Revista de Osteoporosis y Metabolismo Mineral

Our cover: Band of osteoblasts by toluidine blue staining (40X).
Authorship: Group of Bone, Vascular Metabolism and Chronic Inflammatory Diseases. Health Research Institute of the Principality of Asturias (ISPA). Central University Hospital of Asturias. Oviedo (Spain).

Summary

Vol. 13 - Nº 4 - November-December 2021

Indexed in: Scielo, Web of Sciences, IBECS, Scopus, SIIC Data Bases, embase, Redalyc, Emerging Sources Citation Index, Open J-Gate, DOAJ, Free Medical Journal, Google Academic, Medes, Electronic Journals Library AZB, e-revistas, WorldCat, Latindex, EBSCOhost, MedicLatina, Dialnet, SafetyLit, Mosby's, Encare, Academic Keys, ERIH plus, British Library, ROAD.

Revista de Osteoporosis y Metabolismo Mineral has recently been acepted for coverage in the Emerging Sources Citation Index, wich is the new edition of the Web of Science that was launched in november 2015. This means that any articles published in the journal will be indexed in the Web of Science at the time of publication.

Editorial Committee

Sequential treatment: it has come a long way... with a long way to go

González Macías J
Professor of Medicine
Emeritus Professor at the University of Cantabria

Innovations in therapeutic behavior in the face of a certain disease are generally designed to improve effectiveness. But sometimes they may have other reasons. For example, avoiding side effects, lowering the cost or making the form of administration of a drug more comfortable. Sequential therapy represents a therapeutic innovation in the field of osteoporosis.

The first form of sequential therapy used in osteoporosis was probably called ADFR (A = activate remodeling, D = depress resorption, F = free formation, and R = repeat) or "coherence therapy", initially proposed by Frost around 1980[1]. The theoretical approach on which this strategy was based was to synchronize the remodeling units, placing them in the spring phase, by administering an osteoclast-stimulating drug, to then inhibit them. Afterwards, a free time of 2-3 months was left, during which the osteoblasts, activated by the coupling set in motion with the initial stimulus of the osteoclasts, were supposed to develop the osteoforming effect. The ADFR regimen was a therapeutic innovation that was intended to improve the efficacy of osteoporosis treatment, but it was a failed attempt.

The next form of sequential treatment that was considered in osteoporosis did so much later, some 20-25 years later. And, unlike the ADFR guideline, it was not established as a consequence of a theoretical reasoning, but arose as a necessity when it was found that the suspension of the administration of teriparatide was followed by a loss of the effects achieved with it, unless that an antiresorptive drug was administered[2]. It was therefore a regimen that we previously described as intended to improve therapeutic efficacy.

Sequential therapy can also be applied in the field of osteoporosis to avoid side effects. In fact, the knowledge that prolonged treatment with potent antiresorptives (bisphosphonates, denosumab) can lead to serious complications, such as atypical femoral fracture (AFF) and osteonecrosis of the jaws, has led to at least two forms of sequential therapy in the management of the disease. One of them, only applicable to relatively young women (50-60 years) with mild-moderate osteoporosis that does not involve a risk of hip fracture, consists of administering during the first 6-8 years (depending on the age of the patient) a SERM, so that bisphosphonates or denosumab can be introduced later, and the patient can delay her exposure to the risk of the aforementioned complications[3]. The other affects those patients who are already under treatment with these drugs, and have been doing so for at least five years before. In them, the possibility of stopping the drug (specifically in the case of bisphosphonates) should be considered, since it is known that this reduces the risk of AFF rapidly. The risk-benefit ratio must first be assessed. If bisphosphonate is discontinued, the risk of FAF decreases, but that of osteoporotic fracture increases. When this is small, the risk can be assumed. Withdrawal of the drug then places the patient on what we call "therapeutic holidays"[4]. After these, the drug should be reintroduced, to return, perhaps, in the future to a new holiday period (bisphosphonate-holiday-bisphosphonate-holiday cycles, etc., with which the patient could remain adequately treated for the rest of his life). If the risk of osteoporotic fracture is great, what should be done, instead of suspending the treatment, is to administer a drug that does not carry the risk of the complications referred to: specifically, teriparatide (which Nelson Watts, in some ASBMR webinars, has called very graphically "sabbatical period", establishing an analogy with drug holidays). Both the sequence bisphosphonates-drug holidays-bisphosphonates, etc., and the sequence bisphosphonates-teriparatide-bisphosphonates, are two good examples of sequential treatments aimed at avoiding complications. Discussing the extent to which the concept of therapeutic vacation could be applied to denosumab and that of sabbatical to romosozumab requires comments beyond the scope of this editorial.

Another reason that may motivate a change in osteoporosis treatment is perception, when assessing patient evolution. Response is inadequate and a change should be made (it may be debatable whether by changing drug the concept of "sequential therapy" is appropriate –in other pathology fields it would not be considered this way–; however, for the purposes of our discussion here, we can accept it). In such a case, the reported[5] attitude could consist of one of these three possibilities: a) changing the drug considered insufficiently effective for another of the same, more potent type; b) change from an oral drug to an injectable one; c) change from an antiresorptive to an osteoformer. Today we might add, for certain occasions, a fourth possibility: the combination of two drugs, generally an osteoformer and an antiresorptive. Imagine, for example, a therapeutic failure of denosumab in a situation where, due to lack of availability or due to contraindication, romosozumab cannot be used since the administration of teriparatide after denosumab is not recommended. It could be switched to an association of teriparatide with zoledronate or even with denosumab itself. The combination, therefore, can constitute one more link in the therapeutic chain of sequential treatment.

The possible sequential guidelines for the treatment of osteoporosis are numerous. Fortunately, almost all of them have been studied and, consequently, we have a lot of information about them. But precisely their high number and the occasional existence of discrepancies in their results, require, in order to facilitate their knowledge, to carry out a necessarily laborious task of systematization and analysis. And this is exactly the task carried out by Drs. Casado and Neyro in the article published in this issue of the journal[6], facilitating our work in such a complex field.

The therapeutic sequences considered in this article constitute a sound basis on which to decide which specific treatment guidelines are appropriate. In some cases, the information we already have will suffice. But at other times, new studies will be needed to supplement the ones that have existed so far. The latter will occur, for example, when the current data come from studies whose outcome variables are not fractures, but others of a surrogate nature (in general, bone mineral density [BMD]). Or when it is necessary to include aspects not previously considered in the regimen in question, such as economics or those related to the convenience of drug administration.

There are multiple examples. The administration of denosumab after a bisphosphonate increases BMD more, but we lack data regarding the effect that this has on reducing fractures (this idea of reaching a certain BMD value –more specifically the T-index– responds to a concept that is very attractive –that of the "treat-to-target"–, which may possibly play an interesting role in the future, but which is currently not mature enough). In this regard, some authors[7,8] have recently insisted on the similarity of the efficacy of zoledronate and denosumab in reducing the risk of fractures despite the greater effect of the latter on BMD. Osteoformers have been shown to be more effective than oral antiresorptives, but we do not know what would happen if they were compared with denosumab or zoledronate. Starting a treatment with an osteoformer to continue with an antiresorptive may be more effective than starting with the antiresorptive directly when the risk of fracture is very high. It has not been proven, however, that the same occurs when the risk is not so high. Incidentally, it is striking that authors who defend that this guideline is used in a practically generalized way, explicitly indicate in their published findings that this proposal does not take into account economic aspects and the convenient use of the osteoformer[9,10]. Administering cycles of a bone-forming drug separated by that of an antiresorptive has also been considered a particularly effective osteoporosis treatment. The administration of two osteoformers in succession, one after the other, has even been suggested. Again, they are examples of suggestive theoretical approaches pending evaluation in practice both from the point of view of their efficacy and their viability (cost or acceptance by the patient).

