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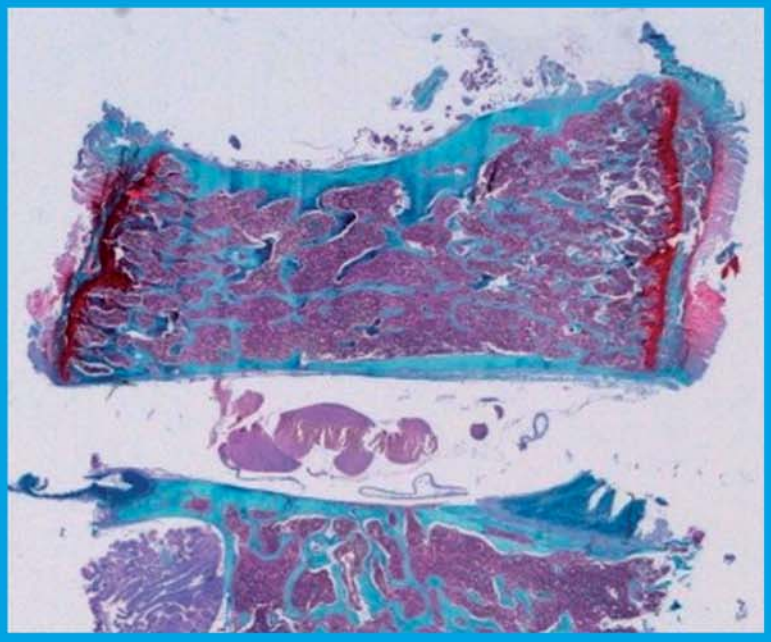

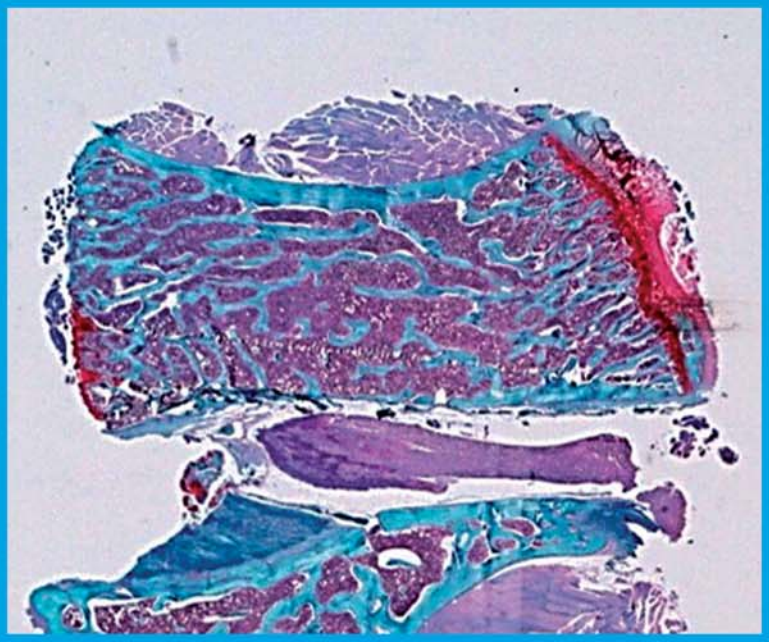

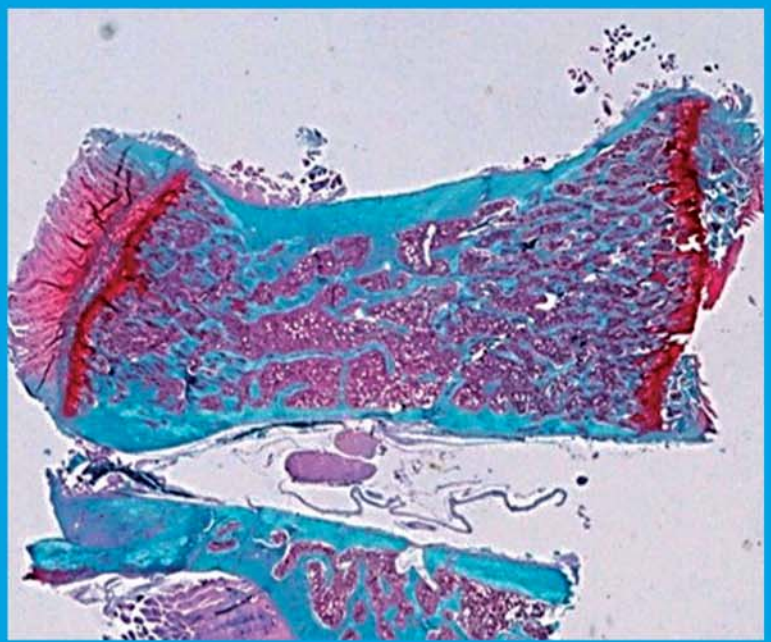

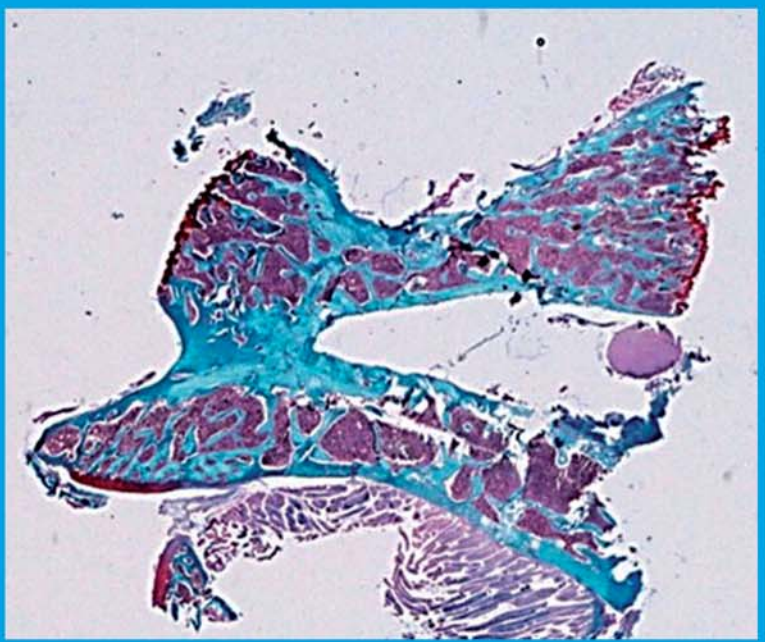

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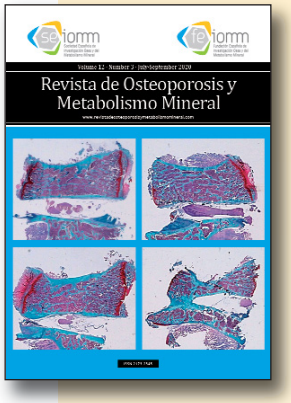

Our cover: Staining with 4x safranin. Trabecular components of the L4 vertebra in orchidectomized, OPG-Fc-treated and testosterone-treated orchidectomized SHAM rats.
Author: Concepción de la Piedra Gordo. Research Biochemistry Department. Institute of Medical Research. Jiménez Díaz Foundation. Madrid (Spain).

Sociedad Española de Investigación Ósea y del Metabolismo Mineral (SEIOMM)

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Velázquez, 94 (1ª planta)
28006 Madrid (Spain)

Telf: +34-648 949 755

seiomm@seiomm.org

www.seiomm.org

Editing

Ibáñez & Plaza Asociados, S. L.
EDITORIAL TÉCNICA Y COMUNICACIÓN

Avda. Reina Victoria, 47
28003 Madrid (Spain)
Telf. +34-915 538 297
correo@ibanezyplaza.com
www.ibanezyplaza.com

Graphic design
Concha García García

English translation
David Shea

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Submit originals:
romm@ibanezyplaza.com

Summary

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The importance of identifying intrinsic and modifiable risk factors for falls in order to act early prevention measures

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Formiga F[1], Tarazona-Santabalbina FJ[2]
1 Internal Medicine Service. Bellvitge Hospital. Bellvitge Biomedical Research Institute (IDIBELL). University of Barcelona. L´Hospitalet de Llobregat. Barcelona (Spain)
2 Center for Biomedical Research on the Net Frailty and Healthy Aging (CIBERFES). Geriatrics Service. University Hospital of the Ribera. Alzira. Valencia (Spain)

Falls in the elderly constitute one of the main public health problems, both due to their prevalence and consequences. One of the most serious is the fracture of the femur. The annual prevalence of falls in the over 65 population ranges between 28% and 35%, and these falls are frequently repeated[1]. The factors responsible for a fall are divided into intrinsic (related to the patients themselves) and extrinsic (derived from the activity or the environment), the cause being multifactorial in most cases[1]. When assessing the intrinsic factors of a fall, we must take into account the physiological disorders related to age (including the presence of nutritional alterations, sarcopenia and frailty), acute and chronic diseases and the prescription of certain drugs[1]. For this reason, when faced with a fall, it is essential to have a comprehensive approach to the adult through a global geriatric assessment that includes a complete assessment of gait and balance.

We mention frailty, which ,may be erroneously considered another word for disability and comorbidity. However, frailty is a potentially reversible situation in which there is a progressive decrease in the physiological reserve capacity and in the adaptation capacity of the body's homeostasis (homeostenosis) that occurs especially with non-physiological aging. Frailty, as a clinical entity, is influenced by (individual) genetic factors and is accelerated by acute and chronic diseases, toxic habits, disuse, and social and care conditioning factors. There are currently two fundamental approaches to frailty: a functional and restrictive one, proposed from the Linda Fried phenotype, according to which frailty would be a state prior to disability but different from it, assessed by five components (weight loss, tiredness, weakness, psychomotor slowdown and hypoactivity). There is another, broader but less defined conception in terms of a less clear differentiation of frailty and disability, and in which frailty would be attributed to an accumulation of deficits (Rockwood cumulative indices of frailty).

Between these two positions, there is an important multitude of intermediate options[2].

By the same token, there may be an interaction, and overlap, between the presence of frailty and sarcopenia. With age, starting at thirty years old, there is a progressive loss of skeletal muscle mass and strength[3]. To advance its understanding, in 2010 the European Working Group on Sarcopenia in the Elderly published a document that set out a practical clinical definition and consensus diagnostic criteria for age-related sarcopenia. Thus, the diagnosis of sarcopenia is based on the confirmation of low muscle mass (criterion 1) plus one of the following: low muscle strength (criterion 2) or low physical performance (criterion 3).

This group has carried out an update of its consensus in which greater attention is given to muscle strength as key data in sarcopenia (relegating the measurement of muscle mass to a point of research rather than of use in clinical practice), it modifies the diagnostic algorithm and establish clear cut-off points for diagnosis. In addition, the use of the SARC-F questionnaire as a screening tool is recommended[3]. Currently, the importance of osteosarcopenia as a phenotype resulting from the combination of sarcopenia and low bone mineral density is being highlighted, and that an increased risk of falls and fractures would be associated[4].

For all these reasons, studies such as the one by Rodriguez-García et al.[5] that assess risk factors, such as frailty and sarcopenia, on the risk of falls and osteoporotic fractures in the real world are always welcome. The authors randomly evaluate 624 inhabitants (308 men and 316 women) older than 50 years (mean age 65 years, with a long follow-up period of 8 years –high percentage of follow-up at the end of the study–) and calculate the incidence of falls and non-vertebral osteoporotic fractures. In the baseline evaluation, the grip strength in the hands was measured and a questionnaire was completed with clinical variables, risk factors related to osteoporosis and questions related to difficulty or inability to carry out daily activities. Falls were reported in 44.9% of women and 23.5% of men, and non-vertebral fractures were reported in 13.2% of women and 2% of men. The incidence of falls increased with age and they were more common in women[1]. Grip strength in hands was not associated with the incidence of falls or fractures.

However, the impossibility or difficulty of: “sitting for more than 1 hour in a hard chair”, “taking off socks or stockings” or “leaning from a chair to pick up an object

Correspondence: Francesc Formiga (fformiga@bellvitgehospital.cat)

from the floor" were associated with the presence of falls. Furthermore, the impossibility or difficulty of "carrying a 10-kilo object for 10 meters" and "lifting a box with 6 bottles and putting them on a table" was associated with fracture. The authors conclude that there is an association between difficulty or inability to perform daily activities and the presence of fractures and between activities related to functional capacity and the presence of falls.

The study has strength (long follow-up, few losses) and some weakness that the authors recognize (especially the dynamic course of both frailty and sarcopenia over time) and others that we will comment on, probably derived from the fact that the initial study protocol did not was designed specifically to answer the question in the title of the article, but to know the prevalence of vertebral fracture at the European level. Thus, it would probably have been more advisable to use tools to diagnose the presence or absence of more consensual frailty or sarcopenia. The article also does not define what was considered a fall or who reported it (participant, caregiver, both?). Or whether there were associated extrinsic factors. It is also important to reflect, when analyzing the results of the study, on the importance of an older age of the participants (for example, 75 years) in the results obtained: greater number of falls, worse results of the evaluations carried out and a greater chance of finding associations.

The positive reality informs us that the number of elderly people who are being evaluated for specialties other than geriatrics is increasing. This implies the need to apply the principles of geriatric medicine to advance together using the same language[6]. The association between falls and frailty or sarcopenia is increasingly recognized in the literature[7,8], but we must speak of the same entities and we must standardize the assessment tests. In this specific case, it is even more important, because both situations present considerable possibilities of reversal, mainly through multi-component exercise programs and through adequate and individualized nutritional intervention. Therefore, to avoid the incidence of falls and its consequences, it is essential to implement multi-factorial assessment and intervention programs[9,10]. The final message that we must not forget is that, in the presence of a fall, we should always evaluate the elderly and intervene whenever necessary and possible.

Conflict of interests: Authors declare no conflict of interests.

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Effect of frailty and sarcopenia on the risk of falls and osteoporotic fractures in an unselected population

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Rodríguez-García M[1], Gómez-Alonso C[2], Rodríguez-Rebollar A[3], Palomo-Antequera C[4], Martín-Vírgala J[2], Martín-Carro B[2], Fernández-Villabrille S[2], Rodríguez-Carrio J[2,5], Cannata-Andía JB[2], Naves-Díaz M[2]

1 Nephrology Clinical Management Area
2 Bone Metabolism Clinical Management Unit
3 Medicine Laboratory
4 Internal Medicine Clinical Management Unit
5 Basic and Translational Research in Chronic Inflammatory Diseases

Central University Hospital of Asturias. University of Oviedo, Institute of Health Research of the Principality of Asturias (ISPA). Renal Research Network of the Carlos III Health Institute (ISCIII). Oviedo (Spain)

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Summary

Objetive: Assess whether grip strength and difficulty in carrying out daily activities could be predictors of falls and osteoporotic fractures.

Material and methods: 624 men and women over 50 years of age were randomly selected and followed for 8 years to determine the incidence of falls and non-vertebral osteoporotic fractures. At the beginning, the grip strength in the hands was measured and a questionnaire was filled out with clinical variables, risk factors related to osteoporosis, and questions related to difficulty or inability to perform daily activities.

Results: Grip strength in the hands was not associated with the incidence of falls and fractures. However, the impossibility or difficulty of "sitting for more than 1 hour in a hard chair", "taking off socks or stockings" and "leaning from a chair to pick up an object from the floor" were associated with falls: 1.83 (1.16-2.89); 1.85 (1.14-3.00) and 1.68 (1.04-2.70), respectively. Similarly, the impossibility or difficulty of "carrying a 10-kilogram object for 10 meters" and "lifting a box with 6 bottles and putting them on a table" was associated with fracture: 2.82 (1.21-6.59) and 2.54 (1.12-5.81) respectively.

