (MCP1 or CCL2) intervene to recruit mesenchymal stem cells to promote tissue repair9,10.

Based on this evidence, our objectives are focused on recreating an in vitro charge model to generate mechanotransduction in a controlled culture environment11 and to study the effect of conditioned medium secreted...
by osteocytes after being mechanically stimulated in the promotion of the proliferative and migratory capacity of mesenchymal cells and bone cells as well as the possible protein expression of certain chemotactic factors involved in proliferation and migration processes.

**MATERIALS AND METHODS**

**Cell cultures.** For our assays, different cell types were used:

- Adipose stromal cells (ASC), obtained by primary culture of human lipoaspirates carried out in the HM Montepríncipe Hospital (HM Hospitals), as described in the work of Zuk et al. in 2001. All donors gave their informed consent, in accordance with the appropriate clinical protocol. The patients were operated in the Department of Plastic Surgery of HM Hospitals (Madrid, Spain) and the tissue sample collection was approved by the Institutional Review Board/Clinical Research Ethics Committee of HM Hospitals (Madrid, Spain). These cells were cultured with DMEM (Dulbecco modified Eagle’s minimal essential medium) + GlutaMAX (Gibco, Life Technologies, Alcobendas, Spain) with 10% fetal bovine serum (fetal bovine serum, FBS) and 1% penicillin-streptomycin (Invitrogen) at 37°C with 5% CO₂.

- Continuous line of MLO-Y4 osteocytes from murine long bones extracted as described in Kato et al. in 1997, courtesy of L. Bonewald, which was cultivated in 100 mm diameter plate (Jet Biofil, Guangzhou, China) previously collagenized with Collagen I (Sigma-Aldrich) with α-MEM (Minimum Essential Medium Eagle - Alpha Modification) at 2.5% calf serum (Calf serum, CS) (Sigma-Aldrich), 25% FBS and 1% penicillin-streptomycin at 37°C with 5% CO₂.

- Continuous line of mouse preosteoblast of the skull vault, MC3T3-E1 subclone 4 (ATCC CRL-2593).

- Continuous line of mouse macrophages capable of differentiating to osteoclasts, RAW 264.7 (ATCC TIB-71), which were cultivated with α-MEM with 10% FBS, 1% penicillin-streptomycin and 2 mM L-glutamine at 37°C with 5% CO₂.

**Mechanical stimulation tests by fluid passage (Fluid Flow, FF).** This technique generates physiologically relevant mechanical stimulation in bone cells in vitro. For this, 250,000 MLO-Y4 cells were seeded on Teflon-bound glass slides leaving a space of 15 cm² previously collagenized and incubated for at least 48 hours at 37°C with 5% CO₂ until they reached the confluence. Subsequently, the cells were subjected to mechanical stimulation or not (static control or SC) with the Flexcell Streamer device of medium cut stress that produces a stress of 10 dynes/cm² for 10 minutes (Flexcell International Corporation, Hillsborough, North Carolina, USA). The cells were then incubated with α-MEM Medium without phenol red (Gibco) with 0.5% CS, 0.5% FBS and 1% penicillin-streptomycin to obtain conditioned media (CM) from the different experimental groups: CM of stimulated cells (FF) collected at 6 hours after the stimulus, CM of SC cells collected at 24 hours after the stimulus and CM of FF cells collected at 24 hours after the stimulus.

**Proliferation assay.** To carry out the proliferation assay, both mouse pre-osteoblast cells and human pre-adipose cells were seeded at a concentration of 6,000 cells/well in 12-well culture plates (Jet Biofil), one plate per condition with each of the lines Cells, and incubated at 37°C with 5% CO₂. The following day, the medium was exchanged for 20% of conditioned medium and 80% of its culture medium, adjusting the FBS to 10%. After 24 hours of incubation at 37°C with 5% CO₂, the cells were raised with Trypsin-EDTA and a cell count was made with Trypan Blue 0.4% in PBS (GE Healthcare, Hyclone, Logan, Utah, USA) in the Neubauer chamber. The process was repeated at 48 and 72 hours, obtaining a proliferation assessment with each of the conditioned media for 3 days and in triplicate.

**Migration trial in Transwell.** Seed in 4 Transwell 6 well culture plates (Corning, Costar, Life Sciences, New York, USA) 75,000 cells/well with its culture medium on the membrane, and 20% conditioned medium was placed underneath and 80% of your culture medium at 1% FBS. After 24 hours of incubation at 37°C with 5% CO₂, both media and the upper cell layer of the membrane were removed with the aid of a cotton swab. The cells remaining at the bottom of the membrane were fixed with 4% paraformaldehyde in PBS (Alfa Aesar, Thermo Fisher) for 10 minutes and stained with 0.1% crystal violet in distilled water (MERCK, Kenilworth, New Jersey, USA) for 15 minutes. Finally, the membranes were mounted on slides and observed in the phase contrast microscope (Leica Microsystems DMS500 CTR6000) from which 20 images were obtained at 50 μm per well to analyze the number of cells that had migrated as a function of the conditioned medium used.

**Western Blot.** The cells were prepared to extract the total protein with RIPA buffer (Sigma-Aldrich) supplemented with protease inhibitors and phosphatases (Calbiochem). On the other hand, the conditioned medium was lyophilized and the pellet was resuspended in MilliQ water. To quantify the amount of protein in each of the samples, both lyophilized conditioned medium and cell lysate, the Varioskan Flash Multimode Reader (Thermo Scientific) with a Comassie template were used. Once quantified, they were separated in 15% acrylamide gels and transferred to nitrocellulose membranes. The membrane was then blocked with 5% bovine serum albumin (BSA) dissolved in TBS with Tween 20 (Sigma-Aldrich) for one hour at room temperature and incubated overnight at 4°C with polyclonal antibodies. of rabbit: anti-HMG1, anti-MCP1 and anti-RANTES (Abcam, Cambridge, UK). As a control, the anti-α-tubulin mouse monoclonal antibody was used. It was then incubated for one hour at room temperature with the corresponding IgG coupled to peroxidase and the membrane was revealed in the transilluminator (Syngene DYV 6-Ε) with the ECL system (Electro-chemo-luminescence, GE-Amersham, Pittsburgh, USA). The intensities of the bands were quantified by densitometry.

**Statistical analysis.** In the statistical analysis of the results, the data are expressed as mean ± standard deviation of at least two experiments carried out in triplicate. It was performed using the GraphPad Prism V 7.0 software (GraphPad software, La Jolla, California, USA), using a non-parametric study using a two-tailed t-test or U-Mann-Whitney test for two-to-two comparisons, and the Kuskal-Wallis test for group comparisons. Outliers were detected and excluded using the GraphPad QuickCalcs®2018 program that uses the Grubb test, and values of p<0.05 were considered as significant results.
RESULTS

Effect of conditioned media of osteocytes mechanically stimulated in the proliferation of pre-osteoblasts and pre-adipose mesenchymal cells

A proliferation study of mouse pre-osteoblastic cells MC3T3-E1 and pre-adipose mesenchymal cells was performed with 20% conditioned media of mouse osteocytic cells MLO-Y4 in the presence (FF 6 hours and FF 24 hours) and absence (SC or control static) of mechanical stimulus by fluid passage.

As shown in figure 1A, there is no significant difference in the proliferation of the cell line MC3T3-E1 after 24 hours or after 72 hours in the presence of conditioned media of 6 and 24 hours. In the case of the pre-adipose mesenchymal cells, the results also showed no significant differences after 24 hours or after 72 hours in the presence of the same conditioned media (Figure 1B).