Thus, sequential treatment, with its various modalities, represents a clear advance in the management of osteoporosis. The possibilities are numerous, but there are still aspects to be clarified. Experience with the ADFR regimen teaches us to be prudent in accepting therapeutic innovations. Reviews such as the one published by Drs. Casado and Neyro help us to proceed in a field that is becoming increasingly complex. We need to know how to distinguish what is already proven from what is not beyond mere speculation.

Bibliography

1. Frost HM. Treatment of osteoporosis by manipulation of coherent bone cell populations. Clin Orthop. 1979;143:227-244.
2. Lindsay WH, Neer R, Pohl G, Adami S, Mautalen C, Reginster J-Y, Stepan JJ, Myers L, Mitlak BH, et al. Sustained vertebral fracture risk reduction after withdrawal of teriparatide in postmenopausal women with osteoporosis. Arch Intern Med. 2004;164:2024-2030.
3. Cosman F. Long-term treatment strategies for postmenopausal osteoporosis. Curr Opin Rheumatol. 2018;30:420-426.
4. Bauer DC, Abrahamsen B. Bisphosphonate Drug Holidays in Primary Care: When and What to Do Next? Curr Osteoporos Rep. 2021;19:182-188.
5. Diez-Perez A, Adachi JD, Agnusdei D, Bilezikian JP, Compston JE, Cummings SR, Eastell R, Eriksen EF, Gonzalez-Macias J, Liberman UA, Wahl DA, Seeman E, Kanis JA, Cooper C; IOF CSA Inadequate Responders Working Group. Treatment failure in osteoporosis. Osteoporos Int. 2012 Dec;23(12):2769-74.
6. Casado E, Neyro JL. Tratamiento secuencial en osteoporosis. Nuevas tendencias. Rev Osteoporos Metab Miner. 2021;13(4):107-116.
7. Reid IR. No more fractura trials in osteoporosis? Lancet Diabetes Endocrinol. 2020;8:650-651.
8. Reid IA, Horne AM, Mihov B, Stewart A, Bolland MJ, Bastin S, Gamble GD. Predictors of fracture in older women with osteopenic hip bone mineral density treated with zoledronate.J Bone Mineral Res. 2021;36:61-66.
9. Cosman F. Anabolic therapy and optimal treatment sequences for patients with osteoporosis at high risk for fracture. Endocr Pract. 2020;26:777-786.
10. Cosman F, Dempster DW. Anabolic agents fos postmenopausal osteoporosis: how do you choose? Curr Osteoporos Rep. 2021;19:189-205.

Sequential treatment in osteoporosis. New trends

Casado E[1,2], Neyro JL[3,4]
1 Rheumatology Service. University Hospital Parc Taulí (UAB). Sabadell (Spain)
2 Parc Taulí Research Institute (I3PT) (Spain)
3 Obstetrics and Gynecology Service. Cruces University Hospital (Spain)
4 University of the Basque Country. Baracaldo (Spain)

Summary
Treatment for osteoporosis is usually long term, so it is often necessary to implement a sequential treatment strategy. The clinician must know not only how to select the best available therapy in each clinical situation, but also how to discontinue or change treatment at a certain point in the evolution of the disease.
Here, we briefly review the mechanisms of action and the consequences of discontinuing each of the drugs for osteoporosis, as well as what happens with the different treatment sequences.
Discontinuation of denosumab has clearly negative consequences for the skeleton, and only bisphosphonates, due to its particular remaining antiresorptive effect, could be discontinued for a limited time (drug holidays).
Switching from antiresorptive to another antiresorptive with a different mechanism of action is an option that may be favorable in the management of some patients with osteoporosis. Switching from antiresorptive to anabolic may be associated with an initial bone mineral density loss that does not appear to have negative consequences on antifracture efficacy. Starting with anabolic (teriparatide or romosozumab) and subsequently switching to antiresorptive is the best treatment sequence, so it could be the preferred option in patients who present a very high risk of fracture.

Key words: sequential therapy, osteoporosis, treatment, antiresorptive, bone formation.

INTRODUCTION

Osteoporosis is a chronic systemic disease characterized by a deterioration in bone density and/or quality, which predisposes to a greater risk of fracture[1].

All treatments for osteoporosis have shown greater or lesser efficacy in reducing the risk of fracture, especially in postmenopausal women[2]. This beneficial effect occurs from the modification of the bone remodeling process with the consequent increase in bone mineral density (BMD) and/or a modification of the bone's microarchitecture, although this requires a prolonged treatment over years in most cases.

Although treatment with antiresorptives, menopausal hormone therapy [THM], selective estrogen receptor modulators [SERM], bisphosphonates [BP] and denosumab [DMAB]) can be maintained for at least 5-10 years, the balance between risk and benefit should always be taken into account, since in prolonged treatments with the highest antiresorptive potency drugs (BP and DMAB) an increased risk of some very infrequent complications such as osteonecrosis of the maxilla or atypical femur fracture has been described[3,4].

In the case of bone-forming drugs (teriparatide [TPTD] and abaloparatide [ABL]) or dual-effect drugs (romosozumab [ROMO]), the duration of treatment is limited to a shorter period of time. TPTD and ABL (the latter not authorized for marketing in Europe) are not recommended to be administered for more than 2 years[5,6], and ROMO should not be administered beyond 12 months[7].

In recent years, the term sequential treatment has been gaining importance in the management of patients with osteoporosis, that is, the sequential use of different treatments to achieve maximum efficacy with the least risk of complications[8].

ADVANTAGES OF A SEQUENTIAL TREATMENT

In managing patients with osteoporosis, a sequential treatment strategy over the years is often required. After using a treatment for a certain period of time, it can be beneficial to discontinue or replace it with a different one, as a sequence.

There are several reasons that justify sequential therapy in patients with osteoporosis:

1. Osteoporosis is a chronic disease that requires long-term treatment, probably for many years.

2. Some treatments for osteoporosis have a maximum recommended duration (for example, 2 years for TPTD and ABL or 1 year for ROMO).

3. Some treatments are associated with some infrequent complications, especially if they are used for more than a certain time (for example, atypical femur fractures are more frequent in patients treated with BP for more than 5 years).

4. With some of the osteoporosis treatments there is a loss in BMD gain if a different one is not administered after its discontinuation.

5. In some cases, greater efficacy is achieved when a sequence of 2 or more drugs is used than if a single drug is used in the same period of time.

6. Patients tend to get tired and lose adherence to treatments, when they are administered very chronically for years, without changes in the administration guidelines[9].

7. Sometimes there is an inadequate response to treatment, either due to a loss of BMD or the development of new fractures despite following the treatment correctly; or simply an insufficient response to reach a therapeutic objective ("treat-to-target" strategy).

In all these situations we should propose a change in the treatment for osteoporosis. Although in some cases a temporary interruption might be better, in most situations it would be more appropriate to substitute a different treatment.

It is thus very important that the clinician knows the advantages and disadvantages of the different sequences of treatments in the patient with osteoporosis.

MECHANISM OF ACTION OF DRUGS FOR OSTEOPOROSIS

For the proper management of the patient with osteoporosis, knowing how to select the best available therapy in each clinical situation is crucial, as well as discontinuing or changing when the risk-benefit balance recommends. It is therefore essential to be very familiar with the mechanism of action, effectiveness and safety of each of the drugs.