Conclusions: No association was found between grip strength and incidence of falls and osteoporotic fractures, but it was found with difficulty or inability to perform daily activities. Those related to greater strength were associated with fracture, while those related to functional capacity were associated with falls. Taking simple questionnaires could help predict events before they happen.

Key words: osteoporotic fracture, sarcopenia, frailty, falls, daily activities.

Introduction

Life expectancy has increased rapidly in the last century due to economic growth. This has led to reduced mortality, improved quality of life, as well as greater availability of health care. In fact, there are more elderly people than at any other time in our history, and it is anticipated that within the next few years there will be more older adults than children. This forecast makes it essential for people to reach this age in good health, to avoid increased healthcare costs due to longer hospital stays, readmissions and demand for healthcare resources. One of the most common disorders associated with aging is osteoporosis, the most fatal consequence of which is fracture. Approximately half of the clinical fractures that occur in postmenopausal women do not present criteria for osteoporosis according to their bone mineral density[1]. In fact, the highest percentage of fractures occur in osteopenic women. Thus, other variables or tools are needed that allow the identification of people at high risk of fractures, a determining factor of morbidity and mortality in the elderly population.

Correspondence: Manuel Naves Díaz (mnaves.huca@gmail.com)

Frailty is a geriatric syndrome characterized by weight loss, tiredness, weakness, slow gait and decreased physical activity. It is secondary to endocrine dysregulation and a proinflammatory and prothrombotic state. Sarcopenia, characteristic of frailty, is a disorder associated with aging that involves a loss of muscle mass and muscle weakness, limiting the mobility of the person and increasing the risk of falls, fragility and fractures[2].

In addition to the natural aging process, other factors, such as genetic factors, inadequate diet, physical inactivity, sedentary lifestyle, excessive bed rest, chronic diseases and/or certain pharmacological treatments, can favor the development of sarcopenia, contributing to the fragility and fractures, which has led to the term osteosarcopenia[3].

A quick way to identify subjects at risk of sarcopenia is by using tools that allow us to assess muscle strength through an evaluation and scoring system in which records strength, ability to walk, get up from a chair, climb stairs and frequency of falls[4].

Our study aims to assess the role that the muscle strength of "grip in the hands" and certain activities of daily life could have as instruments to predict the incidence of falls and osteoporotic fractures. In clinical practice, having simple tools that reveal the degree of sarcopenia and frailty could help to establish preventive measures before falls and fractures occur.

Material and methods

The initial protocol of the study was designed to determine the prevalence of vertebral fracture at the European level (EVOS study), in which 4 centers initially participated from Spain, including our own. To do this, 624 men and women over 50 years of age were randomly selected from the Oviedo city registry. This same cohort was included in a prospective study that involved measurements of muscle grip strength in both hands with a dynamometer, on a scale ranging from 0 to 300 mmHg. They were also asked to fill in a questionnaire, specifically designed for the EVOS study, which had a good reproducibility index[5,6]. This survey contained questions on clinical variables (weight and height, among others, to calculate BMI), risk factors related to osteoporosis and a series of questions related to the difficulty or not to carry out certain daily life activities.

These activities were based on a questionnaire that measures functional disability ("Funktionsfragebogen Hannover, FFbHR")[7] and that has several items with three possible answers: *you can do it without difficulty, you can do it with some difficulty and you are unable to do it or can only do it with assistance*. Likewise, the entire cohort underwent two lateral radiographs (the radiographic study was not completed in only 2 cases) and the collection of anthropometric measurements, such as height and weight to determine BMI. All subjects had sufficient ambulatory capacity to go up two floors without an elevator and 99% lived in their own home.

After measuring muscle strength and administering the questionnaire, this cohort was followed up prospectively for 8 years using 4 postal questionnaires in order to investigate the frequency of falls and the incidence of non-vertebral osteoporotic fracture during that period. All osteoporotic fractures, excluding skull and limb fractures, were confirmed by radiography or medical report. The total number of people who participated in the last follow-up was 427, with a participation percentage in the eighth year of 81.3% (excluding deaths), the percentages of the 3 previous postal follow-ups being 87.1, 87.5 and 82.4%, respectively.

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

Statistic analysis

Data analysis was carried out using SPSS version 17.0 for Windows. Quantitative variables were analyzed using Student's t test. The qualitative variables were analyzed using the chi square.

To analyze, at a multivariate level, the effect of the difficulty or inability to carry out certain activities of daily life without help on the incidence of falls and non-vertebral osteoporotic fracture, a logistic regression adjusted for age, sex, BMI, active smoker, previous fracture and family history of hip fracture. As a reference or comparison value, not having difficulty to carry out these activities was used.

Results

Table 1 shows the baseline characteristics of the study population. The age in both sexes was similar, with a statistically higher BMI in women. The history of previous fractures, the family history of hip fracture in parents or siblings, as well as the prevalence of vertebral fracture, were slightly higher in women than in men, but without statistically significant differences. Smoking habit was significantly higher in men. The incidence of non-vertebral osteoporotic fractures and the incidence of falls was markedly higher in women than in men.

Table 2 shows the muscle strength values in both hands as a function of the presence of falls during the follow-up period. The strength of the hands was lower in those who suffered falls, this effect being similar in both hands. The logistic regression analysis adjusted for age, sex, BMI, active smoker, previous fractures and family history of hip fracture, did not show a protective effect of muscle strength (every 10 mmHg increase), neither in the right hand: 0, 97 (0.90-1.03), nor on the left: 0.99 (0.92-1.05), on the incidence of falls.

Table 2 shows the percentages of people who present inability or difficulty to carry out daily activities according to the incidence of falls. Regarding the subjects who did not have any difficulty, those who had falls presented an impossibility or difficulty in carrying out the following activities: "carrying a 10-kilo object for 10 meters ($p<0.001$)"; "lean forward to pick up an object from the ground ($p=0.019$)"; "wash hair in a sink ($p=0.029$)"; "sitting for more than 1 hour on a hard chair ($p=0.003$)"; "stand in a queue for 30 minutes ($p=0.002$)"; "get up from bed ($p=0.020$)"; "take off socks or stockings ($p<0.001$)"; "leaning from a chair to pick up an object from the floor ($p<0.001$)"; and "lift a box with 6 bottles and put them on a table ($p<0.001$)".

The logistic regression analysis adjusted for age, sex, BMI, smoking, previous fractures and family history of hip fracture showed that the impossibility or difficulty of "sitting more than 1 hour on a hard chair" was significantly associated with an increase in the presence of falls: 1.83 (1.16-2.89). Difficulty or inability to "remove socks or stockings", as well as "leaning from a chair to pick up an object from the floor" was significantly asso-

ciated with an increase in the presence of falls: 1.85 (1.14-3, 00) and 1.68 (1.04-2.70), respectively. The multivariate analysis by sex showed associations in both sexes between the difficulty or inability to carry out certain daily activities with the incidence of falls. Specifically, in women, "sitting for more than 1 hour on a hard chair: 1.74 (1.01-3.02)" was associated with the incidence of falls; "stand in a queue for 30 minutes: 2.45 (1.41-4.25)"; and "take off the stockings": 2.04 (1.18-3.55). On the contrary, in men, an association was only found with "leaning from the chair to pick up something from the ground: 2.57 (1.27-5.21)".

Table 3 shows the muscle strength values in both hands as a function of the presence of incident non-vertebral osteoporotic fractures. The strength of the hands was lower in those who subsequently fractured, this effect being more pronounced in the strength of the right hand. The logistic regression analysis adjusted for age, sex, BMI, active smoker, previous fractures, and family history of hip fracture showed a slight tendency to a protective effect of muscle strength (every 10 mmHg increase) on the incidence of osteoporotic fracture. non-vertebral, although the differences were not statistically significant neither in the right hand: 0.95 (0.86-1.03) nor in the left: 0.97 (0.88-1.06).

Table 3 also shows the percentages of people who were unable or difficult to perform daily activities based on the presence of incident non-vertebral osteoporotic fractures. In relation to the subjects who did not have any difficulty, those individuals with incident fracture presented an impossibility or difficulty in carrying out the following activities: "picking up an object from a high shelf (p=0.004)"; "carry a 10-kilo object for 10 meters (p<0.001)"; "sitting for more than 1 hour in a hard chair (p=0.027)"; "stand in a queue for 30 minutes (p=0.016)"; "getting up from bed (p=0.029)"; and "lift a box with 6 bottles and put them on a table (p<0.001)".

All these univariate associations were analyzed by multivariate analysis. The logistic regression analysis adjusted for age, sex, BMI, active smoker, previous fractures, and family history of hip fracture showed that the impossibility or difficulty of "carrying a 10-kilogram object for 10 meters" was significantly associated with an increase in the presence of incident non-vertebral osteoporotic fracture: 2.82 (1.21-6.59). Similarly, the inability or difficulty to "lift a box with 6 bottles and put them on a table" was significantly associated with an increase in the presence of incident non-vertebral osteoporotic fracture: 2.54 (1.12-5.81). The multivariate analysis separately by sex only showed associations between the difficulty or inability to carry out daily activities with the incidence of non-vertebral osteoporotic fractures in women. Specifically, "take an object from a high shelf: 2.25 (1.00-5.05)"; "carry an object weighing 10 kilos for 10 meters: 3.47 (1.34-9.00)"; "sitting for more than 1 hour in a hard chair: 2.58 (1.11-6.00)"; and "lift a box with 6 bottles and put them on a table: 3.03 (1.23-7.49)".

Discussion

The concept of frailty in relation to osteoporosis is an increasingly accepted concept in the elderly as a predictor of osteoporotic fractures. It is estimated that between 25 and 50% of those over 85 years of age present frailty[8], determined by genetic, epigenetic and environmental factors. Sarcopenia involves the loss of muscle mass and strength related to aging, which is a key component of frailty. Strategies aimed at improving muscle strength and mass, such as increased protein intake and resistance and muscle strength training, have reportedly decreased the prevalence of sarcopenia and frailty, as well as improve strength and physical performance[9-11].

Several plausible mechanisms have been proposed between sarcopenia and the risk of fractures. On the one hand, small changes in muscle mass in muscle proteins such as myokines associated with abnormal glucose metabolism have a great impact on bone metabolism[12]. On the other hand, sarcopenic individuals have a high risk of falls, which leads to a higher incidence of fractures[13]. Therefore, sarcopenia is considered an effective predictor of fracture risk in the elderly[14]. However, the prevalence of sarcopenia is difficult to establish.

A meta-analysis found a great variability in the prevalence in elderly patients admitted to nursing homes from 1% to 29%[15]. In our study, although lower muscle strength was associated with the risk of falls and fractures at the univariate level, the multivariate analysis did not show significant differences. However, there was a modest trend that a 10 mmHg increase in muscle strength in the hands was capable of reducing the incidence of non-vertebral osteoporotic fracture by up to 5%. In reality, the study only collected the grip strength in the hands without any other measurable parameter of strength or muscle mass, so we believe that this instrument alone is not useful to assess the degree of sarcopenia if it is not accompanied by other complementary tests.

Table 1. Sociodemographic characteristics and clinical variables of the study population

Transversal study			
Variables	**Man (308)**	**Woman (316)**	**P value**
Age (years)	65 ± 9	65 ± 9	0.988
BMI (kg/m^2)	27.0 ± 3.3	28.6 ± 4.3	<0.001
Previous fracture	77 (25.1%)	92 (29.2%)	0.197
Family history of hip fracture	18 (5.8%)	26 (8.2%)	0.245
Active smoker	92 (30.1%)	15 (4.7%)	<0.001
Prevalent vertebral fracture	64 (21.1%)	83 (26.3%)	0.127
Prospective study			
Variables	**Man (200)**	**Woman (227)**	**P value**
Non-vertebral fracture incidence	4 (2.0%)	30 (13.2%)	<0.001
Incidence of falls	47 (23.5%)	102 (44.9%)	<0.001

Table 2. Effect of muscle strength and difficulty or inability to perform daily activities on the incidence of falls

Muscle strength measured by dynamometer	Yes falls	No falls	P value
Strength in the right hand	264 ± 42	278 ± 34	<0.001
Strength in the left hand	260 ± 42	275 ± 38	0.001
Inability or having difficulty performing certain activities	**Yes falls**	**No falls**	**P value**
Pick up a book from a high shelf	36 (24.2%)	47 (17.2%)	0.083
Carry a 10 kg object for 10 meters	80 (53.7%)	97 (35.4%)	<0.001
Wash and dry yourself	21 (14.1%)	28 (10.2%)	0.234
Leaning forward to pick up an object from the ground	71 (47.7%)	98 (35.9%)	0.019
Washing hair in a sink	27 (18.1%)	29 (10.6%)	0.029
Sitting in a hard chair for more than 1 hour	60 (40.5%)	72 (26.4%)	0.003
Stand in a queue for 30 minutes	82 (55.4%)	108 (39.4%)	0.002
Getting out of bed	48 (32.2%)	60 (21.9%)	0.020
Take off socks or stockings	64 (43.0%)	69 (25.2%)	<0.001
Leaning from a chair to pick up an object from the floor	70 (47.0%)	80 (29.2%)	<0.001
Pick up a box with 6 bottles and put them on a table	77 (51.7%)	93 (33.9%)	<0.001

Table 3. Effect of muscle strength and difficulty or inability to carry out daily activities on the incidence of non-vertebral osteoporotic fractures

Muscle strength measured by dynamometer	Incident fracture	No incident fracture	Valor de p
Strength in the right hand	248 ± 49	270 ± 41	0.001
Strength in the left hand	250 ± 47	265 ± 44	0.024
Inability or having difficulty performing certain activities	**Incident fracture**	**No incident fracture**	**Valor de p**
Pick up a book from a high shelf	13 (38.8%)	70 (18.0%)	0.004
Carry a 10 kg object for 10 meters	26 (76.5%)	151 (38.8%)	<0.001
Wash and dry yourself	5 (14.7%)	44 (11.3%)	0.553
Leaning forward to pick up an object from the ground	16 (47.1%)	153 (39.4%)	0.384
Washing hair in a sink	7 (20.6%)	49 (12.6%)	0.187
Sitting in a hard chair for more than 1 hour	16 (48.5%)	116 (29.9%)	0.027
Stand in a queue for 30 minutes	22 (64.7%)	168 (44.3%)	0.016
Getting out of bed	14 (41.2%)	94 (24.2%)	0.029
Take off socks or stockings	14 (41.2%)	119 (30.6%)	0.202
Leaning from a chair to pick up an object from the floor	15 (44.1%)	135 (34.7%)	0.271
Pick up a box with 6 bottles and put them on a table	25 (73.5%)	145 (37.3%)	<0.001

There is evidence that the frailty index is predictive of osteoporotic fractures independent of chronological age in patients[16-19]. In fact, a study with data from the Canadian Multicenter Osteoporosis Study (CaMos) conducted in 9,423 adults with a mean age of 62 years and a follow-up of 10 years showed a hazard ratio of 1.18 and 1.30 for hip fractures and clinical vertebral fractures for each 0.10 increase in the frailty index[19]. There are authors who indicate the need to validate fragility instruments before they can serve as a guide when making clinical decisions[20].

In our study, the incidence of falls was not associated with a decrease in muscle strength, but it was associated with daily activities that reflect stability and adequate physical condition, such as being able to remain "sitting for more than 1 hour in a hard chair", "taking an object on the floor from a chair and "being able to remove stockings or socks ". Other authors have also observed that frailty affects the incidence of falls[21].