Effect of conditioned media of osteocytes mechanically stimulated in the migration of pre-osteoblasts, pre-adipose mesenchymal cells and macrophages

The study of migration of pre-osteoblastic cells MC3T3-E1, mesenchymal pre-adipose and macrophage cells RAW 264.7 was performed with culture medium specific to each cell line (control) and conditioned media of osteocytic MLO-Y4 cells in the presence (FF 6 hours and FF 24 hours) and absence (SC) of mechanical stimulation.

The pre-osteoblastic cells doubled and tripled their migration in the presence of the conditioned media of the osteocytes collected after 6 and 24 hours of being subjected to stimuli by fluid passage, respectively (Figure 2).

In the same way, the pre-adipose mesenchymal cells also duplicated their migration in the presence of that media (Figure 3).

In the case of the RAW 264.7 mouse macrophage line, our results indicate a three-fold decrease in their migration under the conditions of mechanical stimulation (Figure 4).

Analysis of chemokine expression and secretion after mechanically stimulating osteocytes

In order to corroborate the results obtained previously, the analysis of chemoattractant protein expression was carried out using the Western Blot technique. For this, the lysates of osteocytic cells MLO-Y4 were obtained in the presence and absence of mechanical stimulation by the passage of fluid and, on the other hand, the lyophilisation of their respective conditioned media, as described in the section on materials and methods.

We studied three possible proteins involved in the migration of mesenchymal cells after a mechanical stimulus, two of them belonging to the C-C chemokine family: MCP1 and RANTES, and the high mobility protein group 1 (HMGB1). Tubulin was used to normalize the cell lysate samples.

As seen in figure 5, in the cell lysates of mechanically stimulated MLO-Y4 there was a two-fold decrease in the expression of the chemotactic protein MCP1 (Figures 5A-5B). In the samples of lyophilized conditioned media it was observed that the secretion of MCP1 also decreased under the conditions of mechanical stimulation, in this case it decreased three times (Figures 5C-5D).

In mechanically stimulated MLO-Y4 cell lysates, a two-fold decrease in the expression of the HMGB1 chemotactic protein was observed in the FF condition at 24 hours (Figures 5E-5F).

Similarly, in mechanically stimulated MLO-Y4 cell lysates, a threefold decrease in the expression of the RANTES chemotactic protein was observed under FF conditions at 6 and 24 hours (Figures 5G-5H).

DISCUSSION

Aging, loss of sex steroids, excess glucocorticoids and certain bone diseases such as osteoporosis, cause a decoupling in bone remodeling and a loss of bone quality due to the accumulation of apoptotic osteocytes that precede the recruitment of precursors osteoclasts and their differentiation to carry out the process of directed bone resorption14,15.

However, physiological levels of mechanical stimulation such as physical exercise maintains the viability of osteocytes and, furthermore, as demonstrated in this work, acts on their behavior by modifying the production of certain chemokines and regulating the migration of different cell types.

Figure 1. Cell proliferation of MC3T3-E1 (A) and ASC (B) (mesenchymal cells of adipose origin) in the presence and absence of conditioned media of 6 and 24 hours after mechanical stimulation. The values are the mean ± standard deviation of 3 independent experiments in triplicate. Results presented as number of cells vs. control.
Figure 2. Migration of MC3T3-E1 cells. Representative images corresponding to the migration of MC3T3-E1 in each of the study conditions (A-C). Number of cells per field of MC3T3 migration in the absence and presence of conditioned study media (D). The values are the mean ± standard deviation of 2 independent experiments in triplicate. **p<0.001 vs. static control

Figure 3. Migration of ASC cells (mesenchymal cells of adipose origin). Representative images corresponding to the migration of ASC in each of the study conditions (A-C). Number of cells per field of ASC migration in the absence and presence of conditioned study media (D). The values are the mean ± standard deviation of 2 independent experiments in triplicate. **p<0.001 vs. static control

Figure 4. Migration of RAW 264.7 cells. Representative images corresponding to the migration of RAW 264.7 in each of the study conditions (A-C). Number of cells per field of RAW migration 264.7 in the absence and presence of conditioned study media (D). The values are the mean ± standard deviation of 2 independent experiments in triplicate. **p<0.001 vs. static control
Figure 5. Secretion and expression of chemotactic proteins measured by Western Blot (A-C). Expression of the MCP1 protein in MLO-Y4 cell lysate in the absence of mechanical stimulation (SC) and after 6 and 24 hours of performing FF for 10 minutes (FF6h and FF24h) (C-D). Secretion of the MCP1 protein in conditioned MLO-Y4 media in the absence and presence of mechanical stimulus (E-F). Expression of HMGB1 in cell lysate of MLO-Y4 in the absence and presence of mechanical stimulus (G-H). Expression of RANTES in cell lysate of MLO-Y4 in the absence and presence of mechanical stimulation. The relative densitometric values are the mean ± standard deviation of 2 independent experiments in triplicate. *p<0.05 vs. static control.
In our results, we found that the exposure of MC3T3-E1 pre-osteoblastic cells and human pre-adipose mesenchymal cells to conditioned media of mechanically stimulated mouse MLO-Y4 osteocytic cells does not affect their proliferation, but increases their migratory capacity. Previous studies have already shown that the conditioned medium of mechanically stimulated osteocytes is able to recruit osteoprogenitors (mesenchymal cells and osteoblasts) and promote the commitment of the osteogenic lineage of these cells to replenish depleted osteoblasts, improve bone formation and strengthen tissue.

On the other hand, our results indicate a decrease in the migration of RAW 264.7 macrophages in the presence of conditioned media of MLO-Y4 cells collected after 24 hours of performing the Fluid Flow. This corroborates what has been observed by other authors who indicate that this conditioned medium is also capable of inhibiting osteoclastogenesis.

All this suggests a negative feedback mechanism mediated by paracrine factors that would regulate the bone formation and resorption process. Therefore, we check whether certain selected chemokines are involved in this process through Western Blot assays of both the mechanically stimulated osteocytic cells and the conditioned media. According to our results, although there is a significant decrease in the monocyte chemotactic protein type 1 (MCP-1), the high mobility protein group 1 (HMGB1) and the RANTES chemotactic protein in the cell lysates of MLO-Y4, mechanically stimulated, do not seem to be directly associated with the migration of bone forming and repopulating cells.

However, contrary to what occurs in our study, there are previous studies that indicate that HMGB1 is released in the extracellular environment through the active secretion of stimulated cells and promotes osteogenic migration and differentiation of MSCs. In the case of MCP-1, it has been observed that mesenchymal stem cells from the bone marrow migrate in response to this chemokine. And there are findings that indicate that RANTES is capable of causing the migration of different cell types, including mesenchymal stem cells from bone marrow, through the induction of autophagy.

For future research, carrying out a proteomic study of the osteocyte conditioned media would be required, both without stimulation and with mechanical stimulation, to deepen the communication processes of the osteocytes with their environment.

Declaration of interests: The authors declare no conflict of interest.
Bibliography


The determining role of a resorption marker, carboxyterminal telopeptide of collagen I, in assessing therapeutic compliance in patients treated with oral bisphosphonates

Summary
Objective: It is estimated that in one year between 50-60% of patients treated with osteoporosis drugs are non-compliant. There are different indirect methods of assessing compliance. Our objective is to test a single determination of the carboxyterminal telopeptide of type I collagen (CTX) to assess compliance in patients treated with bisphosphonates, either on its own or together with the Morinsky-Green questionnaire.