Menopausal hormone therapy (THM)

Treatment with estrogens, alone or in combination with progestogens (depending on whether the patient retains her uterus or not), acts through the activation of nuclear estrogen receptors (ERα and ERβ) distributed in different body tissues. ERα receptors predominate in bone, their stimulation on the one hand inhibits osteoclastogenesis from the inhibition of the ligand of the activating receptor for nuclear factor kappa B (RANKL) and the stimulation of osteoprotegerin (OPG)[10], and, on the other hand, effects on bone formation have been described, through the stimulation of factors such as IGF-1 or the inhibition of sclerostin[11]. In bone remodeling, MHT has an antiresorptive effect, with the consequent increase in BMD and a decrease in the risk of vertebral, non-vertebral and hip fracture, especially when administered in the first 5-10 years after the menopause[12].

However, given the much debated risk of breast cancer and cardiovascular complications in prolonged estrogen treatments only confirmed in treatments started beyond the age of 60, it is currently assumed that MHT is reserved for women with premature ovarian failure and for those under 60 years of age with overt climacteric symptoms, in which the benefits clearly outweigh the risks[13].

Selective estrogen receptor modulators (SERMs)

SERMs are a group of drugs with agonist activity at bone estrogen receptors, inhibiting resorption, and antagonist at estrogen receptors in the breast and endometrium, which is why they can provide the beneficial effect of estrogens in patients with osteoporosis, minimizing the effects of adverse effects on other organs[14]. Raloxifene (RLX) and bazedoxifene are the SERMs indicated in women with postmenopausal osteoporosis, since they increase BMD and reduce the risk of vertebral fracture. The unproven efficacy in reducing the risk of non-vertebral or hip fracture and the increased risk of venous thromboembolism restrict its use to postmenopausal women under 70 years of age at risk of vertebral fracture and low risk of hip fracture and venous thrombosis[15].

Bisphosphonates (BP)

BPs are compounds derived from inorganic pyrophosphate with a high affinity for bone hydroxyapatite, which, when taken up by endocytosis by the osteoclast, inhibit farnesyl pyrophosphate synthetase and lead to cell apoptosis, with the consequent inhibitory effect on bone resorption[16].

Alendronate (ALN) and risedronate (RIS), taken by mouth, and zoledronic acid (ZOL) by intravenous route, are the BP most widely used and recommended by clinical guidelines, given the demonstrated efficacy both in increasing BMD and in reducing the risk of vertebral, non-vertebral and hip fracture[15,17]. Ibandronate (IBN) is another oral BP, which, although it offers the advantage of its monthly administration, has only shown a reduction in the risk of vertebral fracture[17].

Some infrequent complications associated with prolonged treatment with BP have been described, such as osteonecrosis of the maxilla and atypical fracture of the femur, with incidences of 0.1-1/10,000 and 1-2/10,000 patient-years, respectively[15].

Denosumab (DMAB)

DMAB is a completely human monoclonal antibody, administered subcutaneously, with a potent antiresorptive activity, through the blockade of RANKL[18]. DMAB has been shown to continuously increase BMD, for at least 10 years, and reduce the risk of vertebral, non-vertebral, and hip fractures[19]. DMAB has demonstrated superiority to BP in terms of increases in BMD, and its prolonged treatment has also been associated with osteonecrosis of the maxilla and atypical femur fracture, although also with a very low incidence[20].

Teriparatide (TPTD) and abaloparatide (ABL)

TPTD and ABL are synthetic parathormone analogues (PTH 1-34 and PTHrP 1-34 respectively) with bone-forming activity and approved for treating osteoporosis with high risk of fracture (the EMA has only approved TPTD). Both drugs bind to the PTH receptor (although with affinity to different conformational states) and inhibit sclerostin, thereby stimulating osteoblastogenesis,

SPECIAL ARTICLE _Sequential treatment in osteoporosis. New trends_
Rev Osteoporos Metab Miner. 2021;13(4):107-116

109

and decreasing apoptosis of osteoblasts. Later, and to a lesser degree, they increase RANKL secretion and therefore bone resorption[21].

Both treatments produce an increase in bone mass and improve the microarchitecture of the skeleton, especially in the trabecular bone, increasing bone strength and reducing the risk of fracture.

Both drugs are indicated in the treatment of patients with osteoporosis at high risk of fracture and have shown superiority over BP[22], but should not be administered beyond 2 years according to the technical data sheet.

Romosozumab (ROMO)

ROMO is a humanized antibody for subcutaneous administration with a dual effect on bone remodeling, as it inhibits sclerostin and secondarily RANKL, producing a rapid, but transitory, increase in bone formation (osteoformer) associated with a more sustained decrease over time of bone formation. resorption. As a consequence, with this treatment there is a marked increase in BMD (greater than with TPTD) and a decrease in the risk of fracture[23,24].

Unlike what happens with TPTD or ABL, the increase in bone formation that occurs with ROMO is mainly due to a marked increase in bone shaping (bone formation after activation of the lining cells in quiescent areas, without a process of prior resorption).

The beneficial effects of ROMO are both on the trabecular bone and on the cortical bone.

However, in its development, doubts have appeared in relation to cardiovascular safety, which have not yet been resolved. In the ARCH study, comparing with ALN, a higher incidence of serious cardiovascular events was observed in women treated with ROMO[25].

ROMO has been approved by the EMA, although as of the writing of this article it is not yet marketed in Spain.

According to the technical data sheet, it can only be administered for 12 months and is contraindicated in patients with a history of myocardial infarction or stroke[7]. In addition, the cardiovascular risk of patients should be assessed, based on risk factors, before and during its administration.

DISCONTINUING OSTEOPOROSIS TREATMENTS

THM discontinuation

Discontinuation of estrogen treatment involves increased remodeling in some patients, with a rapid decrease in BMD and progressive loss of anti-fracture efficacy, which can be prevented with the administration of BP[26].

SERM treatment discontinuation

Discontinuation of treatment with X-ray is accompanied by a loss of BMD in both the lumbar spine and the femur, although less than that produced with estrogen interruption and proportional to the physiological loss that occurs with age[27].

Discontinuation of BP treatment

BPs are the only drugs for the treatment of osteoporosis with a residual effect on the skeleton after its discontinuation, the duration of which will depend on the affinity for hydroxyapatite of each BP, ranging between 1-2 years for RIS and IBN and 2-3 years for ALN and ZOL[28].

After discontinuation of treatment with BP, there is no immediate increase in remodeling, but rather this increase will appear progressively over time, which implies a stability or slight loss of BMD, while this residual effect lasts[28,29].

This property of BP justifies the possibility of considering a temporary suspension of treatment or therapeutic vacations in patients with low risk of fracture[28].

Discontinuation of DMAB treatment

DMAB has a reversible RANKL inhibition effect, so that discontinuation of treatment produces a rapid and marked increase in bone remodeling, with elevation of formation and resorption markers, as early as 9 months after the last dose, reached levels even higher than those prior to treatment, and which did not normalize until after 24 months[30]. This "rebound" effect on remodeling is accompanied by a rapid loss of BMD, which can reach the values prior to the start of treatment (figure 1)[30], and, in some patients, an increased risk of vertebral fractures, particularly multiple ones[31]. The incidence of vertebral fractures in women with postmenopausal osteoporosis who discontinue DMAB has been estimated between 8.5-10.5% in the 12-18 months after discontinuation[32,33], although some of these fractures could be due not only to the effect "Rebound", but rather the return to a high-risk situation due to previous vertebral fractures that patients had before starting treatment with DMAB.