In this study, frailty was evaluated based on a validated questionnaire that measures functional grade in people with back pain[7]. This questionnaire contains questions that relate to the activities of daily life that are part of many of the instruments for measuring frailty. It is interesting to note that the loss of strength (lifting objects and carrying them a few meters) were the factors that were best associated with increases in the incidence of fracture (up to 2.5 times). The analysis separated by sex showed only positive associations in women, with these activities, as well as others (stretching to pick up an object or sitting in a hard chair for 1 hour), predictive of the incidence of fractures. In men, no effect was observed, contrary to what other authors refer to[22]. This discrepancy could be related to the low number of incident fractures in males (n=4) found in our study. It is worth mentioning that during the 8-year follow-up there were 6 other incident osteoporotic fractures in men, but these subjects were

excluded from the analysis as they did not reach the end of the indicated follow-up period.

A subsequent analysis was carried out (data not shown in the results), using a score to grade the difficulties in carrying out the 11 activities of daily life included in the functional disability questionnaire, with a minimum of 0 (those who had no difficulty in carry out them) up to a maximum of 22 (those unable to do these activities by themselves). It was observed that, when categorizing the score into quartiles, those with the worst condition (quartile 4) had an increase in the incidence of falls in the multivariate analysis of 2.37 (1.25-4.52) compared to those who were in better condition (quartile 1). This effect could not be observed for the incidence of fracture, which may indicate that worse physical condition would be a predictor of the appearance of falls, but not of fractures, although falls are a risk factor for the appearance of fracture. On the contrary, those activities most related to loss of strength, such as lifting objects and carrying them a few meters, were those that were best associated with the incidence of fracture, up to more than 3 times in the case of women.

Based on these results, the information on the evaluation of frailty and sarcopenia can, together or in parallel, be another tool for osteoporosis assessment, providing a more comprehensive view of the risks that these patients may present[23].

This study has limitations in that the muscle fragility or strength was measured at the beginning of the follow-up, so we cannot rule out that the result obtained during a prolonged follow-up period of 8 years has been underestimated. The questions that contained questions related to difficulties in carrying out daily living activities were not self-administered, but administered by an interviewer, which could have biased the participants' responses, especially in those questions related to difficulties for the self. personal cleanliness. The questionnaire used to measure difficulties in carrying out daily activities was focused on evaluating functional disability in people with back pain. However, despite this, we believe its questions give an idea of the degree of the individual's physical and functional deterioration. Information on how the falls were to related to the fracture that occurred would have been very valuable. Unfortunately, this possibility was not included in the EVOS-EPOS study guidelines.

Despite these limitations, we believe our study has important strengths. On the one hand, the analyzed cohort participated in the EVOS-EPOS study, ours being one of the 5 centers that completed all the study guidelines. Furthermore, the response in the four postal follow-ups carried out during the 8 year period showed a greater than 80% response, which broadly supports the representativeness of the analyzed sample.

In summary, we have been able to verify that difficulties in carrying out certain activities of daily or daily life can presage a deterioration in physical capacity and functional status, being able to constitute another tool in the patient's anamnesis that helps to predict and probably avoid falls and osteoporotic fractures.

As conclusions of this work, we can affirm that no associations were found between grip strength and incidence of falls and osteoporotic fractures. Those activities more related to greater strength were associated with fracture, while those related to greater functional capacity were associated with falls.

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Persistence to aromatase inhibitors in the SIDIAP cohort: mortality and influence of bisphosphonates

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Pineda-Moncusí M[1], Vilalta-Carrera A[1], Ovejero D[1], Aymar I[1,2], Servitja S[3], Tusquets I[3], Prieto-Alhambra D[4,5], Díez-Pérez A[1,2], García-Giralt N[1], Nogués X[1,2]

1 Hospital del Mar Institute for Medical Research (IMIM). Center for Biomedical Research Network on Frailty and Healthy Aging (CIBERFES). Barcelona (Spain)
2 Department of Internal Medicine. Hospital del Mar. Autonomous University of Barcelona. Barcelona (Spain)
3. Department of Medical Oncology. Hospital del Mar Institute for Medical Research (IMIM). Barcelona (Spain)
4 Research Group on Prevalent Diseases of the Locomotor System in Primary Care (GREMPAL). Jordi Gol University Institute for Research in Primary Care (IDIAP Jordi Gol) and Center for Biomedical Research Network on Frailty and Aging Healthy (CIBERFES). Autonomous University of Barcelona and Carlos III Health Institute. Barcelona (Spain)
5 Center for Statistics in Medicine (CSM). Nuffield Department of Orthopedics, Rheumatology and Musculoskeletal Sciences (NDORMS). Oxford University. Oxford (UK)

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Summary

Objetive: To assess the persistence of aromatase inhibitor (AI) therapy, mortality associated with treatment discontinuation and the influence of oral bisphosphonates (BP) in routine clinical practice.
Material and methods: Prospective observational study of women with breast cancer undergoing AI treatment between January 2006 and December 2015, registered in the SIDIAP database. Those previously treated with tamoxifen were excluded. AI persistence was studied with a survival analysis: the Kaplan-Meier estimator was calculated, and a proportional hazards model (Cox regression) was performed between users and non-users of BP adjusting for age. A sensitivity analysis was carried out taking into account mortality as a competitive risk (Fine and Gray models). The difference in mortality between groups was compared using a Chi square test.
Results: A persistence to AI of 87% was observed after 5 years of treatment, with an overall mortality of 19.75%. There was 7.7% less mortality in those patients who completed the 5 years of treatment compared to those who did not. Patients with BP showed a decrease in mortality (6.6%) and a decrease in the risk of discontinuing therapy (adjusted SHR: 0.62 [95% CI: 0.55 to 0.70]) compared to non-users.
Conclusions: Persistence to AI and BP use are associated with a decrease in overall mortality. Furthermore, the use of BP increases adherence to AI treatment.

Key words: aromatase inhibitors, bisphosphonates, breast cancer, mortality, persistence.

Introduction

Aromatase inhibitors (AIs) are the recommended adjuvant therapy to treat estrogen receptor-positive breast cancer[1,2]. Its effectiveness in reducing the risk of recurrence and mortality is acknowledged[3]. However, AIs are also associated with various side effects that affect patients' quality of life and therefore compromise adherence to treatment and associated mortality[4].

Reportedly, 30% of patients prescribed with AI discontinue their treatment due to adverse events[5], mainly musculoskeletal[6,7]. Among them, the most frequent are arthralgias[8] and accelerated loss of bone mass[9] associated with an increase in osteoporotic fracture[10,11]. To prevent the loss of bone mass, treating patients with antiresorptives is recommended, with bisphosphonates (BP) being the most used[12-14].

Correspondence: Natalia García Giralt (ngarcia@imim.es)

BP use has been associated with improved mortality associated with reduced bone metastases[13]. Similarly, a study published in a Korean population showed the use of BP was associated with improved adherence[15].

Our study's objective was to evaluate the persistence of AI therapy, the mortality associated with treatment discontinuation, and the influence of oral BPs, in a population-based cohort with data obtained from routine clinical practice.

Material and methods

Data Base

Data from more than 7 million patients, coming from more than 350 Primary Care centers in Catalonia, are registered anonymously by the Information System for the Development of Research in Primary Care (SIDIAP), covering >80% of the total of the Catalan population (http://www.sidiap.org).

This database contains information on sociodemographic variables, lifestyle risk factors (alcohol consumption, obesity, smoking, etc.), comorbidities, and pharmacological dispensations. The data are collected by professionals in the health sector, including the codes of the international classification of diseases and related health problems, 10th edition (ICD-10), as well as structured forms for the collection of clinical variables (tobacco, index of body mass, etc.). SIDIAP also has registered mortality data, obtained from the Central Registry of Insured Persons, as well as migration outside the catchment area[16].

Study design and participants

Prospective observational study of women diagnosed with hormone receptor-positive breast cancer undergoing AI treatment. Patients treated with AI in monotherapy between January 2006 and December 2015 collected in the SIDIAP database were included. AI users were identified using the ATC (European Pharmaceutical Substances and Medicines Coding System) codes: L02BG03 for anastrozole, L02BG04 for letrozole, and L02BG06 for exemestane. Those with a previous history of cancer (except local non-melanoma skin cancer) were excluded.

Patients' follow-up period

For the adherence study, patients were monitored from the start of AI therapy until the first of the following events: cessation or abandonment of AI therapy, death, migration out of the catchment area, or end of availability of data in SIDIAP (December 31, 2015). In the case of mortality, the patients were followed up from their entry into the study until December 31, 2015.

Study variables

The main study variables were adherence to AI, and overall survival. The continued use of AIs was studied through pharmaceutical billing records. Treatment cessation or abandonment was considered in those records without dispensing with intervals of 6 months or more. Overall survival, expressed in mortality, was reported during the follow-up period.

The effect of BPs on persistence and mortality was studied by stratifying in users and non-users: patients with oral BP records (M05BA) were classified as BP users with codes M05BA01 (etidronic acid), M05BA02 (clodronic acid), M05BA04 (alendronic acid), M05BA05 (tiludronic acid), M05BA06 (ibandronic acid), M05BA07 (risedronic acid), and M05BB03 (combination of alendronic acid and cholecalciferol).

Statistical Analysis

Patient characteristics were described using the mean ± standard deviation (SD) in the quantitative variables with normal distribution, and the number and percentage –n (%)– for the categorical variables.

Adherence to AI treatment was studied with a survival analysis: the Kaplan-Meier estimator was calculated and represented by cumulative probability models. A proportional hazards model (Cox regression) was carried out between users and non-users of BP adjusting for age, obtaining the hazard ratio (HR), and its proportionality verified. Additionally, a sensitivity analysis took into account mortality as a competitive risk (Fine and Gray models), estimating the sub-distribution of the risk ratios (SHR).

Finally, the difference in mortality between groups was compared using a Chi square test.

The analyzes were carried out with R 3.5.3 using the foreign, compare groups, splines, survival, and survminer packages. These were defined as significant with $p<0.05$.

Results

18,455 data were collected from women treated with AI. Its baseline characteristics are described in table 1. The persistence [95% CI] to AI treatment was 99.8% [99.7 to 99.9] at 1 year, 98.3% [98.1 to 98 , 5] at 2 years, 95.8% [95.5 to 96.2] at 3 years, 92.9% [92.4 to 93.4] at 4 years, and 87.0% [86.3 to 87.8] after 5 years of treatment (Figure 1).

Mortality was quantified by stratifying the patients taking into account those who completed 5 years of treatment, and, on the other hand, those who did not. An overall mortality of 19.75% (3,644/18,455) was observed: with 21.2% (3,165/14,908) in patients who did not complete 5 years of AI treatment, and 13.5% (479/3,547) in those treated for 5 years or more ($p<0.001$).

Influence of the BP

Of the 18,455 patients included in the study, 21.7% (n=4,009) were treated with oral BP (Table 2). They showed better persistence to AI than those not treated with BP: 99.9% [99.8 to 100] vs. 99.7% [99.6 to 99.8] at 1 year; 99.8% [99.6 to 99.9] vs. 97.8% [97.5 to 98.1] at 2 years; 98.5% [98.1 to 98.9] vs. 94.9% [94.4 to 95.3] at 3 years; 97.2% [96.6 to 97.8] vs. 91.2% [90.5 to 91.8] at 4 years; and 93.3% [92.2 to 94.4] vs. 84.5% [83.5 to 85.5] at 5 years of treatment, respectively (Figure 2). In this way, the risk ratio of abandoning AIs in BP users compared to non-users was as follows: adjusted HR: 0.53 [95% CI: 0.47 to 0.60], and adjusted SHR: 0, 62 [95% CI: 0.55-0.70].

In contrast, mortality in patients with BP was 14.6% (587/4,009), while in non-users it was 21.2% (3,507/14,446) ($p<0.001$).

Discussion

This study evaluates the persistence of AI therapy in a cohort of women diagnosed with hormone receptor-positive breast cancer, as well as mortality and the effect of bisphosphonates, in routine clinical practice. It was observed that the global persistence at 5 years was 87%

with an overall mortality of 19.75%. Mortality in those patients who completed 5 years of therapy was 7.7% lower than those who did not. On the other hand, FB users showed better persistence to AI treatment, with a 47% lower risk of discontinuing therapy, and 6.6% lower mortality than non-users.

Reports indicate the side effects of AI negatively influence adherence to treatment[5]. Several randomized controlled trials (RCTs) have published persistence rates that vary between 76-90%[17,18]. However, the reliability of these percentages may be questioned by the lack of discontinuity results from some RCTs. Several studies of adherence in population databases show values of around 69-88% in short observation periods (one year of adherence)[19-21], and of 61-79% in longer follow-up periods (3-4,5 years)[20,22]. Hershman et al. (2010)[22] observed a 30% discontinuation rate in patients with AI at 4.5 years of follow-up, while Hadji et al. (2013)[23] described a discontinuation between 44-55% at 3 years. Among other factors, the age of the patients (younger, less adherence), and the cost of medicines and/or derived medical expenses –especially in private health systems–, have been described as variables associated with greater discontinuity[21]. The high persistence observed in our population could be explained by a high mean age (mean ± SD: 67.6 ± 11.6) compared to that reported in the RCTs (mean ± SD: 64.1 ± 9.0 in the study ATAC[24], and 64.3 ± 8.1 in the IES study[25]; median [range]: 61 [38-89] in the BIG 1-98 study[26]; and median of 63.9 and 64.3 in patients with exemestane and anastrozole in study MA.27[27]) and a public health system, where the cost of treatment is practically negligible.

In the case of global mortality, there is a certain diversity of results depending on the design of the RCT. In the BIG 1-98 study, a mortality of 12.3% was observed[28]. The ATAC safety study detected a mortality of 23.5% in all AI monotherapy users, and 21.5% in the subpopulation of women with known hormone receptor-positive tumor status[29]. In contrast, study MA.27, published by Goss et al. (2013), showed a mortality of 5.7% at 5 years[27]. Unlike RCTs, our study uses data from the general population visited in primary care, achieving a more representative population of the usual clinic. Interestingly, the mortality values of our study population are similar to those described in the ATAC study. This fact could be attributed to the fact that both report mortality results of up to 10 years of follow-up.

On the other hand, and in agreement with our results, the use of BP was reported by Lee et al. (2014)[15] as a factor that improves adherence to AI treatment. Likewise, the use of BP was associated with a 34% decrease in the incidence of bone metastases and a 17% reduction in mortality[13]. In general, the use of BP decreases overall mortality, increases life expectancy and prevents the appearance of various cancers in the general population[30]. This improvement in life expectancy is not only attributed to the decrease in fractures[31], but also to a possible prevention of frailty and a greater capacity of the individual to cope with different conditions[32].

Table 1. Baseline characteristics of the participants included in the study

Variable	AI users (N=18,455)
Age (mean ± SD)	67.6 ± 11.6
BMI (mean kg/m² ± SD)	29.7 ± 5.36
Not available [n (%)]	*13,555 (73.45%)*
Smokers [n (%)]	
No smokers	10,269 (81.44%)
Smokers	1,343 (10.65%)
Ex-smokers	997 (7.91%)
Not available [n (%)]	*5,846 (31.68%)*
Risk of alcoholism [n (%)]	
Without/Low	2,410 (85.58%)
Moderate	390 (13.85%)
High/Alcoholism	16 (0.57%)
Not available [n (%)]	*15,639 (84.74%)*
Charlson comorbidity index [n (%)]	
0	2,315 (12.54%)
1	704 (3.81%)
2	9,840 (53.32%)
3	3,553 (19.25%)
≥4	2,043 (11.07%)
MEDEA deprivation index [n (%)]	
Rural area	3,450 (20.28%)
Urban area 1	3,498 (20.56%)
Urban area 2	2,960 (17.40%)
Urban area 3	2,692 (15.83%)
Urban area 4	2,399 (14.10%)
Urban area 5	2,012 (11.83%)
Not available [n (%)]	*1,444 (7.82%)*
BP users [n (%)]	4,009 (21.7%)

BP: bisphosphonates; SD: standard deviation; BMI: body mass index; MEDEA: mortality in small Spanish areas and socio-economic and environmental inequalities.