Material and method: A diagnostic assessment study was carried out in 10 centers in Catalonia. Through consecutive sampling, postmenopausal women with osteoporosis were selected and treated with the same antiresorptive drug in the last year. Those treated with a drug other than bisphosphonate, with cognitive impairment, terminal illness, advanced renal failure or fracture in the previous year, were excluded. Data were collected on the diagnosis of osteoporosis and type of treatment. Analysis was requested with CTX determination. As a gold standard, the medication possession rate (MPR) was used. Using the ROC curve methodology, the theoretical CTX cut-off point was established. Sensitivity, specificity and positive predictive values were calculated to estimate therapeutic compliance.

Results: 100 patients were included, of which more than half were being treated with alendronate. According to the MPR, 70% were compliant. The mean CTX value was 0.193±0.146 ng/ml. It was lower in the compliant patients. A value of 0.196 ng/ml was established as a cut-off point to assess compliance. The joint assessment of the CTX together with the Morinsky-Green questionnaire showed greater discriminatory capacity.

Conclusions: Carrying out a single determination of CTX (<0.196 ng/ml) along with the Morinsky-Green questionnaire allows us to more accurately assess the therapeutic compliance in patients treated with bisphosphonates.

Key words: osteoporosis, bisphosphonates, therapeutic compliance, bone remodeling markers.

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INTRODUCTION

Osteoporosis is a metabolic disease characterized by low bone mass and microstructural deterioration of the bone tissue that leads to increased bone fragility. The main complication involves the appearance of fragility fractures. Osteoporotic fractures are an important health problem associated with high healthcare costs. To prevent the appearance of fractures, different drugs are available that act on bone metabolism and are associated with reduced fracture risk. The most commonly used in Spain are bisphosphonates. However, in order to observe this protective effect, adequate therapeutic compliance is required. In osteoporosis, as in all chronic diseases, compliance is low. In a recent study conducted in Spain, the overall persistence per year after commencing osteoporosis drug is 47%, and at two years, close to 27%.

Therefore, correctly assessing therapeutic compliance is necessary in our consultations to ensure an adequate effect in reducing the risk of fracture. Classically, self-administered surveys have been used to assess therapeutic compliance, such as Morisky-Green and Haynes-Sackett questionnaires, although the latter tends to overestimate compliance. In recent years, thanks to health system computerization, it is possible in certain cases to have access to drug dispensing data, so that the medication possession rate (MPR) can be calculated. This is used in many pharmaco-epidemiological studies, but not always available in day-to-day consultations.

Another possible way to assess compliance involves using bone remodeling markers, although there is little evidence in this regard and requires different determinations. Determining carboxyterminal telopeptide of type I collagen (CTX) as a marker of resorption and of the amino terminal propeptide of type I procollagen (P1NP) as a formation marker is recommended. Our aim is to verify the usefulness of a single CTX determination to assess compliance in patients treated with bisphosphonates (the most prescribed drugs) for at least one year, in isolation or together with a classic therapeutic compliance questionnaire, such as Morisky-Green.

MATERIAL AND METHOD

Study design

Diagnostic validation study carried out in 9 urban primary care centers of the Catalan Health Institute in Barcelona and the Hospital del Mar, between January and December 2012. Accepting 95% confidence and assuming 55% of non-compliers a sample of 93 patients would detect a sensitivity of 80% with an accuracy of 10%.

Participants

Through consecutive sampling, all patients with postmenopausal osteoporosis and treatment with a drug for osteoporosis were selected at least during the last year to complete a total of 115 patients, to cover possible losses. Patients who were treated with an anti-resorptive drug different from an oral bisphosphonate, with cognitive impairment, terminal illness, or advanced chronic renal failure (glomerular filtration <35 mL/min), or who had presented a fracture in the year prior to inclusion were excluded.

Study variables

Information was collected on age, diagnosis of osteoporosis, study with bone densitometry and the presence of previous fractures. Regarding the osteoporosis treatment, the type of drug was collected, the dosage and the conditions of intake, as well as the use of calcium and/or vitamin D supplements. To assess the therapeutic compliance, the calculation of the MPR through pharmacy dispensing data in the year prior to inclusion. For its calculation, the following formula was used:

\[
MPR = \frac{\text{number of prescriptions collected in the last 12 months} \times \text{days covered by each prescription}}{365}
\]

In accordance with available pharmaco-epidemiological studies, an MPR ≥0.8 is considered an indicator of therapeutic compliance. The self-administered therapeutic compliance questionnaire of Morisky-Green was also carried out.

CTX plasma determination was requested, measured by ELISA method, an electrochemiluminescence immunoassay (ECLIA) from Roche that uses two monoclonal antibodies, analyzed in the MODULAR ANALYTICS E170 autoanalyzer (Roche). The intraseries coefficient of variation value is 2.5% and the interseries value is 4.1%. The reference values of the test are: 0.01-1.008 ng/mL. Within a one-month period before the visit of the physician, the determination was carried out.

Statistical analysis

The characteristics of the studied population are described by univariate descriptive analysis, calculating mean and standard deviation for continuous variables and absolute frequency and percentage for categorical variables. The Chi-square test was used to compare proportions and the Student’s T test was used to compare means.

The receiver operator characteristics (ROC) curve methodology was used to determine the area under the curve and the theoretical CTX cut-off point with the best sum of sensitivity and specificity. Sensitivity, specificity, and positive (VPP) and negative predictive values (NPV) were calculated to estimate therapeutic compliance by: 1) Morisky-Green questionnaire, 2) CTX cut-off point and 3) joint assessment of the Morisky-Green and the CTX value. To assess the concordance between the different systems to assess compliance, the Kappa coefficient was used.

All statistical tests were carried out with a confidence interval (CI) of 95%. The statistical package SPSS version 13.0 for Windows and EPIDAT (program for epidemiological analysis of data) Version 3.1 was used for all analyzes.

Ethical aspects

The study was carried out following Declaration of Helsinki principles, the standards of good clinical practice, and as proposed in the Guide of Good Practices in Health Science Research of the Catalan Institute of Health (Second edition). Informed consent was requested from patients. The contact and personal data of the participating patients were only accessible to the study investigators.

RESULTS

Of the 115 patients selected, 15 were excluded for taking a drug other than an oral bisphosphonate (9, strontium ranelate and 6, raloxifene). The baseline characteristics are shown in table 1, with a higher proportion of densitometries prior to treatment and a lower proportion of patients treated with alendronate in the group of compliant patients. In 11 women, different errors were iden-
tified in the taking of the medication (an error in the ta-
kling of the medication—not on an empty stomach— an
error in the medication taking method—with milk— and
10 errors in the waiting time of fasting). As in these 11
cases, the MPR was <0.8 and, therefore, they were con-
considered non-compliant, and thus not excluded from
the analysis.

The therapeutic compliance valued by the MPR was
70% (Table 2), with no differences in the proportion of
compliers, according to whether the treatment was weekly
or monthly (68.2% vs. 73.5%, p=0.580). The compliance
assessed by the self-administered Morisky-Green ques-
tionnaire was 73%, with a moderate agreement compared
to the MPR assessment (Kappa coefficient=0.436).

The mean value of the determination of CTX was
0.193±0.146 ng/ml (median=0.158 ng/ml), with lower pa-
tients compared to non-compliant patients (0.182±0.143
ng/ml vs. 0.219±0.152 ng/ml; p=0.247). A cut-off point
of CTX of 0.196 ng/ml was the one that presented a better
sensitivity and specificity for the diagnosis of therapeutic
compliance. Considering this CTX value, the therapeutic
compliance was 64%, with a low concordance compared
to the MPR assessment (Kappa coefficient=0.234). When
considering the result of the Morisky-Green questionnaire
together with the value of the CTX, compliance was 51%,
with a moderate agreement (Kappa coefficient=0.415).