A systematic review of the literature carried out by a European Calcified Tissue Society (ECTS) working group demonstrated that the risk factors for the presentation of multiple vertebral fractures in patients who discontinue DMAB are young age, having prevalent vertebral fractures, a duration of treatment greater than 2.5 years, a greater gain in hip BMD during treatment and a greater decrease in hip BMD after discontinuation[34]. Some case series indicate that previous treatment with BP could mitigate the rebound effect of remodeling (lower increase in markers) that occurs after discontinuation with DMAB, although it is not clear whether this attenuation prevents loss of BMD and fractures[34].

The administration of another potent antiresorptive treatment such as BP in patients who discontinue DMAB appears to have partial efficacy on the rebound effect that occurs. Experts recommend that patients who have received ABDM for less than 2.5 years and are at low risk of fracture can be treated with an oral BP for a minimum of 1-2 years (depending on bone markers and BMD). However, patients who have received DMAB for a longer time, those still at high risk of fracture, or those who cannot tolerate oral BP should receive a dose of ZOL 6 months after the last dose of DMAB, which could be repeated at 3-6 months, depending on the levels of bone markers[34].

Experts conclude that the indication for DMAB should be carefully assessed, especially in young patients, and that in those who discontinue treatment, a BP should be administered 6 months after the last DMAB injection, while there are no more data from new clinical trials.

Based on the data from the systematic review by Tsourdi et al.[34], we present a practical decision algorithm in patients receiving treatment with DMAB (figure 2).

In case an invasive dental procedure is necessary during treatment with DMAB, experts recommend that it be performed after the 5th month from the last dose, and the next dose is administered as soon as the surgical wound has healed[34].

Discontinuation of treatment with TPTD and ABL

Discontinuation of TPTD (and probably ABL) is accompanied by a loss of BMD in the spine and femur in the subsequent 12 months, being more marked in postme-

nopausal women (7.1%) than in men (4.1%)[35]. The administration of an antiresorptive after discontinuation of TPTD maintains or even increases BMD[36,37].

ROMO treatment discontinuation
The effect of ROMO on bone remodeling is reversible, and its discontinuation is accompanied by a normalization of training and a "rebound" effect of resorption, which translates into a rapid loss of BMD in both the lumbar spine and the hip. (figure 3)[38]. The previous administration of ALN seems to mitigate this "rebound" effect and attenuate the loss of BMD.

ANTIRESORPTIVE FOLLOWED BY ANOTHER ANTIRESORPTIVE

The administration of DMAB after having received ALN achieves greater suppression of remodeling and greater gain in BMD than the same treatment time with ALN alone[39]. The ALN-DMAB sequence also appears more efficient than the ALN-ZOL sequence, at least in terms of DMO gain[40].

Therefore, in patients treated with oral BP who present a therapeutic failure or do not reach the goal of treatment, a reasonable option could be the transition to DMAB or ZOL, as long as its indication is clear and the administration of bone former.

On the other hand, and as mentioned above, in order to minimize the rebound effect that occurs on bone remodeling after discontinuation of DMAB, it seems advisable to administer a BP (oral or intravenous) at 6 months of the last injection of DMAB (figure 2).

ANTIRESORPTIVE FOLLOWED BY OSTEOFORMER

Previous treatment with BP, especially with those with a higher affinity for hydroxyapatite (ALN and ZOL), seems to attenuate the BMD gain that occurs with TPTD[41], observing a decrease in the first 6 months of treatment[42], although anti-fracture efficacy seems to be maintained as demonstrated in a post-hoc analysis of the VERO study[43].