Figure 1. Persistence to AI treatment. The graph presents a Kaplan-Meyer curve that shows the risk of AI abandonment in cumulative terms. Abbreviations: AI: aromatase inhibitors

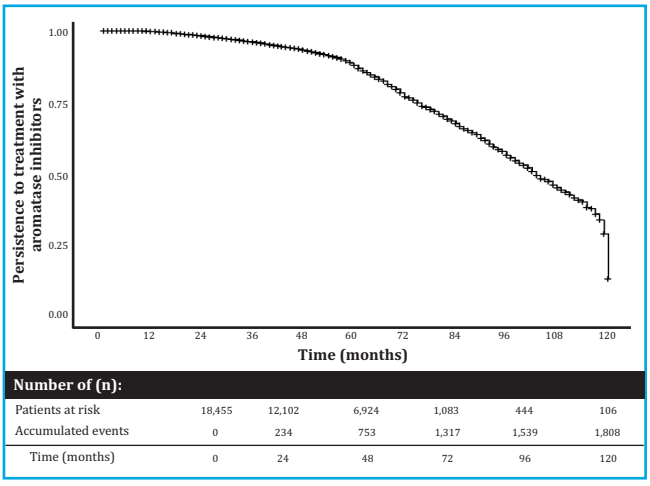
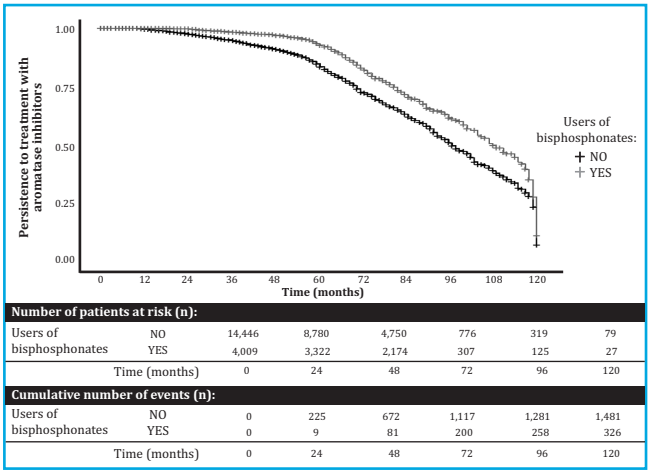


Table 2. Baseline characteristics of women treated with AI according to their use of BP

Variable	Non-BP users (N=14,446)	BP users (N=4,009)
Age (mean ± SD)	67.5 ± 12.0	68.0 ± 10.1
BMI (mean kg/m² ± SD)	29.9 ± 5.45	29.0 ± 4.94
Not available [n (%)]	*10,602 (73.4%)*	*2,953 (73.7%)*
Smokers [n (%)]		
No smokers	8,006 (80.7%)	2,263 (84.2%)
Smokers	1,088 (11.0%)	255 (9.48%)
Ex-smokers	826 (8.33%)	171 (6.36%)
Not available [n (%)]	*4,526 (31.3%)*	*1,320 (32.9%)*
Risk of alcoholism [n (%)]		
Without/Low	1,901 (85.4%)	509 (86.1%)
Moderate	313 (14.1%)	77 (13.0%)
High/Alcoholism	11 (0.49%)	5 (0.85%)
Not available [n (%)]	*12,221 (84.6%)*	*3,418 (85.3%)*
Charlson comorbidity index [n (%)]		
0	1,753 (12.13%)	562 (14.02%)
1	552 (3.82%)	152 (3.79%)
2	7,573 (52.42%)	2,267 (56.55%)
3	2,847 (19.71%)	706 (17.61%)
≥4	1,721 (11.91%)	322 (8.03%)
MEDEA deprivation index [n (%)]		
Rural area	2,809 (21.13%)	641 (17.25%)
Urban area 1	2,680 (20.16%)	818 (22.01%)
Urban area 2	2,304 (17.33%)	656 (17.65%)
Urban area 3	2,040 (15.35%)	652 (17.54%)
Urban area 4	1,890 (14.22%)	509 (13.69%)
Urban area 5	1,571 (11.82%)	441 (11.86%)
Not available [n (%)]	*1,152 (7.97%)*	*292 (7.28%)*

BP: bisphosphonates; SD: standard deviation; BMI: body mass index; MEDEA: mortality in small Spanish areas and socio-economic and environmental inequalities.

Figure 2. Persistence of AI treatment among users and non-users of BP. The graph presents a Kaplan-Meyer curve that shows AI drop out risk in cumulative terms between the study groups: users and non-users of BP. Abbreviations: AI: aromatase inhibitors; BP: bisphosphonates



Taking all this into account, the greater adherence to AI in patients treated with BP could be explained by improved treatment of adverse events that would have a positive impact on the patient, while the decrease in overall mortality derived from the use of the BP could be attributed both to a decrease in bone metastases and to greater adherence to AIs.

One limitation of this study is that the SIDIAP database does not have data referring to the cause of mortality or the reason for discontinuation of treatment. Thus, our study only considers overall mortality, but there is a risk of bias in that mortality before 5 years is not a consequence of discontinuing therapy. Additional studies are needed to verify that the observed difference in mortality is not due to a bias in the populations studied (between those patients who completed 5 years vs. those who did not, and between users and non-users of BP). However, our study corroborates the results observed in previous studies.

In conclusion, a 5-year persistence to AI of 87% has been observed in routine clinical practice, which improves with the use of BP. On the other hand, completing 5 years of AI therapy and the use of BP would be associated with a decrease in mortality.

Ethics statement: This study was approved by the IDIAP Jordi Gol Research Ethics Committee (CEI) and the SIDIAP Scientific Committee (P16/031). The data from the SIDIAP database were anonymized, with a null identification risk, in accordance with Organic Law 15/1999, of December 13. Therefore, the signing of an informed consent by the patients was not required.

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Biocompatibility and osseointegration study of new prosthetic materials

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Giner M[1], Santana L[2], Costa AF[3], Vázquez-Gámez MA[4], Colmenero M[3], Olmo FJ[3], Chicardi E[2], Torres Y[2], Montoya-García MJ[4]

1 Department of Normal and Pathological Cytology and Histology. Sevilla University. Sevilla (Spain)
2 Department of Engineering and Science of Materials and Transportation. Higher Polytechnic School of Seville. Sevilla (Spain)
3 Internal Medicine. Virgen Macarena University Hospital. Sevilla (Spain)
4 Department of Medicine. Sevilla University. Sevilla (Spain)

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Summary

Objetive: Bone implants are increasingly used in clinical practice and, among the materials, Ti or its alloys are offer the best performance given their physicochemical properties. Alloys such as TiNbTa have been shown to improve the biomechanical characteristics of commercial pure Ti (c.p.), however, its osseointegration capacity needs to be evaluated. The objective of the present study was to assess the cytotoxicity and the adhesion, proliferation and differentiation capacity of osteoblastic cells in culture, influenced by discs of TiNbTa material versus Ti c.p.

Material and methods: At 4 and 7 days after culture, we analyzed the MC3T3 cell line, cell viability (AlamarBlue Cell Viability Reagent. Invitrogen, Spain), as well as cell proliferation and differentiation (alkaline phosphatase activity (ALP) and scanning electron microscopy (Fixation for SEM) Student's t test was performed to determine statistically significant differences between the two groups of study discs.

Results: The results obtained show very good cell viability during the study period, with no significant differences for both materials. Likewise, we detected a drop in ALP levels that was significant for both components between days 4 and 7 of the study ($p < 0.05$). Electron microscopy images revealed good adhesion capacity to the material, as well as cell differentiation against both types of discs.

Conclusions: The TiNbTa alloy as a material for bone implants offers good osseointegrative capacity, in addition to solving biomechanical problems that pure titanium presents as a component.

Key words: TiNbTa, cytotoxicity, biocompatibility, osteoblast cells, cell culture, cell adhesion, Young's modulus.

Introduction

The generation of functional tissue through tissue engineering has a high impact in various areas of regenerative medicine, among which is skeletal tissue. The first implants were used in the field of medicine, in 1909, when Kirschner wires and Steinman nails were developed for the fixation of bone fractures, where stainless steel was used. Over the years, steel has been improved, making it more resistant to corrosion and not causing harmful effects on the human body. In 1940, the study of titanium (Ti) began as a biomaterial for bone implantation[1].

The phase change determines the change in the crystalline structure of the material when subjected to temperature changes. Titanium's allotropic transformation occurs at 882ºC and goes from an α phase, which has a compact and hexagonal structure (HCP), little deformable and resistant at room temperature, to a β phase characterized by a cubic structure centered on the body (BCC), which is easily deformable and allows for carrying out heat treatments to optimize the material's properties[2].

Among the characteristics that make titanium one of the best materials for bone implants, its greater specific resistance (resistance/density), its high ductility and lower Young's modulus[3] compared to other elements such as, for example, stainless steel are noteworthy. At the same time, it is a non-ferromagnetic material, which does not present inconveniences when the patient with a Ti implant undergoes magnetic resonance imaging. Anchorage to bone tissue is possible because of the oxide layer formed in the material when passivated[4].

Correspondence: Mercè Giner (mginer@us.es)

Pure commercial Ti (c.p.) and other alloys such as Ti-6Al-4V are currently used mainly for bone implants[5]. However, both elements present a high elastic modulus (E: 100-112 GPa) compared to the elastic modulus of cortical bone (18.6-20.7 GPa) and trabecular (10.4-14.8 GPa) (Table 1) what produces the stress-shielding effect or stress-shielding[6]. This phenomenon is due to the stiffness of the bone implant material being greater than the stiffness of the bone, which places the entire load on the bone implant. Bone remodeling is largely regulated by the mechanical loads to which it is subjected, so that the presence of loads stimulates its bone formation, and the absence of them increases resorption. As a consequence of the bone's reduced load supported, it decreases its density in the area near the implant and, therefore, both premature fracture of the implant and loosening of the implant may occur[5].

At present, to eliminate the voltage shielding phenomenon, reducing the elastic modulus is sought, with several available solutions. One of them, reduce the density of the material used, through porosity. However, as the porosity increases, the mechanical resistance decreases. Another approach would be to search for face-centered cubic structure Ti alloys (BCC) or β-Ti alloys, which are low in elastic modulus and do not show a decrease in mechanical strength. For the stabilization of the β phase, β-stabilizing elements are required: Mo, V, Ta, Nb and Zr as β-isomorphic elements and Cr, Co, Cu, Fe and Ni as β-eutectoid elements. The advantage of β-isomorphic elements is their high amount of substitute solid solution and their inability to form intermetallic compounds of Ti, which have high E. Therefore, it has been shown that elements such as Nb and Ta have a high level of biocompatibility and ability to prevent the increase of particles, which would avoid a bad cohesion interface[7].

Niobium (Nb) and tantalum (Ta) are two transition metals widely used in alloys; in particular, Nb is used in the formation of steel. Until 1866, it was thought that both elements were the same, since they have very similar physicochemical characteristics. The TiNbTa alloy stands out for its high stabilization of behavior, due to the absence of β phase, which allows a decrease in the elastic modulus (49 + 3 GPa), its excellent elastic resistance (σy>1860 MPa) and its high biocompatibility[7,8]. Therefore, a material such as TiNbTa, with a good combination of high strength and low Young's modulus close to that of bone, could be used to prevent loosening of implants to avoid revision surgery (Table 1).

In recent years, a whole series of techniques have been developed with the aim of obtaining porous materials that present a Young's modulus closer to that of cortical bone. Specifically, Chicardi et al. manufactured a TiNbTa alloy with physicochemical properties more similar to those of bone[9]. However, the improvement in the design of materials, destined to be used as bone grafts, must consider their osseointegration capacity, and in this sense, evaluating the cytotoxicity and the adhesion, proliferation and differentiation capacity of osteoblastic cells influenced by the material is required. Our main objective was to evaluate the osseointegrative characteristics of the TiNbTa alloy, with a Young's modulus similar to that of trabecular bone, and to compare them with pure Ti.

Material and methods

1. Cell culture

The MC3T3 cell line (subclone 4) from ATTC (Manassas, Virginia, USA) was cultured in α-MEM medium (Gibco, Thermo Fischer Scientific, Spain) supplemented with 1% L-glutamine (200 mM) and 10 % fetal bovine serum. To induce differentiation, cells were treated with osteogenic culture medium supplemented with 50 g / ml ascorbic acid (Merck, Germany) and 10 nM β-glycerophosphate (StemCell Technologies, Canada). The cells were seeded in discs, 3 mm thick and 7 mm, of titanium c.p. and TiNbTa at a density of $5x10^3$, under conditions of 5% CO_2 at 37°C. Media changes were made every 48 h. Cultures were done in triplicate and readings for cell viability and alkaline phosphatase activity were done after 4 and 7 days.

2. Cell viability

The Alamar Blue assay (AlamarBlue Cell Viability Reagent. Invitrogen, Spain) was carried out according to the manufacturer's instructions. AlamarBlue is a non-toxic, cell-permeable compound, blue in color and non-fluorescent. Viable cells maintain a reducing environment within their cytoplasm. AlamarBlue reagent is an oxidized form of redox and is blue in color. When incubated with viable cells it changes from blue to red and becomes fluorescent. This change can be detected using absorbance methods.

At 4 and 7 days, the cells with cell growth are transferred to a new well and 80 µL of AlamarBlue are added on the disc and subsequently 720 µL of culture medium are added; This medium is incubated for 2 hours at 37 [deg.] C. and the absorbance is measured at the respective excitation and emission wavelengths of 570 and 600 nm (TECAN, Infinity 200 Pro). The results are presented as the percentage of reduction. The experiments were carried out in triplicate on each culture.

3. Alkaline phosphatase

We analyzed the activity of alkaline phosphatase (ALP) according to the manufacturer's protocol (Colorimetric Alkaline Phosphatase Assay Kit, Abcam ab83369, UK) in the culture supernatant. The test was carried out at 4 and 7 days, through the conversion of a colorless p-nitrophenyl phosphate into a colored p-nitrophenol. The absorbance at 405 nm of 4-nitrophenol (TECAN, Infinity 200 Pro) was measured and the ALP activity calculated from a standard curve. The experiments were carried out in triplicate on each culture.

4. Fixation for SEM

To visualize the cells in scanning electron microscopy (SEM), MC3T3 cells were grown on titanium discs c.p. and Ti NbTa for 4 and 7 days in triplicate. The samples were fixed with 10% formalin, followed by a dehydration step with ethanolic solutions and coated by gold plating using a sputum coating (Pelco 91000, Ted Pella, Redding, California, USA). All micrographs were obtained using a Jeol JSM-6330F scanning electron microscope and the acceleration voltage was 10 kV for SEM images.

5. Statistical analysis

All *in vitro* experiments were performed in triplicate for each condition studied. The variables were analyzed for the distribution of normality using the Kolmogorov-Smirnov test. Student's t test was performed to determine statistically significant differences between the two groups.

For the statistical handling of results, the SPSS version 22.0 package for Windows (IBM Corp., Armonk,

Table 1. Physical characteristics of pure titanium, the different phases and bone: elastic modulus and elastic resistance

Group	Compound	Young modulus (GPa)	Yield limit (MPa)
Pure titanium	Ti c.p.	100	650
Phase α+β	Ti-6Al-4V	112	1140
Phase β	TiNbTa	46-52	1860
Bone	Trabecular	0.5-2.0	40-60
	Cortical	20-25	150-180

New York, USA) was used. In all cases, the level of significance was considered to be $p<0.05$. Data are expressed as mean ± standard deviation (SD).