Table 3 shows the values of sensitivity, specificity and
predictive values of the different forms used to estimate
therapeutic compliance. The area under the ROC curve
(95% CI) for the Morisky-Green questionnaire was
0.7119 (0.6127-0.8111), and 0.6238 (0.5185-0.7291)
for the evaluation by a CTX cut-off of 0.196 ng/ml (Fi-
gure 1). When considering the result of the Morisky-
Green questionnaire together with the value of the CTX,
the area under the ROC curve was 0.7452 (0.6573-
0.8332), somewhat higher than if we only consider the
Morisky-Green result (p=0.622) (Figure 2).

**Table 1. Baseline characteristics in the total number of patients and according to treatment compliance**

<table>
<thead>
<tr>
<th>Variable</th>
<th>Patients total (n=100)</th>
<th>Patients compliant (n=70)</th>
<th>Patients non-compliant (n=30)</th>
<th>Value of p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years), mean ± SD</td>
<td>72.04±7.96</td>
<td>72.49±7.64</td>
<td>71.0±8.73</td>
<td>0.366</td>
</tr>
<tr>
<td>Registered diagnosis of osteoporosis, N (%)</td>
<td>99 (99)</td>
<td>69 (98.6)</td>
<td>30 (100)</td>
<td>0.511</td>
</tr>
<tr>
<td>Registered densitometry before treatment, N (%)</td>
<td>94 (94)</td>
<td>69 (98.6)</td>
<td>25 (83.3)</td>
<td>0.003</td>
</tr>
<tr>
<td>Fracture before to inclusion, N (%)</td>
<td>54 (54)</td>
<td>36 (51.4)</td>
<td>18 (60)</td>
<td>0.431</td>
</tr>
<tr>
<td>Prescribed drug: N (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alendronate</td>
<td>51 (51)</td>
<td>31 (44.3)</td>
<td>20 (66.7)</td>
<td>0.004</td>
</tr>
<tr>
<td>Risedronate</td>
<td>30 (30)</td>
<td>24 (23.3)</td>
<td>6 (20.0)</td>
<td>0.153</td>
</tr>
<tr>
<td>Ibandronate</td>
<td>19 (19)</td>
<td>15 (21.4)</td>
<td>4 (13.3)</td>
<td>0.344</td>
</tr>
<tr>
<td>Use of CaD</td>
<td>96 (96)</td>
<td>67 (95.7)</td>
<td>29 (96.7)</td>
<td>0.824</td>
</tr>
</tbody>
</table>

CaD: calcium and vitamin D supplements.

**Table 2. Assessment of therapeutic compliance according to the MPR**

<table>
<thead>
<tr>
<th>Classification of compliance according to MPR; N (%)</th>
<th>≥ 0.8</th>
<th>Between 0.6 and 0.8</th>
<th>Between 0.4 and 0.6</th>
<th>≤ 0.4</th>
</tr>
</thead>
<tbody>
<tr>
<td>General</td>
<td>70 (70.0)</td>
<td>16 (16.0)</td>
<td>7 (7.0)</td>
<td>7 (7.0)</td>
</tr>
<tr>
<td>Alendronate</td>
<td>31 (60.8)</td>
<td>14 (27.4)</td>
<td>3 (5.9)</td>
<td>3 (5.9)</td>
</tr>
<tr>
<td>Risedronate</td>
<td>24 (80.0)</td>
<td>1 (3.3)</td>
<td>2 (6.7)</td>
<td>3 (10.0)</td>
</tr>
<tr>
<td>Ibandronate</td>
<td>15 (78.9)</td>
<td>1 (5.3)</td>
<td>2 (10.5)</td>
<td>1 (5.3)</td>
</tr>
</tbody>
</table>

**DISCUSSION**

In our sample of patients treated with the same bisphos-
phonate for at least the last year, a CTX determination of
less than 0.196 ng/ml is an indicator of therapeutic com-
pliance in the last year, with a moderate discriminating
capacity, lower than the discriminative capacity of the Mo-
risky-Green survey. Their joint assessment (CTX <0.196
ng/ml and Morisky-Green) improves the discriminative
capacity, being a good option to assess the therapeutic
compliance in the consultations. In a recent consensus do-

The initial and three-month determination of bone remodeling markers (CTX and P1NP) was recommended to assess non-compliance based on the observed change (decrease of 56% of CTX and 38% of P1NP). But this requires two determinations of two markers, which are not always accessible for primary care laboratories. In addition, it does not allow for assessing compliance in those patients who have already started treatment and there is no baseline available, nor does it allow analyzing noncompliance over time.

Their specific determination, along with the administration of a classic therapeutic compliance questionnaire, that of Morisky-Green, present the best sensitivity and the best negative predictive value for therapeutic compliance.

In our sample, the observed therapeutic compliance (measured according to the MPR) was high, 70%, much higher than that observed in our environment by different observational studies. One of the possible explanations is that our study was not designed to assess the proportion of therapeutic compliance in our population and, therefore, random sampling was not carried out. In addition, more than half of the patients included had a previous fracture, although there were no significant differences in the percentage of patients with previous fractures between compliant and non-compliant subjects. The presence of previous fractures is associated with higher rates of therapeutic compliance.

One of every ten patients errors was observed in the co-

### Table 3. Sensitivity values, specificity and predictive values for the different tools to estimate therapeutic compliance (95% CI)

<table>
<thead>
<tr>
<th>Tool</th>
<th>Sensitivity</th>
<th>Specificity</th>
<th>VPP</th>
<th>NPV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Morinsky-Green</td>
<td>56.7 (37.3-76.1)</td>
<td>85.7 (76.8-94.6)</td>
<td>62.9 (42.9-83.0)</td>
<td>82.2 (72.7-91.6)</td>
</tr>
<tr>
<td>CTX</td>
<td>53.3 (33.8-72.8)</td>
<td>71.4 (60.1-82.7)</td>
<td>44.4 (26.8-62.1)</td>
<td>78.1 (67.2-89.0)</td>
</tr>
<tr>
<td>MG+CTX</td>
<td>83.3 (68.3-98.3)</td>
<td>65.7 (53.9-77.5)</td>
<td>51.0 (36.0-66.0)</td>
<td>90.2 (81.1-99.3)</td>
</tr>
</tbody>
</table>

VPP: positive predictive value; NPV: negative predictive value; MG+CTX: Morisky-Green and CTX.

### Figure 1. ROC curves to assess therapeutic compliance using the Morisky-Green questionnaire (A), the CTX (B) and the Morisky-Green combination with the CTX (C)
The determining role of a resorption marker: carboxyterminal telopeptide of collagen I...  

Declaration of interests: DML has received personal fees from Eli Lilly, Amgen, Ferrer, Rubió and Novartis; XN has received fees for Amgen and Eli Lilly; ADP has been a speaker or advisory board member for Lilly, Amgen, GSK and UCB; DPA states that their research department/group has received unrestricted research grants from Amgen, Servier Laboratoires and Bioibérica, and has received personal fees from Amgen and UCB; CCA, ASC, POL and RPM declare no conflicts of interest.
<table>
<thead>
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Different development of serum sclerostin compared to other bone remodeling markers in the first year after a liver transplant

DOI: http://dx.doi.org/10.4321/S1889-836X2019000100005

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Summary
Objective: Our main objective was to evaluate the development of sclerostin levels in patients with liver transplantation, and to investigate their relationship with other bone remodeling markers.

Material and method: Prospective observational study of 83 patients with liver transplantation. Sclerostin, β-crosslaps, bone alkaline phosphatase, osteocalcin and C-reactive protein values were determined the week before the transplant and subsequently, at 1, 3, 6 and 12 months. The hydroxy-vitamin D and the parathormone were determined basally. In each revision, the existence of fractures was evaluated. The development of the markers compared to the baseline value was determined by the t-Student test. A p-value less than 0.05 was considered statistically significant.