Figure 1. Changes in bone mineral density of the lumbar spine (A), total hip (B) and 1/3 radius (C) during the 24 months of treatment with denosumab and during the 24 months after discontinuation. (Modified from Bone HG, et al.) [30]
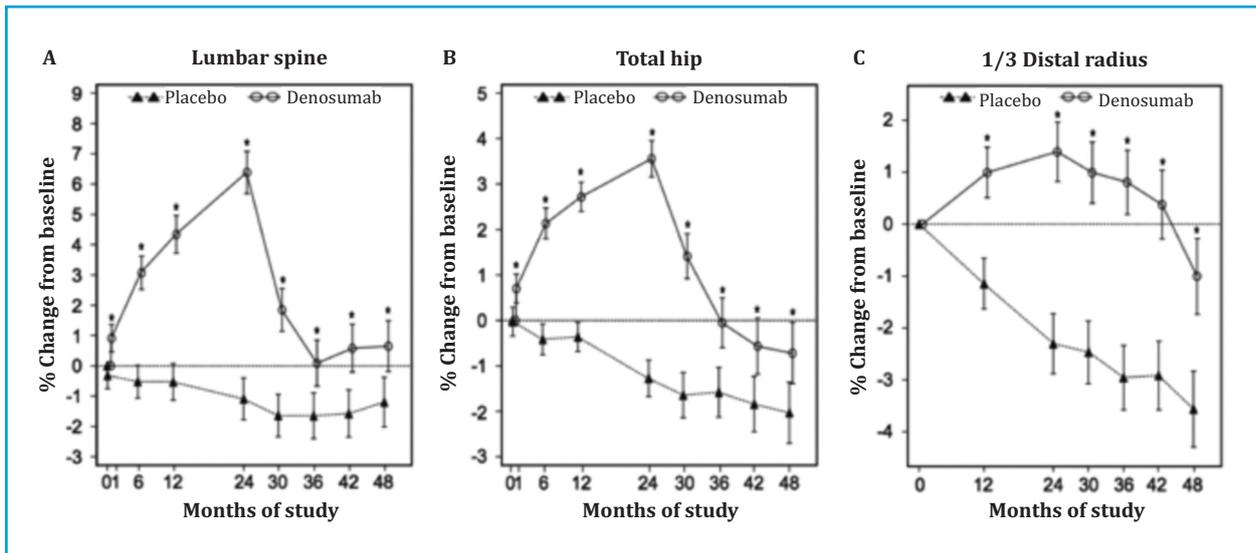
Figure 2. Decision algorithm in patients receiving denosumab treatment for osteoporosis. (Adapted from Tsourdi E, et al.) [34]
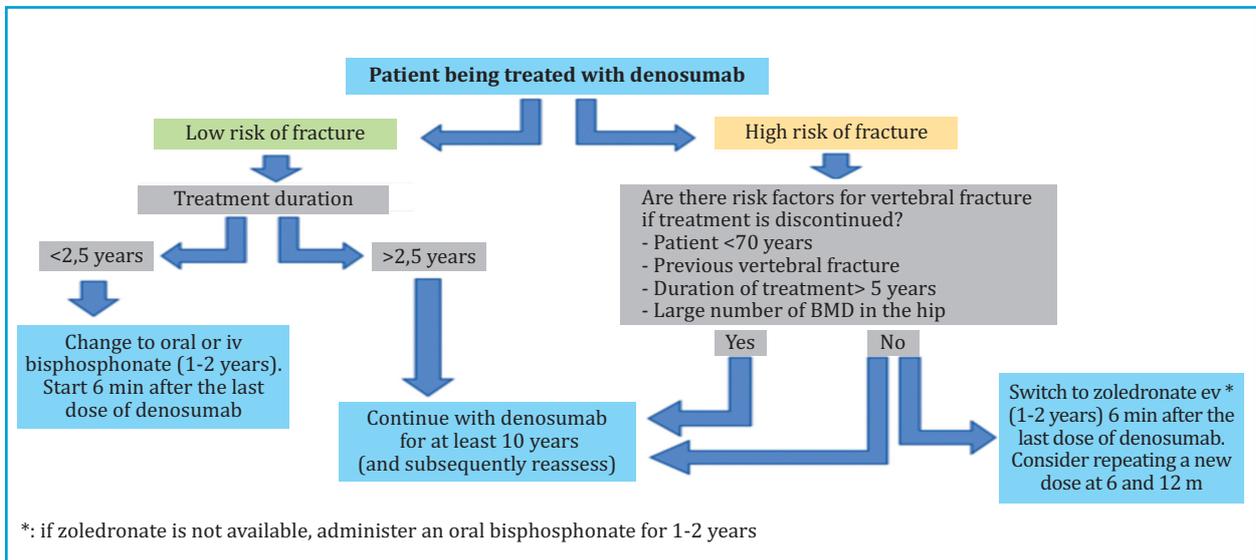
Figure 3. Changes in bone mineral density in the lumbar spine (A) and total hip (C) during treatment with romosozumab and after discontinuation. The loss of BMD is less in patients who had previously received alendronate (B and D). (Modified from McClung MR, et al.)[38]
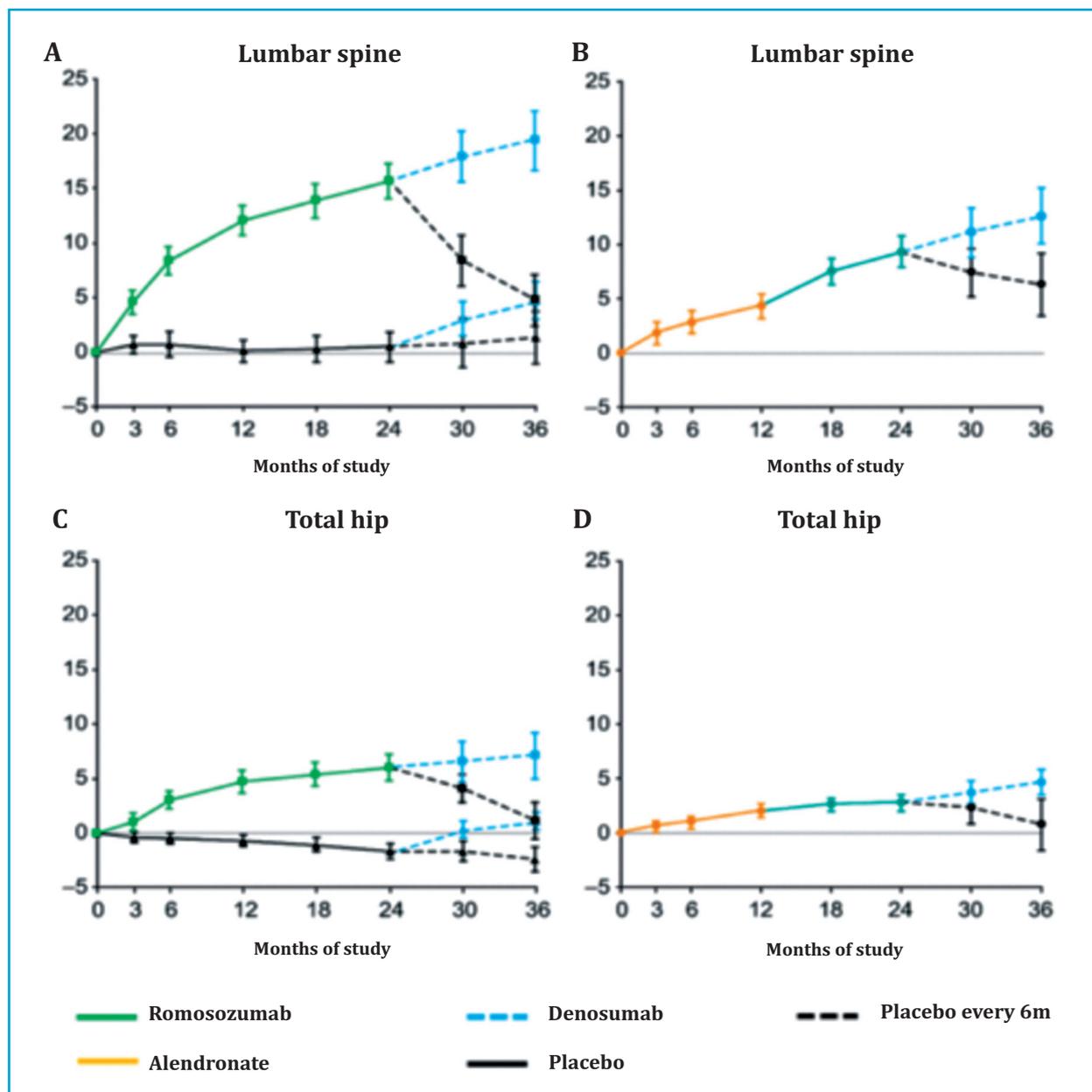
However, the transition from RLX to TPTD does not seem to attenuate the osteoforming effect or the BMD gain of the latter[42].

In the DATA-Switch study, the women who received DMAB for 2 years and switched to TPTD presented an increase in bone remodeling, with a marked decrease in BMD in the hip in the first year, but which recovered in the second year, returning to BMD values at the onset of TPTD (figure 4)[44]. Although there is no evidence whether this transient loss of BMD is accompanied by an increased risk of fracture, some experts suggest not discontinuing treatment with DMAB if TPTD (or ABL) is to be indicated, that is, combination therapy[45].

In the STRUCTURE study, it was observed that, as occurs in the transition to TPTD, patients who received ALN and switched to ROMO had a lower increase in BMD than patients who received ROMO without prior antiresorptive treatment[46].

Although anabolic treatment with TPTD or ROMO seems more effective in patients not previously treated with antiresorptive drugs, patients who, despite receiving antiresorptive treatment, present a high risk of fracture, probably obtain a greater benefit with the change to bone-forming treatment, except in the case of DMAB, which seems better to maintain a combination therapy with TPTD[46].

The DMAB-ROMO sequence could be more favorable, since it has been reported that a second 12-month cycle of ROMO in patients who had received 12 months of DMAB after a first 2-year cycle of ROMO does increase spinal BMD lumbar (2.3%) and maintain hip BMD (figure 5)[47]. However, it must be taken into account that

these results have been extracted from a cohort of only 14 patients, and in whom DMAB had only been administered for one year.

OSTEOFORMER FOLLOWED BY ANTIRESORPTIVE

Sequential TPTD-RLX treatment has been shown to be beneficial. RLX manages to maintain or even increase the BMD gain achieved with previous treatment with TPTD[48].

Equally beneficial is the TPTD-BP sequence. The BMD gain achieved with TPTD can be increased if, after its discontinuation, a BP is administered, also maintaining the anti-fracture efficacy[49].

But the TPTD-DMAB sequence is probably the one that provides the highest BMD gain (18% in the lumbar spine and 8% in the femoral neck after 2 years of treatment with TPTD followed by another 2 years of treatment with DMAB)[44].

Depending on the clinical characteristics of the patient and the risk of fracture, we could select one antiresorptive or another after completing the two years of treatment with TPTD (figure 6).

A study with 68 postmenopausal women has recently been published comparing the efficacy of 3 repeated cycles of 6 months of TPTD followed by 6 months of DMAB (total 36 months), versus a standard treatment of 18 months of TPTD followed by 18 months of DMAB. After 3 years of treatment, the patients who received standard sequential therapy achieved greater gains in BMD in the lumbar spine (16% vs 12%; p=0.04), with no changes in hip and radius BMD. Cyclical therapy achieved better results in hip and radius BMD at 18 months, so according to the authors, repeated cyclical treatment of TPTD and DMAB could be potentially useful in patients at imminent risk of fracture, especially in patients at higher risk of non-vertebral fracture[50].