Results

The cell cultures on the Ti discs c.p. and in the alloy they had a similar cell growth. At 7 days of growth, the cells showed columnar and basophilic morphology under the light microscope (O.M.) compatible with the beginning of differentiation (Figure 1). The surface of the disc does not let light through, making it difficult to see cells clearly. However, in the highlighted parts, it could be observed that some cellular adhesion had occurred in the material, which would indicate that the alloy has good qualities to be used in implants.

Cell viability

Figure 2 represents the viability of the cell line as a function of the cell growth time (4 and 7 days) on the Ti discs c.p. and TiNbTa. At 4 days of culture, cell viability in the TiNbTa discs was similar to that of the Ti c.p. While at 7, we observed a slight increase in viability in the TiNbTa samples and a decrease in the Ti samples c.p., although without significant differences. In all cases, the percentage of viability was always higher than 150%. A toxic effect is considered when the viability is less than 75%, therefore, with the results obtained, it can be deduced that the cell culture is viable throughout the entire duration of the culture under all conditions.

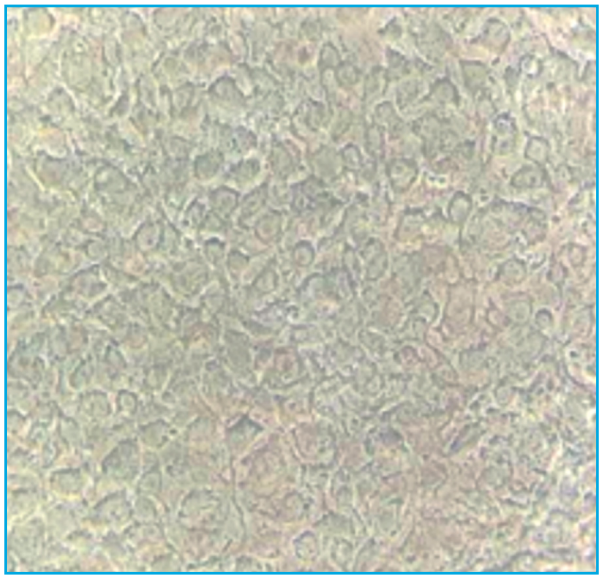
Alkaline phosphatase activity

We quantified the alkaline phosphatase (ALP) activity values in MC3T3 cell cultures at 4 and 7 days. Figure 3 represents the mean and standard deviation of the results obtained in both culture conditions. In the Ti discs c.p., the results obtained showed a decrease in the enzymatic activity, the difference found between days 4 and 7 being statistically significant ($p=0.001$). In the TiNbTa discs, a decrease in activity was also observed with culture time and there was a significant difference between days 4 and 7 of culture ($p=0.006$). In both conditions, at 4 days, the maximum enzymatic activity was obtained, since there is a greater proliferation and cell growth, as the viability values corroborated, and from day 7, the cells presented a more differentiated phenotype, as observed in the SEM images, and ALP activity decreased.

Cell morphology by scanning electron microscopy

A preliminary study was carried out to study the morphology and cell adhesion on the TiNbTa study material compared to the growth in Ti c.p. To do this, we visualized the samples of both types of disc at 4 and 7 days of culture. At 4 days, small cell clusters were observed on both types of surface and of similar density. At 7 days, the

Figure 1. Inverted optical microscope photograph (Olympus CKX53) with a 40X medium magnification objective, of cells grown at 7 days in the TiNbTa discs



images showed a monolayer growth over the entire surface of the disk, being similar in both materials. Cell-cell and cell-biomaterial junctions were also observed. The cells adhered through filopodia (thin cell projections, yellow arrow) and lamelopodia (broader extensions, yellow asterisk), thus demonstrating the connection of the cells with the biomaterial. We began to observe the presence of small vesicles with a hexagonal structure, suggesting a possible nucleation of hydroxyapatite, on the cell surface, suggesting the beginning of the mineralization process development (Figure 4).

Discussion

Inflammatory and degenerative problems of the bones and joints affect millions of people around the world. In fact, they represent half of the chronic diseases in people over 50 years of age in developed countries[10]. These diseases often require surgery, including replacement of the entire joint in cases of deterioration. This fact, accompanied by the increase in life expectancy and the aging of the population, entails a great demand for healthcare derivatives, among which the development of surgical implants and materials with a longer useful life period stand out.

Biomaterials constitute one of the most important advances in current medicine, improving the patient's quality of life and reducing the healing and convalescence time. In 2009, Bjursten highlighted the importance of increasing implant-bone bonding, given that the half-life of an implant is between 10 and 15 years[7,8], which implies an increase in the number of revision surgeries.

Figure 2. Cell viability of cells cultured in Ti discs c.p. or the TiNbTn alloy at 4 and 7 days of cell growth. Results presented as mean ± standard deviation

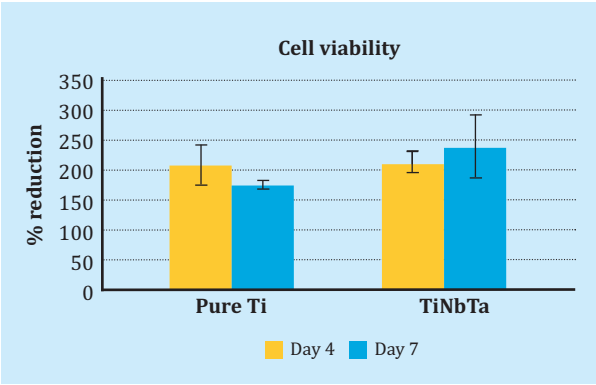


Figure 3. Activity of alkaline phosphatase in cell growth on Ti discs c.p. or TiNbTa for 4 and 7 days. *Ti c.p. 4 days vs. 7 days; ** TiNbTa 4 days vs. 7 days. P<0.05

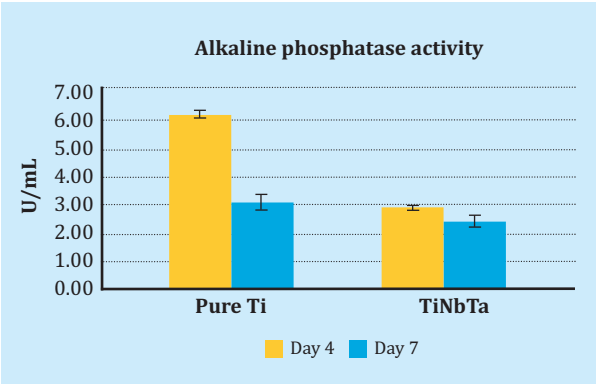
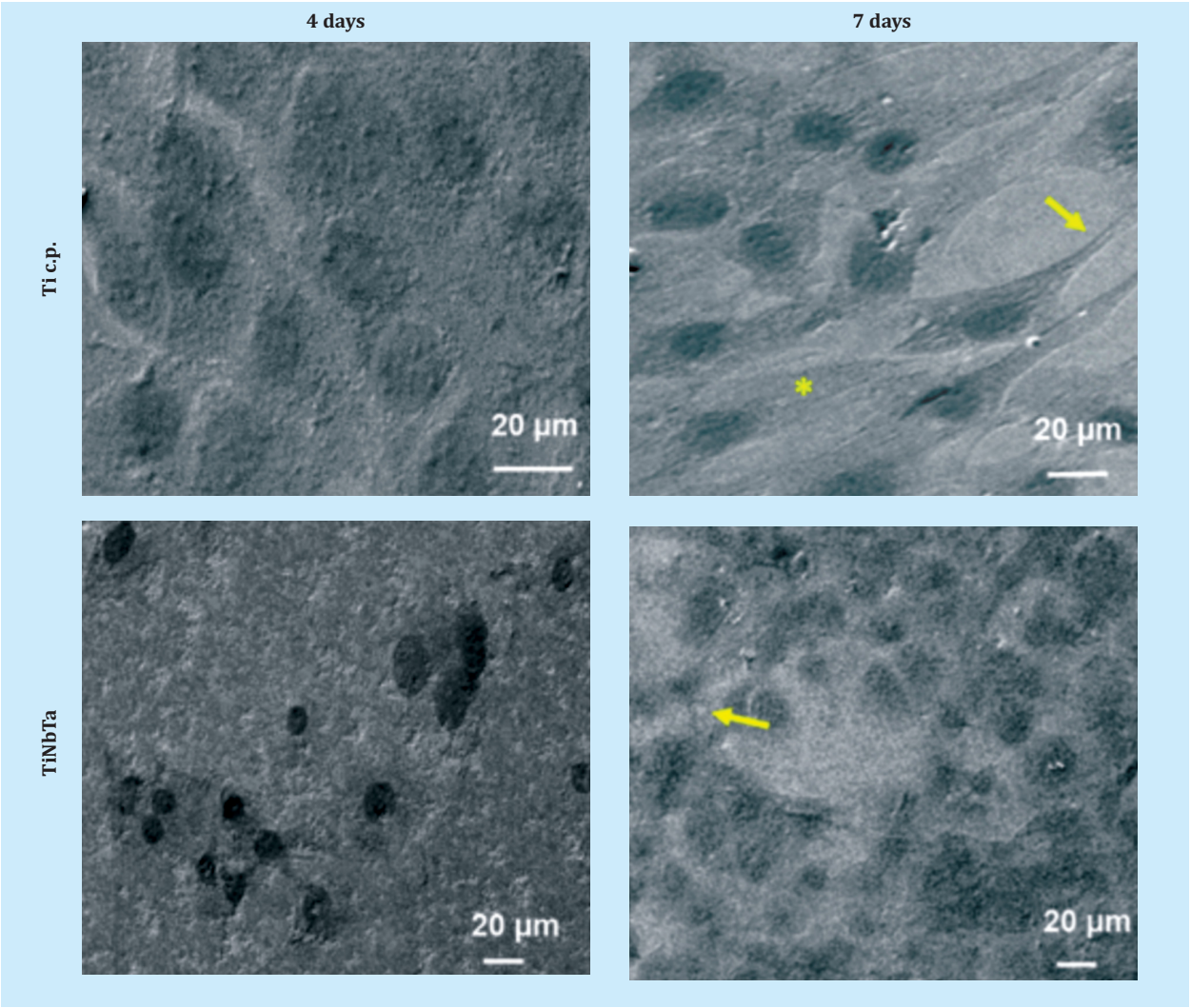


Figure 4. Scanning electron microscope micrographs at 4 and 7 days of osteoblast cultures on the surface of Ti c.p. or TiNbTa. Cell morphology and proliferation is shown on the two surfaces tested. Cell-cell interactions through filopodia (yellow arrows) and cell-surface interactions through lamellipodia (yellow asterisk)



In our study, we have carried out tests to assess a new material, TiNbTa alloy, with a Young's modulus similar to that of trabecular bone. Numerous studies have shown the decrease in elastic modulus in alloys with Nb and Ta, such as Ti35Nb5Ta7Zr, which shows an elastic modulus of 55GPa[11]. The advantage compared to the Ti c.p. or the Ti-6Al-4V alloy would be the lower Young's modulus of the alloy studied, which would considerably reduce the voltage shielding.

The percentage of viability is largely related to the biocompatible and cytotoxic properties of the material; various authors[12,13] have shown how high percentages of

viability are optimal for considering a candidate material to be used as a bone implant in humans. Our cultures, at different study times, presented a high degree of biocompatibility, as well as an absence of toxicity. Positive results were obtained between the cell line and the material. It could be suggested that if this material were implanted, it would tend to synthesize the bone matrix, adhere well to the bone, and be biocompatible[14-15].

The viability values indicate that there are no differences in cell growth between the TiNbTa and Ti c.p alloy, and in all conditions the values are above 75%. Therefore, both materials would be non-cytotoxic and therefore viable. On the other hand, the adequate activity of cellular metabolism confirms that the alloy does not release toxic residues for our cells[15-18]. Previous studies of animal implantation of materials such as Nb and Ta in soft and hard tissues of rats have shown the high biocompatibility of metals and osteogenesis[19,20].

We also quantify cellular activity by measuring ALP activity. Increased ALP activity is directly related to cell proliferation and is a marker of differentiation of the osteoblastic phenotype. In tissues such as bone and cartilage, ALP is expressed early in the calcification process and later in development, ALP expression is decreased. It has been shown that when ALP activity decreases, cell differentiation increases[14].

Our values confirm that the culture on the discs with the new material presents ALP activity that varies according to the study time. At the beginning of the culture, at 4 days, the MC3T3 cells showed a higher cell proliferation in Ti c.p. than those grown on the alloy material, indicating a slower growth in the TiNbTa discs during the first days of culture. At 7 days, the ALP values were similar in both samples and lower than the initial ones, which shows that the culture is behaving with similar characteristics in terms of proliferation and differentiation. In the images obtained by SEM, we observed that at 7 days the cells covered the entire surface of the discs, cell growth occurred equally in both samples and they reached cell confluence, and the lowest activity was consistent with the images cells that are starting the mineralization process. In addition, we began to observe the secretion of material that will form the extracellular matrix by the osteoblasts, and an increase in molecules with the appearance of hydroxyapatite was observed on the surface of the cells[21]. On the other hand, osteoblasts presented filopodia and lamelopodia, essential cellular structures during the cell adhesion process, which indicates that the union to other cells and to the material is direct; other authors describe these junctions in MC3T3 cells and Ti discs c.p.[22,23].

In conclusion, our *in vitro* study allows us to conclude that the novel alloy that combines elements such as Nb and Ta with Ti, in addition to improving the mechanical properties of the material, is, in the short term, biocompatible with osteoblastic cells, behaving with characteristics of viability, proliferation, differentiation and adhesion capacity of osteoblastic cell lines, in a very similar way to that of pure Ti.

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Conflict of interests: The authors declare no conflict of interest.

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Study of bone factor expression in murine model in the absence of pleiotrophin and its changes in the inflammatory situation

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Portal-Núñez S[1], Messa L[1], Sevillano J[2], Herradón G[3], Ramos MP[2], Gortazar AR[1]
1 Institute of Applied Medicine. Department of Basic Medical Sciences. Faculty of Medicine
2 Department of Chemistry and Biochemistry. Faculty of Pharmacy
3 Department of Pharmaceutical and Health Sciences. Faculty of Pharmacy
San Pablo-CEU University. Madrid (Spain)

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Summary
Pleiotrophin (PTN) is a peptide involved in the development and maintenance of bone tissue with important functions in inflammatory processes. However, the deletion of PTN in murine models does not produce a significant bone deterioration, but the mechanisms that compensate for its loss have not been studied to date. Our study was aimed at verifying how the deletion of *PTN* and acute inflammation affect the expression of bone factors. To this end, we used three-month-old female mice deficient for PTN (PTN^{Ko}) to which we induced acute inflammation by administration of lipopolysaccharide (LPS). Vertebrae and tibiae were isolated to measure gene expression and carry out an osteocyte count. In cell cultures, we checked whether PTN could protect MC3T3 (osteoblast) and MLOY4 (osteocyte) cells from the induction of cell death caused by etoposide. Our results show that the expression of osteocalcin is increased in the vertebrae of PTN^{Ko} mice, and that inflammation increased the expression of podhalanin (E11), connexin 43 (Cox43) and the parathormone-related peptide (PTHrP) in the PTN^{Ko} mice treated with LPS. Administering PTN significantly reduced etoposide-induced death in MC3T3 and MLOY4 cell cultures. Thus, PTN deficiency induced increased expression of OCN, and acute inflammation produced overexpression of E11, PTHrP, and Cox43 in PTN^{Ko} mice. PTN increased the viability of osteoblastic cells and osteocytes compared to etoposide treatment.