Results: 56 men and 27 women (mean age: 56.2±10.4 years). Baseline sclerostin levels (0.76±0.35 ng/ml) decreased significantly early (0.55±0.22 ng/ml in the first month, p=0.034), a trend that remained until 12 months (0.62±0.22 ng/ml, p=0.047). On the contrary, the basal levels of osteocalcin (17±10.3 ng/ml) and β-crosslaps (0.44±0.3 ng/ml) increased significantly throughout the study; in the case of osteocalcin, up to 12 months (37.27±26.84 ng/ml, p<0.01) and β-crosslaps, up to 6 months (0.62±0.34 ng/ml, p<0.01), with a subsequent decrease (0.47±0.31 ng/ml, p=0.2).

Conclusions: There is a decrease in the levels of sclerostin after liver transplantation, as opposed to the elevation of other markers of remodeling, β-crosslaps and osteocalcin. More studies are needed to determine if these changes have an impact on the occurrence of osteoporosis in patients undergoing transplantation.

Key words: sclerostin, liver transplant, bone resorption, bone formation, vitamin D deficiency.

INTRODUCTION
Solid organ transplantation is an effective alternative in the final stage of multiple chronic diseases, increasing patients’ survival. However, this improvement is associated with certain complications, such as a higher incidence of osteoporosis and an increased risk of fractures. Numerous studies have concluded that there is a loss of bone mass after transplantation, more marked between the first three and six months, which lasts up to a year after the same. Subsequently there is a stabilization and even recovery of bone mass in the two subsequent years.

Liver transplantation is considered an independent risk factor in the development of osteoporosis. In the case of patients with a liver graft, the incidence of fracture is estimated at 10-43%, with the spine location being the most frequent. Among the factors that contribute to the increased risk of osteoporosis and fractures in these patients are: prolonged treatment with immunosuppressants (mainly calcineurin inhibitors) and glucocorticoids, vitamin D deficiency (very common due to malnutrition) and alterations in liver function found in most patients with cirrhosis.

The biochemical markers of bone remodeling offer information based on the dynamic prediction of the same, its accepted clinical application being the evaluation of the therapeutic response with antiresorptives.
and its potential relationship with the risk of fracture. However, at present, there is no consensus regarding the determination of biochemical markers of bone remodeling in patients with liver transplantation. Sclerostin (SOST) is a protein synthesized by the osteocyte that plays a central role in the regulation of bone remodeling, since it simultaneously acts as a negative regulator of bone formation and stimulates bone resorption through the RANK-ligand. Its usefulness as a biochemical marker of bone remodeling, particularly in liver transplant patients, has not been established.

Thus, our study aims to assess the development of sclerostin levels in patients with liver transplantation, and investigate their relationship with other markers of bone remodeling.

**Patients and Methods**

**Study design and patient selection**

This is a prospective observational study, developed from 2015 to 2017, in a single center: the University Hospital 12 de Octubre (Bone Metabolism Unit of the Endocrinology and Nutrition Service). We included 83 Caucasian patients, fulfilling the condition of being candidates for a liver transplant (regardless of the etiology of the liver disease). Patients who had received drugs that could interfere with bone remodeling prior to transplantation were excluded. The center’s Ethics Committee approved the study and a signed informed consent was obtained from all the participants. In all patients, a descending steroid regimen was used up to a maintenance dose of prednisone of 20 mg over the first six months (as part of the usual center transplant protocol). The SOST, β-crosslaps (CTX), bone alkaline phosphatase (BAP), osteocalcin (OC) and C-reactive protein (CRP) values were determined the week before the transplant and subsequently, at 1, 3, 6 and 12 months. The determination of 25 hydroxy-vitamin D [25 (OH) D] and intact parathyroid hormone (PTH) was carried out basally. Likewise, in each of the reviews, the existence of fractures was evaluated.

**Biochemical determinations**

The patients’ serum samples were obtained between 8:00 and 9:00 hours, after an overnight fast, and they were kept frozen at -70°C. Bone metabolism markers included: OC (Cobas e602, electrochemiluminescence, normal range: 8-48 ng/ml) and BAP (IDS, Roche Diagnostics, normal range: 4.0-20.0 ng/ml) as parameters of bone formation, and CTX (Cobas e602, electrochemiluminescence, normal range: 0.200-0.704 ng/ml) as a resorption marker. Likewise, SOST was determined by enzyme immunoassay (Human Sclerostin, TECO Medical Group, normal range: 0.22-1.1 ng/ml). PTH levels were determined by electrochemiluminescence (Cobas e602, normal range: 7.0-57.0 pg/ml). Serum levels of 25 (OH) D were determined by chemiluminescence (Architect 2000, Abbot Diagnostics). Although there is currently no criterion on the optimal serum levels of 25 (OH) D, most authors define a deficiency of values below 20 ng/ml as deficiency. Serum levels between 21 and 29 ng/ml can be considered as relative insufficiency, and higher than 30 ng/ml indicate sufficiency of the same. PCR was determined by immunoturbidimetry (C-Reactive Protein Gen.3, Roche Diagnostics, normal range less than 0.1-0.5 mg/dL).

**Fractures**

In each of the revisions made to the patients, the existence of fractures was evaluated by means of clinical examination made to the patient and re-evaluation of risk factors for them. In the case of suspicion of asymptomatic or pauci-symptomatic osteoporotic fractures (such as vertebral crushing), a thoracolumbar radiography was carried out.

**Statistical analysis**

The statistical analysis was carried out using the Statistical Package for the Social Sciences, SPSS (version 21.0, IBM, Armonk, New York, USA). The normal distribution was confirmed by the Shapiro Wilks test. The evolution of the markers with respect to the baseline value was determined by the t-Student test. All results were expressed as mean ± standard deviation (SD). A p-value of less than 0.05 was considered statistically significant.

**Results**

A total of 83 patients receiving a liver transplant (56 males and 27 females) were included in the study. The average age of the patients was 56.2±10.4 years. The group’s average weight was 72.1±18.7 kg and its BMI was 27.7±7.5 kg/m².

**Evolution of markers of bone remodeling**

The basal levels of SOST were 0.76±0.35 ng/ml and decreased significantly early (0.55±0.22 ng/ml in the first month, p=0.034), a trend that was maintained until the end of the study. Of the study, the basal (0.62±0.22 ng/ml, p=0.047) (Table 1). There were no significant differences between both sexes in the evolution of the aforementioned marker. The SOST levels did not correlate with the development of fractures. On the contrary, OC levels (1.77±0.35 ng/ml) showed a progressive and significant increase from the 3rd month after transplantation (31.85±26 ng/ml, p<0.01), which it was maintained until the end of the follow-up period (37.27±26.84 ng/ml, p<0.01). In both sexes, the evolution of OC levels was similar.

In relation to CTX, the levels prior to transplantation were 0.44±0.35 ng/ml. One month after transplantation, a significant increase was observed (0.81±0.47 ng/ml, p<0.01) that persisted until 6 months (0.62±0.34 ng/ml, p<0.01) compared to basal level. At 12 months, there was a marked decrease in CTX towards the value before transplantation (0.47±0.31 ng/ml, p=0.2). There were no differences regarding CTX development between both sexes but, since the third month, the group of women had significantly higher levels than men (0.94±0.62 ng/ml and 0.61±0.34 ng/ml, respectively, p<0.01). The levels of PA presented discrete variations throughout the study, without showing significant changes in any of the determinations (Table 1), nor differences between sexes.