The ABL-BP sequence is also effective. In the extension of the ACTIVE study, the administration of ALN after ABL was shown to increase the BMD gain achieved with ABL and to maintain anti-fracture efficacy[51].

There are also data on the ROMO-antiresorptive sequence. In the ARCH study, it was observed how the BMD gain achieved with ROMO was maintained after switching to ALN. Regarding anti-fracture efficacy, the reduction in the risk of vertebral fracture was maintained and the reduction in the risk of non-vertebral fracture was increased[52].

In the FRAME study, the sequence 1 year of ROMO followed by 1 year of DMAB was equally beneficial in terms of gain in BMD and reduction of the risk of vertebral fracture. Although a lower incidence of clinical fractures and non-vertebral fractures was also observed with this sequential treatment than with a single year of ABMD, it must be said that the differences were not significant[24].

THERAPEUTIC HOLIDAYS

In order to minimize the risk of long-term complications or improve patient adherence to treatment, a temporary interruption of antiresorptive treatment may be considered in the management of osteoporosis, but only with BP, both oral and intravenous, given its remaining effect on the skeleton[29,52,53].

The working group on the management of patients with osteoporosis on prolonged treatment with BP of the American Society for Research in Bone Metabolism

(ASBMR) recommends considering therapeutic holidays after at least 5 years of treatment with oral BP or 3 years of ZOL, but only in patients under 70 years of age, without fractures before or during treatment and who do not have a hip BMD in the osteoporosis range or present risk factors for fracture or a high risk according to FRAX[28].

The duration of the therapeutic holiday will depend on the BP used, being greater in the case of BP with greater affinity for hydroxyapatite such as ALN (2 years) or ZOL (3 years), and lower (1-2 years) in the case of BP with lower skeletal affinity such as RIS or IBN[28].

It should be remembered that a temporary interruption cannot be considered with any other antiresorptive, and that doing so with DMAB could expose the patient to a situation of high risk of fracture, especially vertebral, especially among patients with a previous vertebral fracture, as already mentioned. previously. It is precisely in these cases that one might ask the reasons for a suspension or interruption of treatment.

COMBINATION THERAPY

The combination of two antiresorptives has not been shown to be more effective than treatment with a single antiresorptive[54].

Nor does the combination of osteoformer with BP provide greater efficacy. The combined treatment of TPTD and ALN appears to be even less effective than TPTD alone[55]. In any case, it could be somewhat more beneficial to add TPTD to treatment already started with BP or to add BP to treatment already started with TPTD[56], although more studies are needed to confirm this assumption.

Combined TPTD and ZOL therapy has been shown to achieve a faster increase in BMD in both the lumbar spine and the hip, although with no greater gain in BMD at 12 months than TPTD in monotherapy[57].

The most promising combination therapy is the simultaneous administration of TPTD and DMAB. In the DATA-Switch study, it was observed that the patients treated with the combination had greater increases in BMD in the spine and hip than the patients treated with each drug alone (figure 4)[44].

According to the latest osteoporosis recommendations of the Spanish Society of Rheumatology, this combination could be justified in very selected cases of severe osteoporosis[12].

CONCLUSIONS

• Osteoporosis is a chronic disease that usually requires treatment for many years.

• For optimal management of the disease, the clinician must know very well the mechanism of action, efficacy and safety of each of the drugs, as well as the differences that exist according to the order of administration of the same.

• With the exception of BPs, which have a residual anti-fracture effect, discontinuation of treatment for osteoporosis is not favorable for the bone, being especially negative in the case of DMAB.

• For this reason, therapeutic holidays can only be considered in patients treated with BP with a low risk of fracture.

• The change from an antiresorptive to another antiresorptive with a different mechanism of action is an option that may be favorable in the management of some patients with osteoporosis.

SPECIAL ARTICLE   *Sequential treatment in osteoporosis. New trends*
Rev Osteoporos Metab Miner. 2021;13(4):107-116