Key words: pleiotrophin, bone homeostasis, murine model.

Introduction
Pleitropin (PTN) is a cytokine secreted by multiple tissues during embryonic development, and which in adulthood is abundantly expressed in the brain and bone[1,2]. PTN is composed of 136 amino acids and its sequence is very rich in lysine and cysteine. Together with midkine (Mdk), with which it shares 50% homology, this cytokine constitutes the heparin-binding family of growth and differentiation factors, both having affinity for bone extracellular matrix[3-5]. PTN is also known as osteoblast stimulating factor 1 (OSF-1) or heparin-linked growth factor (HB-GAM)[6]. This cytokine was initially isolated from the bone and neuronal tissues of newborn rats[2,7,8] and subsequently its homologues have been found in many species including humans, with 90% homology between the different species[9,10].

PTN reportedly exerts its effects through its binding to glucosamin-glucans of several receptors such as N-syndecan, also called syndecan 3[11], syndecans 1 and 4[12], integrin $\alpha_v\beta_3$[13] and the receptor protein tyrosine phosphatase beta/zeta (PTRPβ/ζ)[14]. It has also been suggested that nucleolin may be a low-affinity receptor for PTN[15] and that anaplastic lymphoma kinase (ALK) may play a role in PTN signaling[16].

Correspondence: Sergio Portal Núñez (sergio.portalnunez@ceu.es)

Probably the best-studied receptor for PTN is PTRPβ/ζ that has also been shown to be expressed in osteoblasts[17]. This receptor is a protein tyrosine phosphatase whose activation by PTN produces its destabilization and, therefore, the cessation of its phosphatase activity. This triggers the increased phosphorylation of its substrates (e.g. fyn kinase) leading, among other effects, to the activation of the nuclear factor that enhances the kappa light chains of activated B cells (NFκβ)[18]. The presence of this receptor in bone tissue and its activation through PTN and another ligand, such as insulin-like growth factor binding protein 2 (IGFBP-2)[19] has recently been confirmed. Conversely, the expression of N-syndecane in osteoblasts and its relationship with bone regeneration have also been demonstrated[6]. In this case, the binding of PTN to N-syndecane would produce the phosphorylation of src, which in turn would lead to reorganizations in the cell cytoskeleton that would allow an increase in cell migration[20].

Among PTN's most prominent functions is its role as a promoter of angiogenesis and endothelial cell migration[13,21], the growth of neurites[22] and its role as a modulator of inflammatory processes governed by microglia in the central nervous system[23]. The functions of PTN in bone tissue are diverse. Thus, PTN has been described as capable of inducing the proliferation of osteblastic cells in a manner dependent on its concentration and the expression of its receptors[24]. It promotes the differentiation of mesenchymal cells to chondrocytes during bone development[25] and increases migration and adhesion to the extracellular matrix of bone cells[6,26]. In fact, the role of PTN in relation to bone mass has been investigated by carrying out gene deletion or overexpression experiments in murine models. In the first case, the absence of the gene reportedly did not produce a decrease in bone mass or significant changes in the biomechanical properties of these bones[27]. Later studies also found that the bone structure of mice without PTN was not altered, but that there was a delay in bone maturation in 2-month-old mice[28].

The role of PTN in osteocyte mechanotransduction has also been studied with different results. In *in vitro* studies, mechanical loading was found to lead to a decrease in PTN expression in SaOs-2 bone cells and in primary osteoblasts subjected to this stimulus[29]. However, Imai et al. demonstrated that MLOY-4 cells (osteocyte cell line), treated with mechanical loading, increased their production of PTN[28]. These data are consistent with those found in an animal model of female C57BL/6J mice subjected to mechanical loading, in which there was an increase in the expression of this cytokine[29]. Furthermore, in the same study, the absence of PTN (using a mouse with the deleted PTN gene) did not influence the increase in bone mass produced by mechanical loading.

Additionally, the effects of overexpression of PTN in bone tissue do seem to have a protective effect in situations of loss of bone mass, such as weightlessness. Mice transgenic for PTN are partially protected against the loss of mass produced by the state of weightlessness when subjected to a stay in the international space station, this protection being related to an increase in osteoblastic activity[30].

As has been commented, the absence of PTN in bone does not seem to significantly influence the skeletal structure of murine models, in this work we endeavored to ascertain how the expression of factors associated with the correct maintenance of bone metabolism was altered in the absence of PTN. Furthermore, we wanted to investigate how the expression of these genes was regulated in a situation of acute inflammation produced by injection of LPS, both in the presence and in the absence of PTN. Finally, we verify the protective effect of PTN on osteoblasts and osteocytes in the presence of a death stimulus such as etoposide.

Material and methods

Animal model

Three-month-old female mice deficient in PTN (PTN^{Ko}) and with normal genotype (WT) were used, 9 mice for WT and 9 for PTN^{Ko} per group. All the mice came from our animal facility, where they are routinely raised. To induce a state of acute inflammation, a dose of 7.5 mg/kg of lipopolysaccharide was injected intraperitoneally 16 hours before sacrifice in 6 WT mice and 5 PTN^{Ko} mice. The animal protocols were approved by the Animal Welfare Committee of the CEU-San Pablo University and the Ministry of the Environment of the Community of Madrid, in accordance with Royal Decree R.D. 53/2013 and European guideline 2010/63/EU.

Processing of bone samples and RNA purification

Total RNA was extracted from the vertebrae after removing non-bony tissues from the lumbar vertebrae 1 to 3, by means of a tissue spray and by dissolving this spray in trizol (Invitrogen, Groningen, The Netherlands). RNA was extracted by means of purifications by the chloroform: isoamyl method (Sigma-Aldrich), followed by precipitation with isopropanol, subsequent washes with 70% ethanol and resuspension in sterile RNAase-free water. RNA reverse transcription to obtain complementary DNA was carried out from 2 µg of RNA using the cDNA High capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, California, USA) in an Eppendorf mastercycler thermocycler, following the following sequential protocol: 10 minutes at 25ºC, 120 minutes at 37º C and 5 minutes at 85ºC.

Quantitative PCR

Quantitative PCR was carried out in an AB 7500 HD thermal cycler (Applied Biosystems). Using TaqMan MGB probes (Assay-by-Design™ System, Applied Biosystems) to measure the expression of the following genes: osteoprotegerin (OPG), activator receptor ligand for nuclear factor κB (RANKL), osteocalcin (OCN), related peptide parathyroid hormone (PTHrP), podoplanin (E11), connexin 43 (Cox43), vascular endothelial growth factor receptor 2 (VEGFR2) and dickkopf-1 (Dkk1). To produce the PCR reaction, the polymerase included in the Taqman mastermix kit (Applied Biosystems) was used applying the following protocol of 1 minute at 95ºC, followed by 40 cycles of 15 seconds at 95ºC and 1 minute at 60ºC, collecting the fluorescence data in each step of 60ºC. Expression of the 18S ribosomal gene was used as a reference gene to normalize expression. The variation of expression of each of the genes in the various groups was calculated in relation to the expression obtained in the WT mice following the following formula:

$$\text{Expression of the gene of interest} = 2^{-\Delta\Delta Ct},$$

where $\Delta\Delta Ct = \Delta Ct$ treatment - ΔCt baseline[31,32]. All determinations were made in duplicate.

Osteocyte count

The tibiae of the mice were also extracted at the time of sacrifice and preserved in 10% formalin for later histo-

logical processing. Once processed and stained with hematoxylin-eosin, the osteocytes embedded in the trabeculae were counted in 2 to 4 non-serial sections corresponding to the region immediately below the epiphyseal plate of each of the tibiae. The number of osteocytes was normalized by their corresponding area of bone tissue calculated using the Image J program (ImageJ 15.3a, National Institutes of Health, USA), and following the recommendations of the American Society for Bone and Mineral Research[33] . The number of resulting osteocytes was calculated by taking the mean of each count per mouse, and was expressed as the mean ± standard error of the mean (SEM).

In vitro cell cultures and viability assay

MC3T3-E1 murine osteoblast cells were cultured in alpha MEM medium supplemented with 10% fetal bovine serum (FBS) (Sigma-Aldrich) and 1% penicillin-streptomycin. The stable line of murine MLOY-4 osteocytes was maintained in culture plates previously collagenized and with alpha MEM medium with 2.5% calf serum (Sigma-Aldrich), 2.5% FBS and 1% penicillin-streptomycin. Both cell lines were incubated at 17ºC in a 5% CO_2 atmosphere. For viability assays, both cell lines were incubated in 75% subconfluence in 6-well plates and were pre-treated or not for one hour with PTN (5.5 nM), and subsequently incubated in the presence of 50 µM etoposide (apoptotic agent) in 1% fetal bovine serum for 48 hours. At 48 hours, the cells were counted, including those in the supernatant of the wells and the trypsin from each well, and a cell count was made with Trypan Blue 0.4% in PBS in the Neubauer chamber, distinguishing the living cells from the dead. The percentage of dead cells from each of the experiments was calculated. Three experiments were carried out with each condition in triplicate and the result was expressed as the mean ± standard error of the mean (SEM).

Statistics

The results were expressed as mean ± standard error of the mean (SEM). The comparison between various groups was made using the non-parametric Kruskall-Wallis test with *a posteriori* U Mann-Whitney test, if appropriate. A $p<0.05$ was considered significant. Analyzes were carried out with the Graphpad InStat software (San Diego, California, USA).

Results

Gene expression in bone tissue in the absence of PTN

In the first place, we wanted to verify how the expression of genes related to bone metabolism varied in the absence of PTN and in the presence of inflammatory conditions after the administration of LPS. Among the expression of all the genes analyzed, we found that OCN was highly overexpressed in PTN^{Ko} mice (Figure 1A). However, the expression of an early osteocyte differentiation marker such as podoplanin (E11) did not undergo significant changes (Figure 1B). In the same way, the expression of the modulators of osteoblast and osteoclastic activity OPG and RANKL did not undergo significant changes in the mouse PTN^{Ko} (Figures 1C, 1D); the Wnt pathway inhibitor, Dkk1 (Figure 1E); VEGFR2 (Figure 1F); and the levels of PTHrP (Figure 1G) and Cox43 (Figure 1H). On the other hand, when normal WT mice were treated with LPS, we found that the levels of OCN, Dkk-1 and VEGFR2 were significantly decreased (Figures 1A, 1D, 1F). With respect to the PTN^{Ko} mice treated with LPS, we found a significant increase in the expression of E11 (Figure 1B), PTHrP (Figure 1G) and Cox43 (Figure 1H) with respect to the WT mice treated with LPS.

The number of osteocytes was unchanged in PTN^{Ko}, WT+LPS and PTN^{Ko}+LPS mice

After observing that the only gene whose expression was regulated was OCN, a gene related to very advanced stages of osteoblastic maturation[34]. As this was upregulated, we thought that this expression could reflect an increase in the number of osteocytes in PTN^{Ko} mice. (We also extend this count to the WT and PTN^{Ko} groups treated with LPS). To do this, we stained the tibiae isolated from the mice and performed an exhaustive count of the osteocytes found in the tibial bone sections. As can be seen in Figures 2A, 2B, the number of osteocytes was similar in all groups of mice, not finding significant differences in any case.

Protective effect of PTN against the induction of death in bone cells

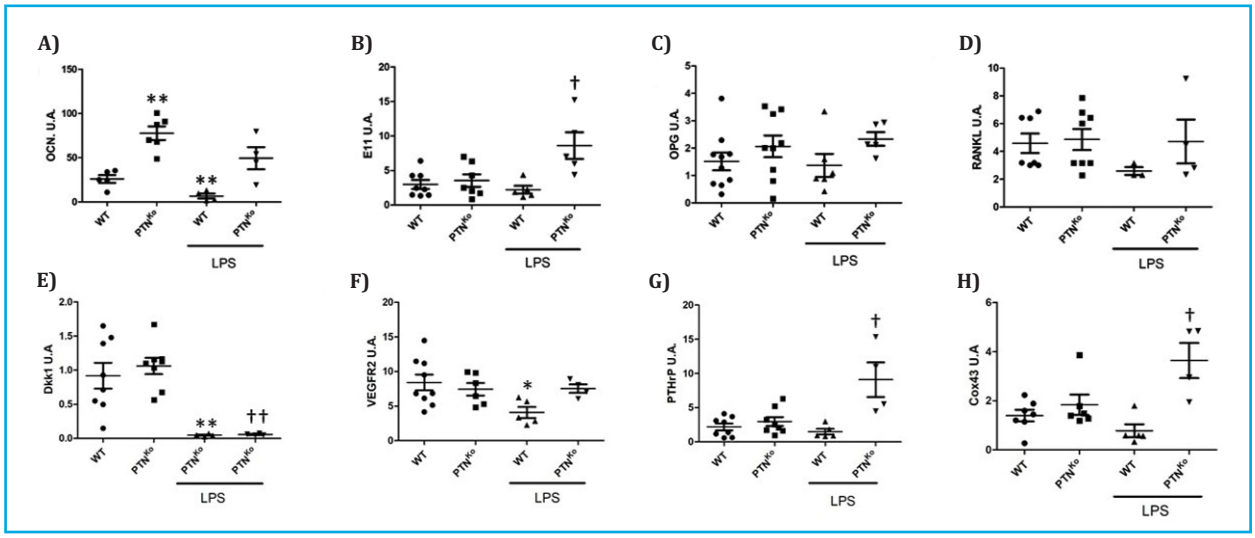
Given that the proliferative effect of PTN on MC3T3 osteoblasts has been previously described[24], we wanted to verify the protective effect of PTN against the induction of death by etoposide in two murine cell lines, one of osteoblasts (MC3T3) and the other of osteocytes (MLOY-4), *in vitro*. The administration of PTN prior to treatment with etoposide produced a protective effect, since the cell death induced by this agent was significantly reduced in the MC3T3 line from 20.8% to 11.5% and in the MLOY4 line from 27, 5% to 18.1% (Figures 3A, 3B).

Discussion

It is noteworthy that deleting a gene as important for the development and maintenance of bone tissue as PTN does not produce a phenotype marked by bone alterations. First of all, one might think that since PTN and Mdk have parallel expressions and similar functions, the lack of PTN could be compensated by the increase in Mdk. However, this does not appear to be the case, since it has been shown that Mdk-deficient mice have an increase in bone mass[29], indicating that both cytokines do not share the same functions fully in bone tissue. In our study, we wanted to verify the expression of genes related to bone metabolism and whether this variation could explain, at least in part, the lack of bone effects in PTN^{Ko} mice.

Recent research indicates that OCN is a protein that acts by regulating the correct alignment of hydroxyapatite crystals with collagen, which is directly related to bone quality[35], although it is also used as a marker of osteoblastic maturation[36], and its current role as a bone-produced hormone is under debate[37-39]. The fact that it is increased in PTN^{Ko} mice led us to think that perhaps the number of osteocytes in parallel could be increased in these mice, as it is a marker of late maturation of the osteoblasts just before they become osteocytes. This may be one of the cellular mechanisms of compensation for the lack of PTN. However, our results (Figure 2A, 2B) show that the number of osteocytes does not vary significantly in the PTN^{Ko} mice compared to the WT mice, nor in the rest of the experimental groups. These results are consistent with those shown by Lehman et al, where an increase in OCN expression was observed in primary cultures from PTN^{Ko} mice and with an equivalent number of osteocytes between normal mice and PTN^{Ko} [27].