There were no statistically significant correlations between the different markers of bone metabolism in the study.

**Vitamin D and PTH**

At the time of transplantation, 25 (OH) D levels were in the deficiency range: 10.44±6.5 ng/ml. 82.1% of the patients had deficiency (levels of 25 (OH) D <20 ng/ml) and 17.9% levels of relative insufficiency. As for the PTH, the initial mean value was slightly above the high limit...
of the normal range (78.8±52 pg/ml). No correlations of interest were found between the serum values of PTH and 25(OH)D and the markers of bone remodeling included in the study.

### Inflammation
C-reactive protein (CRP) levels were elevated before transplantation (4.77±3.8 mg/dL). After the intervention, there was a progressive and significant decrease during the first 3 months of the study, up to a figure of 1.3±3.5 mg/dL (p<0.005).

### Fractures
Throughout the year of follow-up, 3 fractures were observed: a vertebral crush, and 2 Colles fractures (one of them after a trauma in an accident). There was no statistically significant correlation between the development of fracture after transplantation and the different markers of bone metabolism.

**DISCUSSION**
In recent years it has been proposed that SOST, glycoprotein of 213 amino acids secreted by the osteocyte, and that produces an inhibition of bone formation by suppressing the Wnt/β‐catenin intracellular signaling pathway, could be a biomarker of central importance in the evaluation of bone remodeling. However, there is little information about SOST levels after a solid organ transplant, although patients undergoing this procedure suffer osteoporosis very often and, in particular, vertebral fractures. Thus, in a study carried out on bone biopsies of patients undergoing different types of transplantation (kidney, liver, heart), an increase in SOST expression evaluated by immunohistochemistry has been described.

In the present observational prospective study, the evolution of the levels of sclerostin (SOST) and other markers of bone remodeling during 12 months after a liver transplant was evaluated. Our results show a significant decrease of SOST after a solid organ transplant, although patients undergoing this procedure suffer from osteoporosis very often and, in particular, vertebral fractures. Thus, in a study carried out on bone biopsies of patients undergoing different types of transplantation (kidney, liver, heart), an increase in SOST expression evaluated by immunohistochemistry has been described.

In a study previously carried out in kidney transplant patients, men had a similar SOST level than women before surgery. Similarly, in our study we did not observe any difference between SOST serum concentrations in both sexes, neither at the beginning of the study nor during the follow-up.

### Table 1. Development of sclerostin and the rest of the markers of bone remodeling throughout the study (mean ± standard deviation)

<table>
<thead>
<tr>
<th></th>
<th>Basal</th>
<th>1st month</th>
<th>3rd month</th>
<th>6th month</th>
<th>12th month</th>
</tr>
</thead>
<tbody>
<tr>
<td>OC (ng/ml)</td>
<td>17 ± 10.35</td>
<td>17.95 ± 12.40</td>
<td>31.85 ± 26</td>
<td>35.75 ± 32.63</td>
<td>37.27 ± 26.84</td>
</tr>
<tr>
<td>AP (ng/ml)</td>
<td>34.87 ± 17.8</td>
<td>30.16 ± 13.77</td>
<td>27.97 ± 11.93</td>
<td>42.07 ± 21.23</td>
<td>31.05 ± 11.41</td>
</tr>
<tr>
<td>CTX (ng/ml)</td>
<td>0.44 ± 0.35</td>
<td>0.81 ± 0.47</td>
<td>0.72 ± 0.48</td>
<td>0.62 ± 0.34</td>
<td>0.47 ± 0.31</td>
</tr>
<tr>
<td>SOST (ng/ml)</td>
<td>0.76 ± 0.35</td>
<td>0.55 ± 0.22</td>
<td>0.63 ± 0.23</td>
<td>0.63 ± 0.30</td>
<td>0.62 ± 0.22</td>
</tr>
</tbody>
</table>

OC: osteocalcin; AP: alkaline phosphatase; CTX: β-crosslaps; SOST: sclerostin.

events, although it does occur with the presence of vascular calcifications.

In renal transplantation, one of the main factors that justify the initial reduction of SOST could be an increased loss of urine due to tubular dysfunction due to overload, typical of the initial period after transplantation. In the case of the liver, previous studies have shown that in certain diseases that may require transplant, such as primary biliary cirrhosis, patients had increased sclerostin levels. Among the possible factors that would justify the initial decrease in SOST could be the improved pro-inflammatory situation after surgery and immunosuppression.

Regarding inflammation, in our study CRP levels were basally elevated and decreased significantly throughout the year (although there was no correlation between the value of SOST and that of CRP). However, in the literature there are examples in which the possible relationship between SOST and inflammation has not been confirmed. Thus, in a previous study in patients with rheumatoid arthritis treated with TNF inhibitors (tumor necrosis factor) no effect of anti-inflammatory treatment on the levels of SOST was evidenced.

The decrease in SOST observed in the patients of our study could be one of the causes that justifies the improvement in mineral metabolism after the intervention, just as it has been considered in patients undergoing a kidney transplant. One of the proposed hypotheses is that the changes in the SOST would reflect the optimization of osteocyte function after transplantation.

In a study previously carried out in kidney transplant patients, men had a similar SOST level than women before surgery. Similarly, in our study we did not observe any difference between SOST serum concentrations in both sexes, neither at the beginning of the study nor during the follow-up.

Regarding the rest of the bone remodeling markers, in our study we observed an increase in markers of bone formation (OC) as well as those of bone resorption (CTX). These results are consistent with those already presented in the literature, after liver transplantation. Recently, an increase in CTX and N-terminal propeptide of type I procollagen (P1NP) was observed 6 months after liver transplantation. These results, similar to those of our group, seem to confirm the existence of a high bone remodeling in patients undergoing a transplant. In this context, it is worth highlighting the influence of steroids (as part of the treatment after transplantation), favoring resorption and suppressing bone formation, especially during the first six months after surgery.
In addition to the determination of bone remodeling markers, in our study a high 25 (OH) D deficiency rate prior to transplantation (82.1%) was also confirmed. After adequate supplementation, a significant improvement in 25 (OH) D levels was observed, until the mean value was placed in the insufficiency range. In parallel, slightly elevated levels of PTH were observed (in a probable context of hyperparathyroidism secondary to vitamin D deficiency) before surgery, with normalization at 12 months, after improvement in the 25 (OH) D figures. There are multiple factors that influence the insufficient levels of 25 (OH) D prior to transplantation: proinflammatory state, higher prevalence of malnutrition, loss of liver contribution by hydroxylation of its precursor, etc. Several previous studies have reported high rates of 25 (OH) D deficiency, although not as high as those shown in our cohort. Thus, in a group of patients undergoing liver transplantation, a deficiency rate of 25 (OH)D of 37% was observed, improved at 6 months, with a deficiency rate of 17%.25

Our study presents several strengths. First, its optimal sample size (n=83) and the fact that this is a longitudinal and prospective study. Finally, it is the first study that includes SOST determination after liver transplantation. Despite this, the study has certain limitations. Bone mineral density was not evaluated, nor the etiology of the liver disease that motivated the transplant. On the other hand, the low number of fractures observed does not allow us to draw conclusions about the real impact of these changes on the risk of post-transplant osteoporosis26.

In summary, our results show a decrease in SOST levels after liver transplantation, which goes in the opposite direction to the variations observed in other remodeling markers such as CTX and OC. Deficiency of 25 (OH) vitamin D pre-transplant is high and improves after supplementation. More studies are needed to determine if these changes have a significant impact on the occurrence of osteoporosis or long-term cardiovascular disease in patients undergoing transplantation.