113

Figure 4. DATA-Switch study. The denosumab-teriparatide sequence produces a marked decrease in bone mineral density in the hip in the first year of transition, which recovers in the second year (red). The teriparatide-denosumab sequence achieves a marked gain in BMD in the hip (blue), similar to the combination therapy followed by denosumab (green). (Adapted from Leder BZ,et al.)[44]
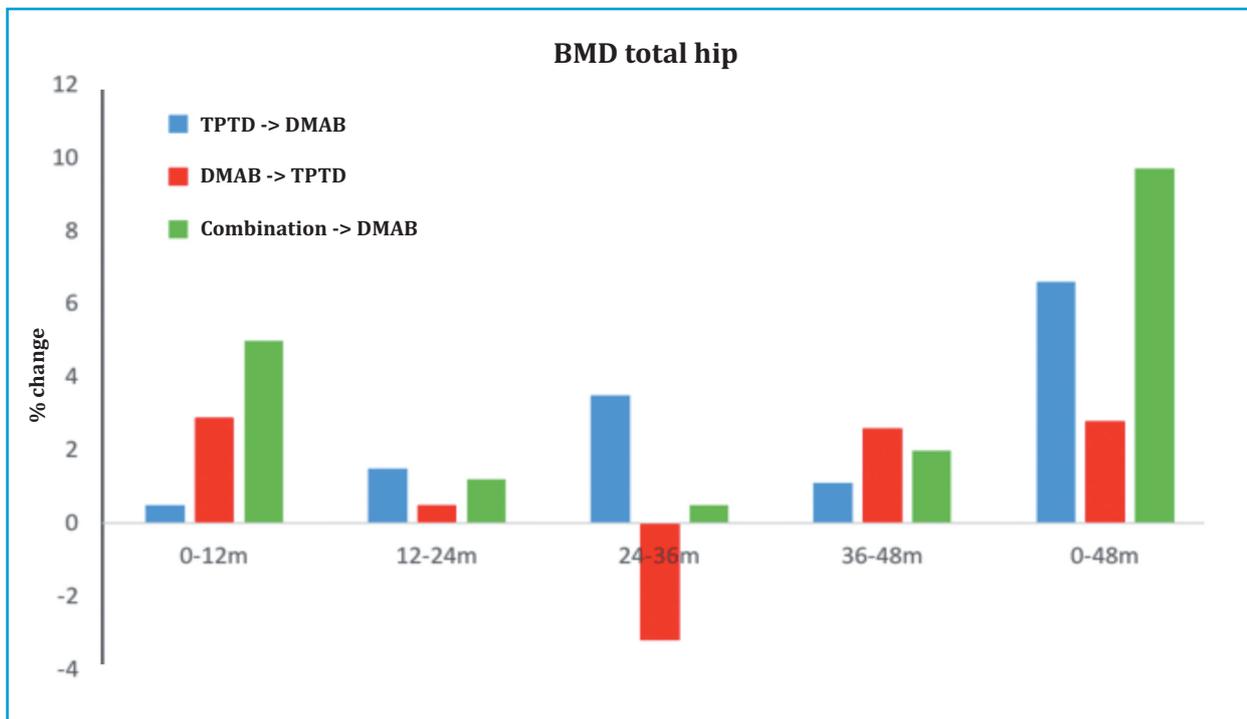
Figure 5. Sequential denosumab-romosozumab treatment after a 2-year cycle of romosozumab succeeds in increasing bone mineral density in the lumbar spine and hip. (Adapted from Kendler DL, et al.)[47]
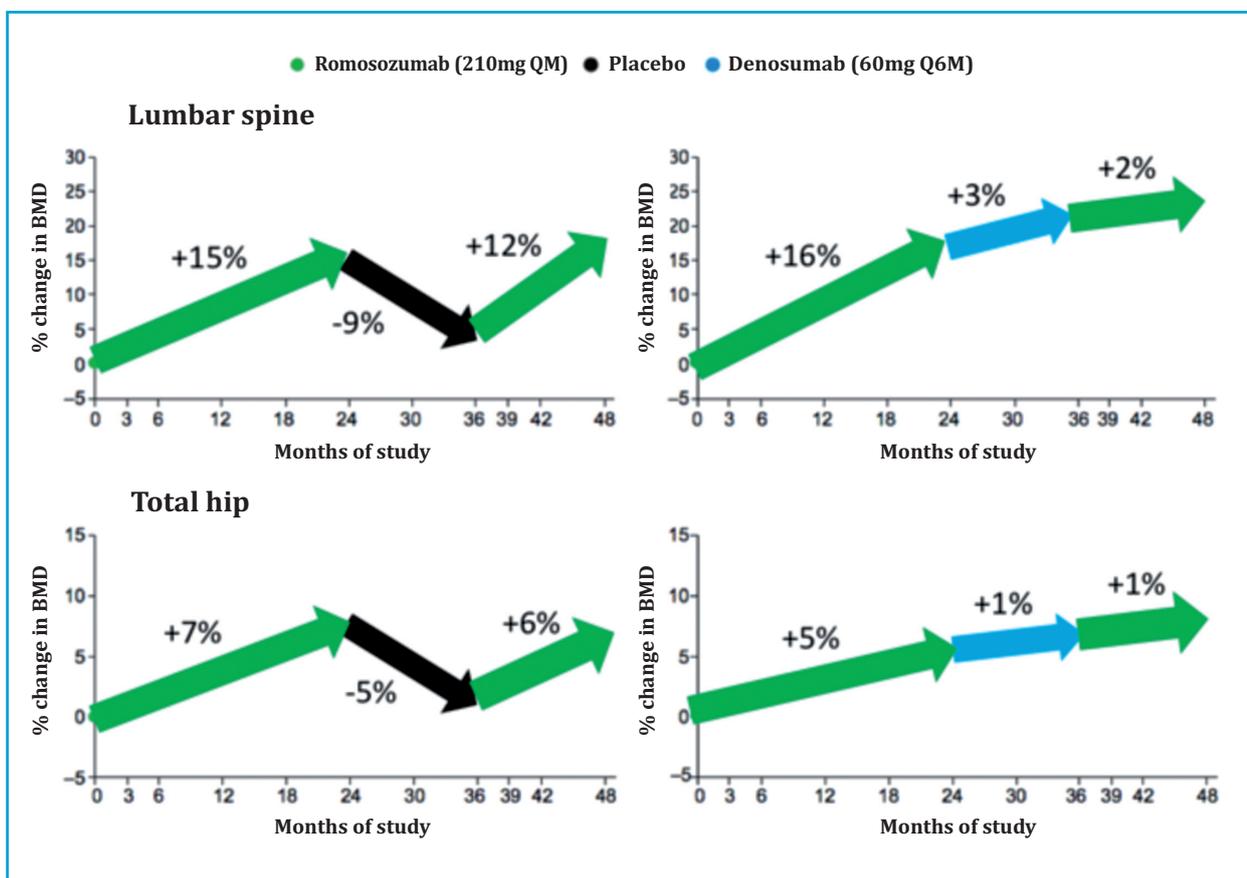
Figure 6. Decision algorithm in patients who have completed two years of treatment with teriparatide for osteoporosis
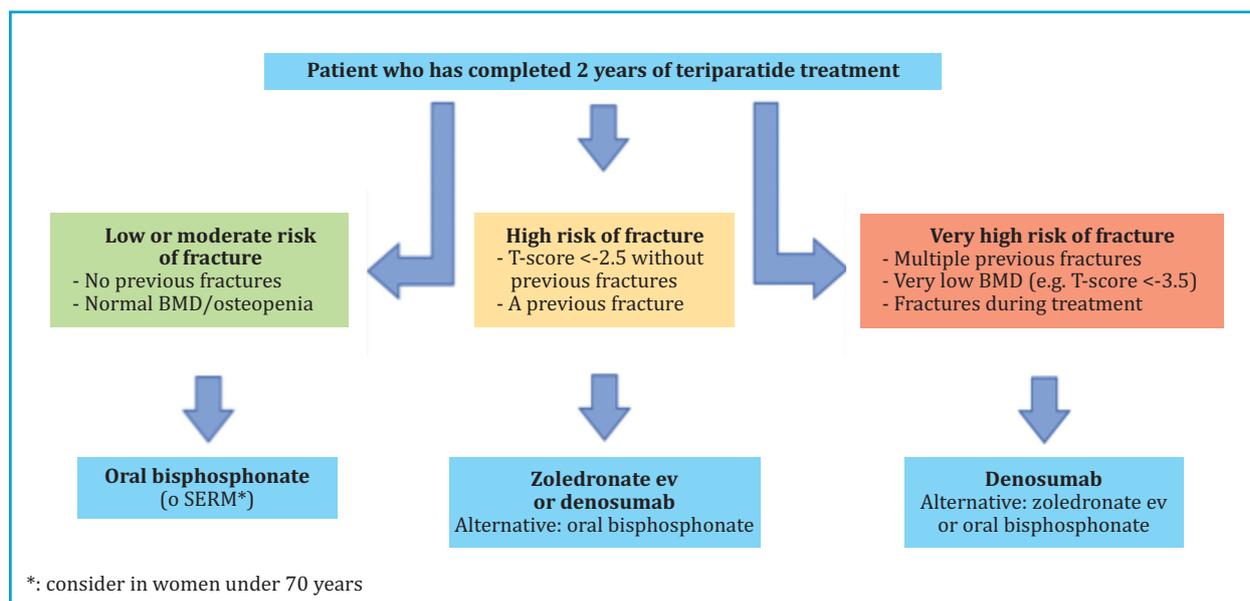
- Although exchanging an antiresorptive for an osteoformer may be associated with a lower initial BMD gain (or even loss in the case of ABMD), this does not appear to have negative consequences on antifracture efficacy.

- Starting with bone-forming treatment (TPTD or ROMO) and then continuing with an antiresorptive is the best treatment sequence, so it could be the preferred option in patients with a very high risk of fracture.

SPECIAL ARTICLE *Sequential treatment in osteoporosis. New trends*
Rev Osteoporos Metab Miner. 2021;13(4):107-116