Figure 1. Expression of different genes in the vertebrae of WT or PTN^{KO} mice treated or not with injection of LPS



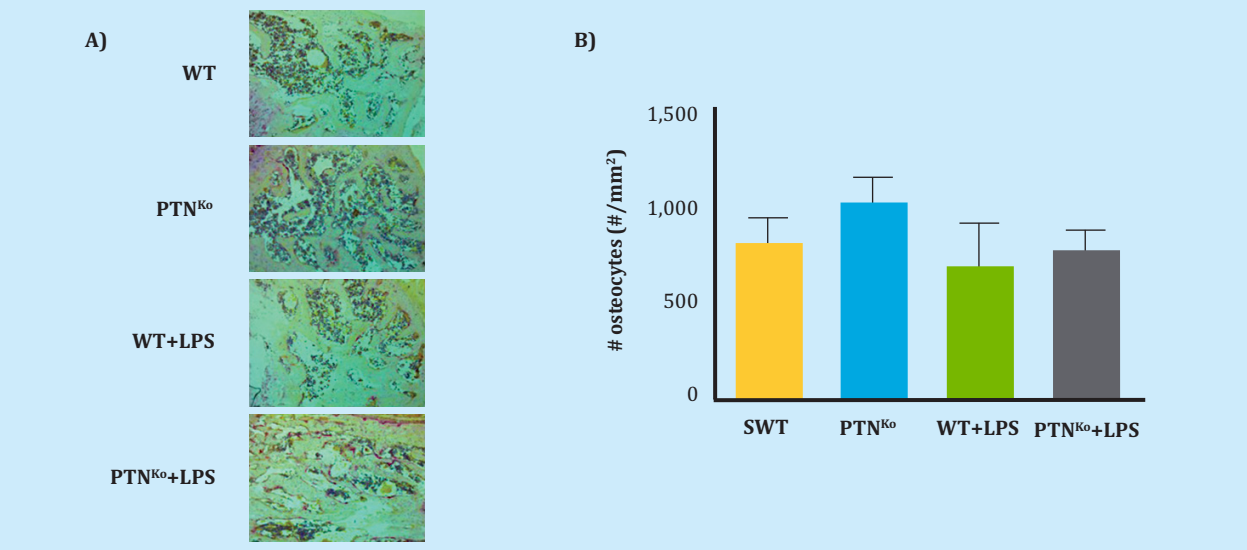
A) Osteocalcin (OCN) vs. WT, **p<0.05; B) Podoplanin (E11) vs. WT+LPS, †p<0.05; C) Osteoprotegerin (OPG); D) Activating receptor ligand for nuclear factor κB (RANKL); E) Dickoppf1 (Dkk1) vs. WT, **p<0.01; Dkk1 vs. WT+LPS, ††p<0.01; F) Vascular endothelial growth factor receptor 2 (VEGFR2) vs. WT, *p<0.05; G) Parathormone-related peptide (PTHrP) vs. WT+LPS, †p<0.05; H) Conexin43 (Cox43) vs. WT+LPS, †p<0.05. The qPCR results are expressed in arbitrary units (A.U.) once normalized with the 18 S ribosomal control gene.

The increase in osteocalcin probably has other effects on bone and the bone matrix of these mice presents an altered composition. To verify this, subsequent nuclear magnetic resonance studies would be necessary to analyze the quality and composition of the bone of the PTN^{Ko} mice. In contrast, and given the importance of PTN in certain inflammatory processes, we also wanted to investigate its role in the expression of these genes in the face of acute inflammation caused by injection of LPS. In this case, we discovered that the expression of E11, PTHrP and Cox43 was increased in the PTN^{Ko} mice compared to the WT mice treated with LPS. PTHrP is a cytokine that modulates bone remodeling, locally resembling the actions of parathormone[40]. This cytokine is involved in the inflammatory processes that occur in the bone marrow, promoting inflammatory factor MCP-1 expression in endothelial cells and osteoblasts[41]. E11 governs the first steps of the osteoblast-osteocyte transition[42], its overexpression is a marker of bone tumors[43] and of inflammation in other tissues and various tumors[44]. On the other hand, Cox43 is part of the communications established by osteocytes among themselves to form the osteocyte network. The role of connexins in inflammatory processes is diverse and variable depending on the different tissues[45]. Increases in the expression of Cox43 have been described that are related to diseases such as osteoarthritis[46], and a greater expression in synoviocytes in response to stimuli such as LPS[47]. The decrease in Cox43 levels could be related to a decrease in arthritic inflammatory processes[47]. In our study, the absence of PTN, at least in the bone compartment, would have a detrimental role from an inflammatory point of view, since it would allow the overexpression of the three genes mentioned (Figures 1B, 1G, 1H) contrary to what occurs in the LPS-treated WT mouse, and thus would lead to undesired effects. Obviously, the ultimate verification of these observations requires subsequent studies both *in vivo* and *in vitro* to unravel the molecular mechanisms triggered by the absence of PTN in this context.

One of the limitations of this study is that the age of the mice (3 months), rather far from the 5 months in which a mature mouse can be considered from the skeletal point of view. It must be taken into account that murine models do not suffer from closure of the growth plates or estrogen depletion, which means that they always have little bone growth throughout their life. In this regard, it should be noted that severe bone defects have not been described in 50-week-old PTN-deficient mice[27], although other authors have found small growth delays in 4-month-old mice[28]. In addition, in this latest study, it has been shown that the biomechanical characteristics of long bones are altered by having decreased the parameters of stiffness, resistance to breakage, and bone hardness[28]. Since PTN contributes to the correct alignment of the crystals of hydroxyapatite, future studies in the bones of adult or even aged mice with absence of PTN should include analysis of these bones by means of nuclear magnetic resonance to determine if the absence of PTN can cause brittle bone in advanced ages. Another study limitation is that, given that cortical areas have not been included when counting osteocytes, we cannot establish whether there are differences between the cortical and cancellous compartments, so this type of analysis must be carried out in subsequent studies.

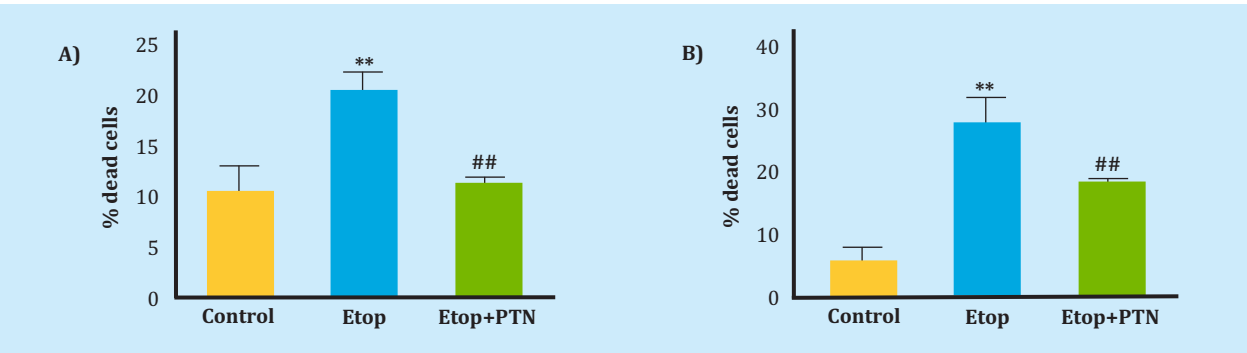
Notably, it has been previously verified that PTN administration *in vitro* promotes the proliferation and differentiation of osteoblast cells[24]. Therefore, we wanted to verify, as far as we know for the first time, the protective effect of PTN against a pro-apoptotic stimulus such as etoposide in osteoblastic cells (MC3T3) and osteocytes (MLOY4), observing that PTN has a protective action. This result points to a possible additional role of PTN in situations of fracture or chronic inflammation in which it could protect, at least partially, bone cells. It is true that in this study the protective role that PTN can play in terms of death measured by LPS has not been proven. In this sense, previous studies have shown that this agent produces a senescent phenotype in osteocytes and an increase in bone resorption mediated

Figure 2. Number of osteocytes in the trabecular section of the tibiae of the WT and PTN^{Ko} mice



A) Representative images of the tibia sections where the osteocyte count was carried out under the epiphyseal plate of the tibiae of the different experimental groups. B) Quantification of the number of osteocytes per mm^2 of bone tissue in the WT, PTN^{Ko}, WT+LPS and PTN^{Ko}+LPS mice.

Figure 3. Protective effect of PTN against the induction of death with etoposide in bone cells



A) MC3T3 cells vs. control, **p<0.01; MC3T3 cells vs. etoposide, ##p<0.01; B) MLOY4 cells vs. control, **p<0.05; MLOY4 cells vs. etoposide, #p<0.05.

by the production of IL-6 and RANKL by osteocytes, rather than death in a short period of time. as is the LPS administration time that has been carried out in this study. However, it would be interesting to study the role of PTN in these contexts in subsequent studies and in suitable models[48,49].

Based on our findings, we can conclude that PTN deficiency is accompanied by an increase in OCN expression and that the induction of acute inflammation by means of LPS in PTN-deficient mice produces the overexpression of E11, PTHrP, and Cox43. Similarly, we have demonstrated the protective role of PTN in osteoblastic cells and osteocytes in the face of a cell death stimulus.

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Fibrous dysplasia mimicking rib metastasis

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García-Gómez FJ, de la Riva-Pérez PA, Calvo-Morón MC
Nuclear Medicine Service. University Hospital Virgen Macarena. Seville (Spain)

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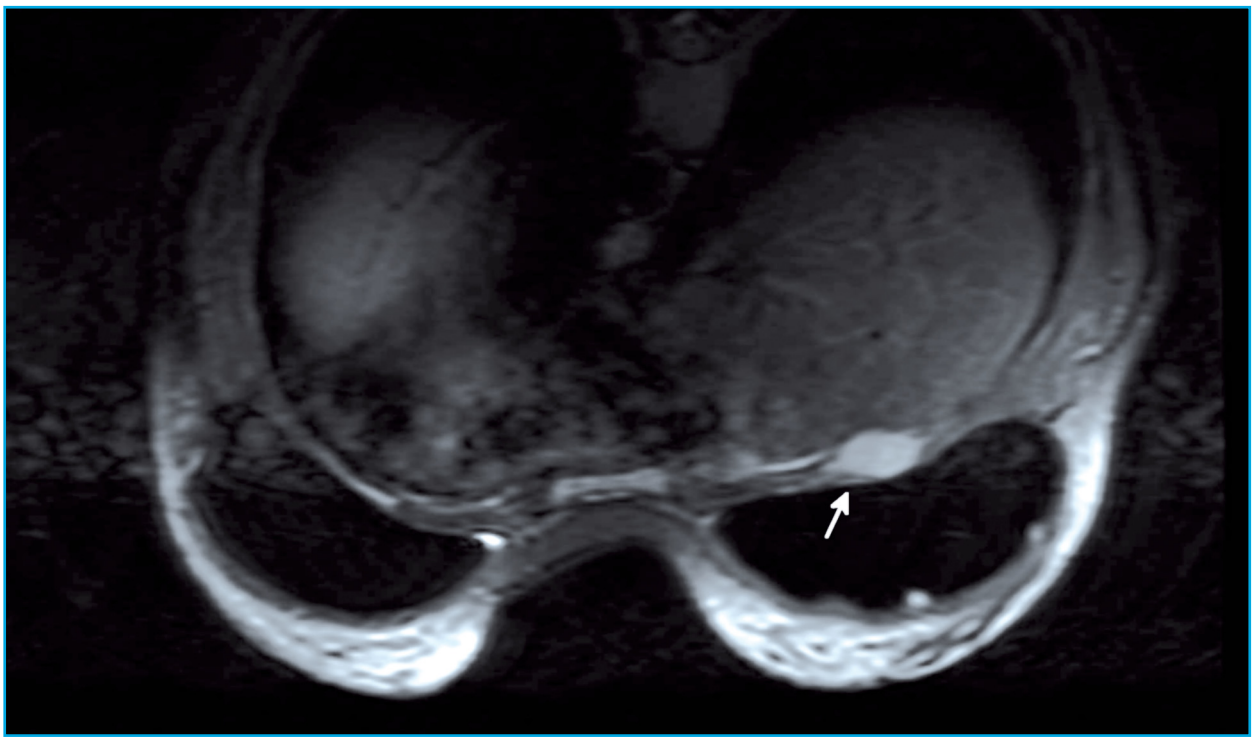
We present the diagnostic images of a 30-year-old woman, an asymptomatic BRCA1 mutation carrier and undergoing clinical-radiological follow-up for bilateral mammary fibroadenomas. The control MRI (Figure 1) highlighted the appearance of a nodular lesion posterior to the right breast prosthesis, relatively well defined and with lobulated contours. Given the suspicion of metastatic bone disease, a positron emission tomography (PET/CT) with 18F-fluorodeoxyglucose (18F-FDG) was carried out to assess its metabolic activity and extent of the disease. This was the only active lesion, with a 2.6 cm diameter and high metabolic activity, located in the fourth right costal arch (Figure 2). In this context, the lesion was excised to rule out neoplastic etiology. Pathology studies showed it was fibrous dysplasia, a benign and slowly progressive pseudotumoral disease, which represents less than 5% of bone tumors.

Fibrous dysplasia is characterized by the replacement of normal bone tissue by osteofibrous connective tissue, adopting a sclerotic, cystic-lytic or mixed pattern[1,2]. The disease is due to an imbalance in the function of osteogenic cells, triggering expansive osteolytic lesions that affect adjacent normal bone and fibrous tissue[1]. The monostotic variant accounts for 70% of cases, and can be asymptomatic and detected incidentally[2]. In monostotic forms, the bones most frequently affected are, in decreasing order, the maxillae, the proximal femur, the tibia, the humerus, the ribs, the skull, the radius and the iliac[1,2].