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Different development of serum sclerostin compared to other bone remodeling markers in the first year after a liver transplant


Free vitamin D: an increasing determination

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Summary

Vitamin D has long been a familiar concept for any professional working in clinical biology. Nowadays it is becoming known to a large sector of the population. The great research efforts developed over the past decade have led to an explosion in the number of determinations requested of the most qualified metabolite to express the body state of vitamin D, 25-OHD free, represents the small fraction not bound to transporter proteins. According to the free hormone hypothesis, it should be considered the best representation of the body state of vitamin D. Unfortunately, limited attention has been paid to this determination since, until recently, the scientific community only had tedious, indirect methods to measure it.

A few years ago, a direct measurement method of free 25-OHD was already available to carry out research studies with promising results.

Key words: colorectal cancer, aging, osteoporosis, pregnancy, infertility, 25-hydroxyvitamin D, 25 hydroxyvitamin D free, 1,25-dihydroxyvitamin D.

INTRODUCTION

In recent decades, vitamin D has attracted growing interest, not only in the medical field, but also among the general population. Initially, the evaluation of vitamin D was part of bone metabolism assessment when, for example, rickets or osteomalacia were suspected, or in populations at risk of osteoporosis¹. 25-hydroxyvitamin D (25-OHD) is the circulating metabolite of higher concentration and longer half-life, used to monitor the body status of vitamin D. Patients with chronic kidney disease and undergoing dialysis treatment are also controlled by measurements of the evaluation of this state². In this case, in addition to 25-OHD, the active metabolite of vitamin D, 1,25-dihydroxyvitamin D (1,25-(OH)²D), produced mainly in the cells of the proximal tubule of the nephron.

In the past, vitamin D measurements made by laboratories were limited and almost always for research purposes. For about ten years the situation has changed. The request for determinations has drastically increased and more than 4,500 articles³ on vitamin D are currently published every year, and the general public has been sensitized. This is due to the knowledge of the involvement of vitamin D metabolites in multiple physiological processes⁴, its association with various diseases and the dissemination of clinical studies, as well as the dissemination of the concept of ‘vitamin of the sun’ for the general population.

Currently, about 90% of the determinations requested and carried out in the laboratory refer to the total 25-OHD form. A concentration below 20 ng/mL is accepted as a deficiency, and a concentration below 30 ng/mL is considered insufficient. The ideal levels are higher than 30 ng/mL, while the toxicity levels are above 100-150 ng/mL. This topic is currently the subject of debate, and real cases of intoxication have been described, mainly due to formulation errors and/or errors in the daily intake⁵,⁶. The remaining 10% of the requests for metabolites of vitamin D are from the active metabolite, 1,25-(OH)²D, which are largely requested by mistake, due to confusion between both metabolites by the prescriber⁷. These test results, carried out to evaluate the vitamin D endocrine system in healthy and sick populations, remain controversial for numerous reasons⁸.

Several new trials have been developed to facilitate the work of researchers and clinicians. The C₃ epimer, a stereo-isomer of 25-OHD, can now be easily measured by liquid chromatography-mass spectrometry (HPLC-MS/MS), although its clinical importance is not clearly understood⁹. 24,25-dihydroxyvitamin D (24,25-(OH)²D), which is a catabolite of 25-OHD, can also be measured by HPLC-MS/MS methods, and is useful in the diagnosis of idiopathic childhood hypercalciemia¹⁰. The measurement of bioavailable and free 25-OHD levels have also been incorporated as new markers of vitamin D status and, although the concept has been known since the 1980s, it has not been used regularly, due to the lack of an adequate method for its quantification¹¹.
Physiology of Free Vitamin D

The free 25-hydroxyvitamin D (free 25-OHD) represents the fraction of 25-OHD that is not bound to vitamin D binding proteins. Due to its hydrophobic nature, the metabolites of vitamin D, especially 25-OHD, bind to transporter proteins. The main one is the vitamin D binding protein (VDBP or DBP), also known as GC-globulin, which binds to all the metabolites of vitamin D but with greater affinity for 25-OHD, binding approximately 90% of its circulating concentration. Albumin, due to its high concentration in blood, although with a much lower affinity than VDBP for 25-OHD, binds the remaining 10%12. A small fraction, less than 0.1% of the total, circulates freely, and not bound13. The sum of free 25-OHD and the bound fraction to albumin is called bioavailable 25-OHD, since it is believed that the low-affinity albumin-25-OHD complex allows 25-OHD molecules to be readily available for produce their biological effects14.

However, this concept tends to be abandoned in favor of the free hormone hypothesis (Figure 1).

Free Hormone Hypothesis

The hypothesis of the free hormone states that a hormone’s biological activity is affected by its concentration not linked to protein (free) instead of the concentration bound to plasma proteins. In 1989, Mendel proposed that this hypothesis “is probably valid with respect to all tissues for thyroid hormones, cortisol and hydroxylated metabolites of vitamin D”15. Later, Chun et al.16 conjectured that “the binding of 25-OHD to VDBP hinders the delivery of 25-OHD to the target tissues, which ultimately prevents its metabolism to the active form, 1,25-(OH)2D. On the contrary, it is the unbound and free form that can pass through the cell membrane and, therefore, exercise the biological actions” (Figure 2).

Figure 1. Vitamin D free and bioavailable: 25-OH free vitamin D, bioavailable, and total. Union of 25-OH vitamin D to albumin and DBP (vitamin D carrier protein)

Figure 2. Transport mechanisms: Mechanisms mediated by membrane receptor and receptor-independent for the cellular uptake of vitamin D. Vitamin D metabolites bind to DBP in serum and extracellular fluid. The entrance to the cell of metabolites vitamin D can occur through one of the different mechanisms of the four described in the scheme.
This free hormone hypothesis has been validated clinically for thyroid hormones, with the emergence of trials for free T3 and T4. These trials have replaced in practice most of the total T3 and T4 determinations that were previously made in clinical laboratories. The story is similar, although to a lesser extent, for testosterone and cortisol. For vitamin D, the situation is somewhat different, as the metabolite measured (25-OHD) is not the hormonally active form (it is 1,25-(OH)2D). However, as Chun et al. previously reported, the conversion of 25-OHD to 1,25-(OH)2D and, consequently, the biological activity of vitamin D, is highly influenced by the concentration of 25-OHD free in the plasma.

**Methods for the measurement of free 25-OHD**

Historically, free 25-OHD and 1,25-(OH)2D metabolites were measured by centrifugal ultrafiltration and equilibrium dialysis using the tritiated metabolite, which constitutes the classical immunoassay for measuring total concentrations. Simple equations have been used to calculate the free hormone concentration and, although these methods provide reliable results, they are extremely complicated to assemble. They require considerable time and a large sample volume, in addition to involving the handling of material marked with tritium and/or carbon-14. Therefore, other methods of quantification have been developed over time, based on the measurement of the total metabolite of vitamin D and the binding protein concentrations, using the related affinity constants. While in clinical laboratories the quantification of albumin and total 25-OHD are part of the routine analysis, the quantification of VDBP is based on kits for use only in research (Research Use Only, RUO), usually not validated. Trials using monoclonal antibodies have proved unreliable, since they do not measure the different polymorphisms of VDBP equally.

However, trials and techniques that employ radial immunodiffusion using polyclonal antibodies produce excellent results. As different VDBP forms are found in the different serum samples to be evaluated, and although this is still a matter of debate since these different forms have different binding coefficients for 25-OHD, the calculation of each sample should ideally include the genotyping of the patient to use the appropriate coefficient. In clinical practice, this is done very exceptionally.

In 2017, a new direct method was developed and made available to the scientific community. The amount of free 25-OHD is measured by the enzyme-linked immunosorbent assay (Enzyme-Linked ImmunoSorbent Assay). The separation of the free and bound forms, as well as the capture of the former, is achieved through the use of a monoclonal antibody (anti-25-OHD), altering as little as possible the balance between both forms, having validated the precision, sensitivity, accuracy and specificity of the assay.

On the other hand, this methodology generates results similar to those obtained by centrifugal ultrafiltration and has been used successfully in many clinical studies. The test obtained the European Union (CE) European Union mark of conformity in 2018, which allows its use in *in-vitro* diagnostic laboratories (IVD).

**25-OHD free in clinical studies**

Recently, Tsuprykov et al. published a direct measurement method for free and total 25-OHD in a cohort of 297 healthy pregnant Caucasian women (gestational age ranged from the fourth week to the fortieth week), along with the 25-Total OHD, and other parameters (Table 1). Free 25-OHD correlated better than the total with various parameters, so it was concluded that optimal monitoring of vitamin D status in pregnant women should consist of free 25-OHD measurements at the beginning and end of pregnancy.

The same year, Franasiak et al. studied free 25-OHD in a small group of infertile patients. Free 25-OHD was calculated using the DBP data obtained with an antibody-based assay, and showed statistically significant differences between the groups of infertile and control (6.3±2.9 pg/mL vs. 4.3±1.8 pg/mL), differences that were not observed for total 25-OHD (30.3±9.8 ng/mL vs. 28.9±8.7 ng/mL). These results are explained by the lower concentration of DBP in the infertile group, which influences the balance between the free and bound forms of 25-OHD. Although these data only represented a pilot study, they concluded that the quantification of total 25-OHD can be misleading when assessing the status of vitamin D in situations of infertility.

Free 25-OHD has also been studied in elderly healthy African-American women over 60 years of age, analyzing the relationship between physical performance and osteoporosis prevention with vitamin D in this population. This is a 3-year, randomized, double-blind, placebo-controlled study that examined the effect of vitamin D on physical performance and bone loss in 260 women. Free 25-OHD significantly predicted the grip strength in a linear regression model (R²=0.02, F=5.22, regression coefficient [β]=1.52, p=0.023), suggesting that for each increase of 1 pg/mL of free 25-OHD produced an increase in the grip strength of 1.52 lb, this association not being found for the total 25-OHD. These results suggest the usefulness of free 25-OHD as a predictor of physical performance with the aging of African-American women. The association of free 25-OHD with performance measures of the upper and lower extremities supports further examination of the role of serum-free 25-OHD in physical performance to prevent frailty and fractures in older adults.

Another application, in oncology, was proposed by the Yang group. The aim of this study was to exhaustively evaluate the prognostic value of VDBP, total and free 25-OHD and its bioavailability in patients with colorectal cancer in stages I-III. The results showed that the elevation of free 25-OHD and its bioavailability was significantly associated with a better survival at 5 years, after...
Table 1. Metabolites of vitamin D 25-OH total vitamin D, 25-OH free vitamin D and 1,25 (OH) 2 vitamin D with pregnancy parameters and other biochemical parameters. In bold, the results are statistically significant

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Total 25(OH)D (ng/mL)</th>
<th>25(OH)D free (pg/mL)</th>
<th>1,25 (OH) 2D total (pg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gestational age (days)</td>
<td>p=0.957</td>
<td>p=0.003</td>
<td>p&lt;0.001</td>
</tr>
<tr>
<td>Maternal age (years)</td>
<td>p&lt;0.001</td>
<td>p&lt;0.001</td>
<td>p=0.564</td>
</tr>
<tr>
<td>PTH (pg/mL)</td>
<td>p&lt;0.001</td>
<td>p=0.010</td>
<td>p=0.014</td>
</tr>
<tr>
<td>Calcium (mmol/L)</td>
<td>p=0.238</td>
<td>p=0.006</td>
<td>p&lt;0.001</td>
</tr>
<tr>
<td>Phosphate (mmol/L)</td>
<td>p=0.119</td>
<td>p=0.920</td>
<td>p=0.867</td>
</tr>
<tr>
<td>Alkaline phosphatase (µg/mL)</td>
<td>p=0.037</td>
<td>p&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>Albumin (g/dL)</td>
<td>p=0.101</td>
<td>p=0.010</td>
<td>p&lt;0.001</td>
</tr>
<tr>
<td>LDL (mg/dL)</td>
<td>p=0.527</td>
<td>p&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>Urea (mg/dL)</td>
<td>p=0.860</td>
<td>p=0.028</td>
<td>p&lt;0.001</td>
</tr>
<tr>
<td>Adiponectin (µg/mL)</td>
<td>p=0.302</td>
<td>p=0.009</td>
<td>p=0.001</td>
</tr>
<tr>
<td>Sodium (mmol/L)</td>
<td>p=0.335</td>
<td>p=0.505</td>
<td>p=0.588</td>
</tr>
<tr>
<td>Vitamin B12 (pg/mL)</td>
<td>p=0.055</td>
<td>p&lt;0.001</td>
<td>p&lt;0.001</td>
</tr>
<tr>
<td>TSH (uU/mL)</td>
<td>p=0.319</td>
<td>p=0.089</td>
<td>p=0.816</td>
</tr>
<tr>
<td>Free thyroxine (ng/dL)</td>
<td>p=0.183</td>
<td>p=0.033</td>
<td>p&lt;0.001</td>
</tr>
<tr>
<td>Triiodothyronine (pg/mL)</td>
<td>p=0.028</td>
<td>p=0.001</td>
<td>p=0.401</td>
</tr>
<tr>
<td>HDL (mg/dL)</td>
<td>p=0.001</td>
<td>p=0.449</td>
<td>p&lt;0.001</td>
</tr>
<tr>
<td>LDL/HDL ratio</td>
<td>p=0.161</td>
<td>p=0.003</td>
<td>p&lt;0.001</td>
</tr>
<tr>
<td>Vitamin B6 (ng/mL)</td>
<td>p=0.024</td>
<td>p&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>Zinc (µmol/L)</td>
<td>p=0.822</td>
<td>p=0.090</td>
<td>p&lt;0.001</td>
</tr>
<tr>
<td>Hemoglobin (g/dL)</td>
<td>p=0.382</td>
<td>p=0.065</td>
<td>p=0.001</td>
</tr>
<tr>
<td>Hematies (10⁶/µL)</td>
<td>p=0.841</td>
<td>p=0.313</td>
<td>p=0.002</td>
</tr>
<tr>
<td>Leukocytes (10⁷/µL)</td>
<td>p=0.789</td>
<td>p=0.024</td>
<td>p&lt;0.001</td>
</tr>
</tbody>
</table>

Figure 4. Relationship between free 25-OHD and grip strength

r=0.14, p=0.023
carrying out a univariate statistical analysis. Upon completion of the study using a multivariate Cox analysis, they also found that high levels of free 25-OHD (HR=0.442, 95% CI: 0.238-0.819, p<0.010) could be identified as an independent factor to predict better survival. In conclusion, the study suggested that higher levels of free and bioavailable 25-OHD were associated with greater survival in patients with colorectal cancer in stages I-III. In addition, free 25-OHD could be considered as an independent prognostic biomarker for survival. More recently, many other articles have been published, including reviews and clinical studies, which highlight the importance of free 25-OHD.\(^\text{21}\)

**Declaration of interests:** Nicolas Heureux works for DIASource ImmunoAssays. José Manuel Quesada Gómez declares no conflict of interest.

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3. Búsqueda en el PubMed realizada el 1 de marzo de 2018, con la palabra clave ‘Vitamin D’.