Figure 1. MRI showing nodular lesion in the right anterior thoracic wall (arrow), posterior to the breast prosthesis, with intense enhancement with contrast, intense rapid uptake and early lavage, compatible with a possible primary or secondary neo-formative nature, without ruling out other diagnostic options



Correspondence: Francisco Javier García-Gómez (javier191185@gmail.com)

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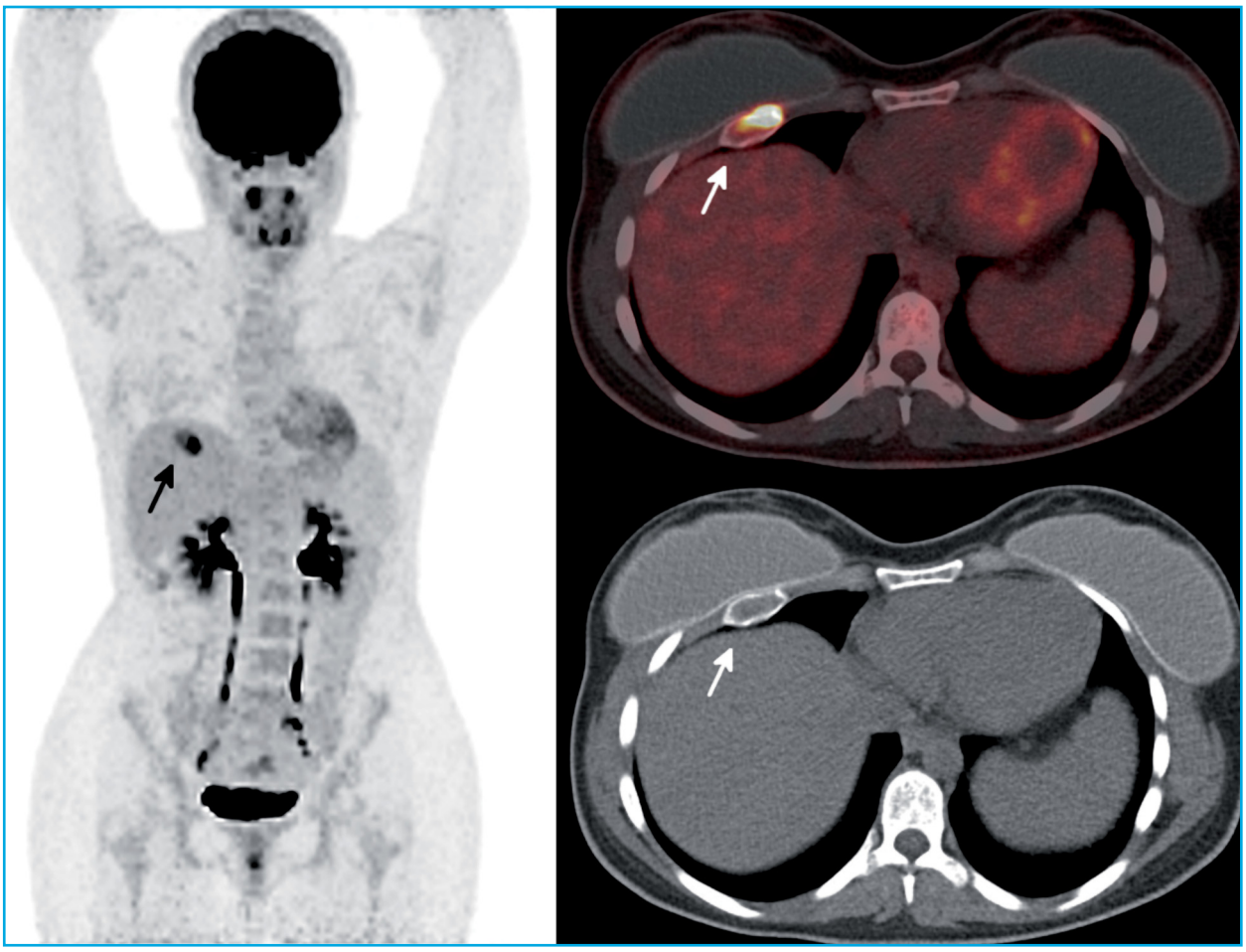
In its diagnosis, diagnostic imaging techniques will be useful. As these lesions present a high rate of bone turnover, they will show high uptake in both bone scintigraphy and PET/CT, making them crucial techniques in the objective determination of the extension, metabolic activity and predicting the evolution of the disease[3,4]. When there are doubts concerning the diagnosis, a bone biopsy or excision with mutational study may be carried out.

Figure 2. PET/CT with 18F-FDG, which highlights a single hypermetabolic lesion circumscribed to the anterior third of the fourth right costal arch (arrow), with very high metabolic activity (SUVmax:15.5)



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Olive oil and bone health

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Rubert M[1], Torrubia B[2], Díaz-Curiel M[3], de la Piedra C[2]
1 Hospital Support Team. Palliative Care. Móstoles University Hospital. Móstoles. Madrid (Spain)
2 Biochemical Research. Medical Research Institute. Jiménez Díaz Foundation. Madrid (Spain)
3 Department of Internal Medicine. Medical Research Institute. Jiménez Díaz Foundation. Madrid (Spain)

Summary
Objetive: A series of studies in the literature indicate that the incidence of osteoporosis and associated fractures is lower in countries where the Mediterranean diet is predominant. Olive oil is characteristic of the Mediterranean diet, a third of the intake of vegetable fats. We carried out an extensive review of studies showing that the ingestion of olive oil, both in experimental animals, especially ovariectomized rats, and in humans, produces positive actions on the bone. The effects of different components of virgin olive oil such as oleuropein, a phenolic compound, and other phenolic alcohols such as tyrosol and hydrotyrosol have been reviewed. Oleuropein not only exerts actions on the bone of ovariectomized rats, but also enhances the formation of osteoblasts and decreases the formation of "osteoclast-like" cells. The phenolic compounds in olive oil exert anti-oxidant actions *in vitro* and *in vivo*. Tyrosol and hydrotyrosol exert actions on bone loss in ovariectomized rats and inhibit dose-dependent osteoclast formation. Our group's research has shown that virgin olive oil also exerts actions on the biomechanical parameters of the bone such as Young's modulus and fractal dimension in ovariectomized rats. The results of this review indicate that olive oil has a positive action on bone health. Its components have antioxidant and anti-inflammatory properties. Thus they are potential candidates for preventing osteoporosis.

Key words: osteoporosis, virgin olive oil, oleuropein, tyrosol, hydrotyrosol.

Introduction
Osteoporosis is the bone disease that most affects humans and predisposes a person to fractures. It constitutes a serious public health problem due to its impact on patients' quality of life and the economic burden it represents. Osteoporosis reportedly affects more than 200 million people[1]. Therefore, it is extremely important to take all possible measures to mitigate its development.

Along with other factors, bone modeling and remodeling are determined by nutritional status[2]. Nutrition has relevant effects on peak bone mass, bone loss with age, and muscle strength[3]. Of course, the main nutrients for bone are calcium and vitamin D[4], since calcium is the major component of bone and its contribution is regulated by vitamin D, thus optimizing peak bone mass. However, the European Union has indicated the relevance of other nutrients on bone development and the advisability of conducting research into these on bone development[5]. The main advantage of nutrition in assessing its importance for bone health is that it can be modified.

The Mediterranean diet is characterized by a high intake of fruits, vegetables, and olive oil. The incidence of osteoporosis and associated fractures seems to be less in countries where the Mediterranean diet is predominant[6].

In this work we are going to focus on olive oil, which is the main common characteristic of the entire Mediterranean diet, assuming a third of the vegetable fat intake[7].

Olive oil contains oleic acid (C1 8:1) (55 – 83%), palmitic acid (C1 6:0) (7.5 – 20%), linoleic acid (C1 8:2) (3.5 – 21%), and more than 200 additional chemical compounds[8]. Besides triglycerides, we are interested in highlighting phenolic compounds here. Oleuropein is the main phenolic compound in olive leaves, olives and olive oil, with an amount in it between 1 ppb and 11 ppm. A group of very important bioactive compounds in olive oil are phenolic alcohols such as tyrosol and hydrotyrosol[9]. Flavonoids are also abundant, one of which is lutein[10]. In general, in this work we focus on virgin olive oil, because refined olive oil does not contain polyphenols, which, as we will see later, have been shown to exert important positive actions on the bone.

Effect of the Mediterranean diet on bone
Savanelli et al.[11] conducted a study in 418 healthy people (105 men and 313 women) between 50±14 years of age. The results showed a positive correlation between bone health and adherence to the Mediterranean diet (higher consumption of virgin olive oil, vegetables, fruit, legumes, fish), being negatively associated with the consumption of red meat, suggesting that greater adherence to the Mediterranean diet favors bone health.

Silva et al.[12] studied 105 healthy postmenopausal women between 45 and 65 years of age. Those who showed greater adherence to the Mediterranean diet had

Correspondence: Concepción de la Piedra (cpiedra@fjd.es)

higher lumbar bone mineral density (BMD) values (1.076±0.146 *vs.* 0.997±0.143 g/cm^2, p=0.007). Thus, adherence to the Mediterranean diet is positively associated with higher BMD values in a non-Mediterranean region, since this work was carried out in Brazil. Adherence to the Mediterranean diet has been associated with a decrease in the incidence of fractures in the European Prospective Investigation into Cancer and Nutrition Study, which included 188,795 subjects followed over 9 years[13]. Keiler et al.[6] described that the incidence of osteoporosis and associated fractures is lower in countries where the Mediterranean diet is predominant. Kontogianni et al.[14] showed that adherence to the Mediterranean diet was positively related to bone mass, suggesting its potential bone-preserving properties.

Adherence to the Mediterranean diet has also been shown to have beneficial effects on BMD in the calcaneus, measured by dual X-ray absorptiometry (DXA) in a sample of healthy women from southern Spain[15]. In the same way, a lower incidence of fractures has been shown in Greece, where a higher proportion of olive oil is consumed than in the USA or in northern European countries[16]. However, in an elderly population in France, a Mediterranean-type diet was not associated with a decrease in the fracture risk[17].

The problem with the interpretation of these data is that we cannot be sure that it is the olive oil that produces the effects of the Mediterranean diet with complete certainty. It contains many fruits, vegetables and fish but there are authors such as Keiler et al.[6] who are almost completely certain that these positive effects are due to the active compounds of virgin olive oil and especially to phenolic compounds. In the works that we present below, we focus on the effects of the oil itself or of its components.

Extensive literature demonstrates the positive effects of olive oil on bone in experimental animals. Ostrowska et al.[18] administered virgin olive oil (19% w/w) to pigs and observed an increase of 6.28 mg/cm^2 of BMD/day in these animals. Bullon et al.[19] demonstrated that a diet based on virgin olive oil prevented alveolar resorption due to age in rats through a mitochondrial mechanism. In an interesting work, Saleh et al.[20] administered 12-14-month-old female Wistar rats with virgin olive oil (1 ml/kg body weight) for 12 weeks, 4 before oophorectomy and 8 weeks after. The ovariectomized rats showed a significant decrease in plasma calcium and an increase in alkaline phosphatase, malondialdehyde, and nitrate levels (the latter two indicating a reduction in oxidative stress). These changes were tempered by olive oil. The tibia of the ovariectomized rats showed a decrease in cortical width and trabecular thickness and a significant increase in the number of osteoclasts. These parameters improved considerably in the group treated with olive oil.

Rezq et al.[21] observed that replacing dietary lipids with olive oil for 6 weeks increased femoral length, volume, and BMD in mice. Liu et al.[22] compared the effectiveness of treatment with oil (1 ml/100 g of diet) and with diethylstilbestrol (25 µg/kg of diet), a synthetic estrogen, to mimic hormone replacement therapy in humans. Both treatments produced an increase in lumbar and femur BMD in ovariectomized rats. This could be attributable to a decrease in oxidative stress in the treated groups, indicated by malondialdehyde and nitrate levels.

In contrast to these results, Tagliaferri et al.[23] found that to alleviate bone loss induced by ovariectomy in rats, virgin olive oil is not enough. Rather, an additional vitamin D supplement is required.

In a study carried out in humans, Roncero Martín et al.[24] administered virgin olive oil to 523 women with a mean age of 50 years (between 23 and 81). The women were divided into two groups: those who ingested more than 18.32 g/day of oil and those who ingested less than that amount. They observed a significant increase in BMD (p<0.001) in the group with the highest olive oil intake.

Liu et al.[22] carried out a study in women between 30 and 50 years old who had undergone a hysterectomy. One group was treated with 50 ml of olive oil daily and another control group received no supplement. After 1 year, the BMD of the L2, L3,L4 and of the left femur decreased significantly in the control group and not in the oil-treated group.

Effects of the different components of olive oil on bone health

Puel et al.[25] evaluated the effects of oleuropein in a model of ovariectomized rats with and without inflammation. This phenolic compound (0.15 g oleuropein/kg/day) was able to exert positive effects on bone loss in rats with inflammation, but not in those without inflammation.

Oleuropein increases the formation of osteoblasts from bone marrow stem cells and decreases the generation of adipocytes and fat cells, suggesting that oleuropein intake could have preventive effects against bone loss associated with osteoporosis and age[26].

In terms of bone resorption, oleuropein at 10 µM decreased the formation of "osteoclast-like" cells (positive tartrate-resistant acid phosphatase) in a spleen cell culture. At a concentration of 50 µM and 100 µM, oleuropein completely suppressed the formation of these cells *in vitro*[27].

García Martínez et al.[28] investigated the effects of the phenolic extracts of Sicilian virgin olive oil on the growth of osteoblasts, using the MG-63 osteosarcoma line. Treatment of osteoblast cells with phenolic extracts increased the number of cells between 13.77 and 30.98%, compared to controls.

Cells of the same MG-63 line were cultured for 24 h with 10^{-6} M of the phenolic compound phenyl acid, caffeic acid, coumaric acid, apigenin or luteolin. The expression by MG-63 cells of growth markers and differentiation/maturation was modified after treatment with 10^{-6} M of the aforementioned phenolic compounds, increasing the gene expression of transforming growth factor β1 (TGF-β1), the TGF1, 2 and 3 receptor, the bone morphogenetic protein 2 and 7, the transcription factor-run 2, the alkaline phosphatase, osteocalcin, type I collagen and osteoprotegerin. The phenolic components of virgin olive oil reportedly have a beneficial effect on the bone by modulating the osteoblast's physiology, which supports its protective effect against bone diseases[29].

The phenolic compounds in olive oil have been shown to possess antioxidant properties *in vivo* and *in vitro*[30,31]. Taking phenols can influence BMD by acting as free radicals, preventing oxidation-induced damage to bone cells.

An extensive review carried out from 2001 to 2014 in the databases of MEDLINE L´EMBASE and the Cochrane Library, using as entries "Mediterranean diet", "virgin olive oil", "phenols", "bone", "osteoblasts" and "osteoporosis," suggest that phenols in olive oil may be beneficial

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in preventing bone loss. They are reported to induce the proliferative capacity and cell maturation of osteoblasts by increasing alkaline phosphatase activity and depositing calcium ions in the extracellular matrix[28].

We previously mentioned phenolic alcohols, tyrosol and hydroxytyrosol, as components of olive oil. Hydroxytyrosol has been shown to eliminate trabecular bone loss in femurs of ovariectomized rats[27]. On the other hand, hydroxytyrosol at concentrations between 50 µM and 100 µM inhibits the formation of multinucleated osteoclasts in a dose-dependent manner. In a culture of spleen cells, hydroxytyrosol (50 and 100 µM) and tyrosol (100 µM) reduced the formation of acid phosphatase-tartrate-cell resistant[27].

Although, as we have mentioned, there are various studies in the literature that show that treatment with olive oil increases BMD, it has not been possible to demonstrate that there has been an increase in biomechanical parameters[32,33]. However, in a recent work carried out by our group[34] we have treated a group of ovariectomized 6-month-old Wistar rats with olive oil by oral gavage for 3 months (100 µl/day or 200 µl/day). Our results show that the treatment with 100 µl of olive oil recovered the value of Young's modulus in the x-axis, which had decreased with oophorectomy, and the treatment with 200 µl of oil produced an improvement in the z-axis of Young's modulus. with respect to the ovariectomized rats, that is to say, that the olive oil influenced the biomechanical parameters. In this same work, we found that the groups treated with 200 µl of olive oil presented a value of the fractal dimension D2D and D3D greater than that of the ovariectomized rats. The fractal dimension expresses the degree of complexity of the outline of a structure in filling a surface or volume. These results indicate that the bone composition of rats treated with 200 µl of virgin olive oil is more complex and more irregular and, thus, more similar to normal bone. Despite these improvements in bone health, in our work we did not find differences in the BMD of the treated rats or in the micro-morphometric parameters, but the results obtained in Young's modulus and in the fractal dimension that indicate an improvement cannot be disregarded in the bone quality of the treated ovariectomized rats. It is important to highlight that in our work we give the rats 100 µl or 200 µl of virgin olive oil/day. Taking into account that the rats weighed 320 g at the beginning of the study, this would be equivalent to giving 18.7 or 37 ml of olive oil/day to a 60 kg person, a dose that could be consumed normally. Many of the published experimental works supply rats with a very high quantity of oil relative to what a normal human diet might be.

The results of this review show, without a doubt, that virgin olive oil exerts a positive action on bone health. This is possibly due to the action of its phenolic components, which include oleuropein, tyrosol and hydroxytyrosol. These agents have been shown to have anti-oxidant and anti-inflammatory properties, and therefore may be potential candidates for the prevention of osteoporosis.

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