115

Bibliography

1. NIH Consensus development panel on osteoporosis prevention, diagnosis, and therapy. JAMA. 2001; 285(6):785-95.
2. Yang L, Kang N, Yang JC, Su QJ, Liu YZ, Guan L, et al. Drug efficacies on bone mineral density and fracture rate for the treatment of postmenopausal osteoporosis: a network meta-analysis. Eur Rev Med Pharmacol Sci. 2019; 23(6): 2640-68.
3. Lu L, Lu L, Zhang J, Li J. Potential risks of rare serious adverse effects related to long-term use of bisphosphonates: An overview of systematic reviews. J Clin Pharm Ther. 2020; 45(1):45-51.
4. Pittman K, Antill YC, Goldrick A, Goh J, de Boer RH. Denosumab: Prevention and management of hypocalcemia, osteonecrosis of the jaw and atypical fractures. Asia Pac J Clin Oncol. 2017; 13(4): 266-76.
5. Ficha técnica de Forsteo. https://www.ema.europa.eu/en/documents/product-information/forsteo-epar-product-information_es.pdf.
6. Ficha técnica de Tymlos. https://www.accessdata.fda.gov/drugsatfda_docs/label/2017/208743lbl.pdf.
7. Ficha técnica de Evenity. https://www.ema.europa.eu/en/documents/product-information/evenity-epar-product-information_es.pdf.
8. Guañabens N, Moro-Álvarez MJ, Casado E, Blanch-Rubió J, Gómez-Alonso C, Díaz-Guerra GM, et al. The next step after anti-osteoporotic drug discontinuation: an up-to-date review of sequential treatment. Endocrine. 2019; 64(3): 441-55.
9. Cadarette SM, Burden AM. Measuring and improving adherence to osteoporosis pharmacotherapy. Curr Opin Rheumatol. 2010; 22(4): 397-403.
10. Hofbauer LC, Schoppet M. Clinical implications of the osteoprotegerin/RANKL/RANK system for bone and vascular diseases. JAMA. 2004; 292(4): 490-5.
11. Mirza FS, Padhi ID, Raisz LG, Lorenzo JA. Serum sclerostin levels negatively correlate with parathyroid hormone levels and free estrogen index in postmenopausal women. J Clin Endocrinol Metab. 2010; 95(4): 1991-7.
12. Anderson GL, Limacher M, Assaf AR, Bassford T, Beresford SA, Black H, et al. Effects of conjugated equine estrogen in postmenopausal women with hysterectomy: the Women's Health Initiative randomized controlled trial. JAMA. 2004; 291(14): 1701-12.
13. de Villiers TJ, Pines A, Panay N, Gambacciani M, Archer DF, Baber RJ, et al. Updated 2013 International Menopause Society recommendations on menopausal hormone therapy and preventive strategies for midlife health. Climacteric. 2013; 16(3): 316-37.
14. Nelson ER, Wardell SE, McDonnell DP. The molecular mechanisms underlying the pharmacological actions of estrogens, SERMs and oxysterols: implications for the treatment and prevention of osteoporosis. Bone. 2013; 53(1): 42-50.
15. Naranjo Hernández A, Díaz Del Campo Fontecha P, Aguado Acín MP, Arboleya Rodríguez L, Casado Burgos E, Castañeda S, et al. Recommendations by the Spanish Society of Rheumatology on osteoporosis. Reumatol Clin. 2019; 15(4): 188-210.
16. Russell RG. Bisphosphonates: the first 40 years. Bone. 2011 ; 49(1): 2-19.
17. González-Macías J, Del Pino-Montes J, Olmos JM, Nogués X; en nombre de la comisión de redacción de las guías de osteoporosis de la SEIOMM. Clinical practice guidelines for posmenopausal, glucocorticoid-induced and male osteoporosis. Spanish Society for Research on Bone and Mineral Metabolism (3rd updated version 2014). Rev Clin Esp. 2015; 215(9): 515-26.
18. Dempster DW, Lambing CL, Kostenuik PJ, Grauer A. Role of RANK ligand and denosumab, a targeted RANK ligand inhibitor, in bone health and osteoporosis: a review of preclinical and clinical data. Clin Ther. 2012; 34(3): 521-36.
19. Cummings SR, San Martin J, McClung MR, Siris ES, Eastell R, Reid IR, et al. Denosumab for prevention of fractures in postmenopausal women with osteoporosis. N Engl J Med. 2009; 361(8): 756-65.
20. Ismail A, Hassan BA, Ibrahim AA, Mirbaha S, Baratloo A. Denosumab and atypical femoral fractures: A scoping literatura review. Trauma Mon. 2018; 23(1): e42869.
21. Silva BC, Costa AG, Cusano NE, Kousteni S, Bilezikian JP. Catabolic and anabolic actions of parathyroid hormone on the skeleton. J Endocrinol Invest. 2011; 34(10): 801-10.
22. Kendler DL, Marin F, Zerbini CAF, Russo LA, Greenspan SL, Zikan V, et al. Effects of teriparatide and risedronate on new fractures in post-menopausal women with severe osteoporosis (VERO): a multicentre, double-blind, double-dummy, randomised controlled trial. Lancet. 2018; 391(10117): 230-40.
23. McClung MR, Grauer A, Boonen S, Bolognese MA, Brown JP, Diez-Perez A, et al. Romosozumab in postmenopausal women with low bone mineral density. N Engl J Med. 2014; 370(5): 412-20.
24. Cosman F, Crittenden DB, Adachi JD, Binkley N, Czerwinski E, Ferrari S, et al. Romosozumab treatment in postmenopausal women with osteoporosis. N Engl J Med. 2016; 375(16): 1532-43.
25. Saag KG, Petersen J, Brandi ML, Karaplis AC, Lorentzon M, Thomas T, et al. Romosozumab or alendronate for fracture prevention in women with osteoporosis. N Engl J Med. 2017; 377 (15):1417-27.
26. Ascott-Evans BH, Guanabens N, Kivinen S, Stuckey BG, Magaril CH, Vandormael K, et al. Alendronate prevents loss of bone density associated with discontinuation of hormone replacement therapy: a randomized controlled trial. Arch Intern Med. 2003; 14; 163(7): 789-94.
27. Neele SJ, Evertz R, De Valk-De Roo G, Roos JC, Netelenbos JC. Effect of 1 year of discontinuation of raloxifene or estrogen therapy on bone mineral density after 5 years of treatment in healthy postmenopausal women. Bone. 2002; 30(4): 599-603.
28. Adler RA, El-Hajj Fuleihan G, Bauer DC, Camacho PM, Clarke BL, Clines GA, et al. Managing 0steoporosis in patients on long-term bisphosphonate treatment: Report of a task force of the American Society for Bone and Mineral Research. J Bone Miner Res. 2016; 31(1): 16-35.
29. Black DM, Schwartz AV, Ensrud KE, Cauley JA, Levis S, Quandt SA, et al. Effects of continuing or stopping alendronate after 5 years of treatment: the Fracture Intervention Trial Long-term Extension (FLEX): a randomized trial. JAMA. 2006; 296(24): 2927-38.
30. Bone HG, Bolognese MA, Yuen CK, Kendler DL, Miller PD, Yang YC, et al. Effects of denosumab treatment and discontinuation on bone mineral density and bone turnover markers in postmenopausal women with low bone mass. J Clin Endocrinol Metab. 2011; 96(4): 972-80.
31. Cummings SR, Ferrari S, Eastell R, Gilchrist N, Jensen JB, McClung M, et al. Vertebral fractures after discontinuation of denosumab: A post hoc analysis of the randomized placebo-controlled FREEDOM trial and its extension. J Bone Miner Res. 2018; 33(2): 190-8.
32. McClung MR, Wagman RB, Miller PD, Wang A, Lewiecki EM. Observations following discontinuation of long-term denosumab therapy. Osteoporos Int. 2017; 28(5): 1723-32.
33. Zanchetta MB, Boailchuk J, Massari F, Silveira F, Bogado C, Zanchetta JR. Significant bone loss after stopping long-term denosumab treatment: a post FREEDOM study. Osteoporos Int. 2018; 29(1): 41-7.
34. Tsourdi E, Zillikens MC, Meier C, Body JJ, Gonzalez Rodriguez E, Anastasilakis et al. Fracture risk and management of discontinuation of denosumab therapy: a systematic review and position statement by ECTS. J Clin Endocrinol Metab. 2020; 26: dgaa756.
35. Leder BZ, Neer RM, Wyland JJ, Lee HW, Burnett-Bowie SM, Finkelstein JS. Effects of teriparatide treatment and discontinuation in postmenopausal women and eugonadal men with osteoporosis. J Clin Endocrinol Metab. 2009; 94(8): 2915-21.
36. Adami S, San Martin J, Muñoz-Torres M, Econs MJ, Xie L, Dalsky GP, et al. Effect of raloxifene after recombinant teriparatide [hPTH(1-34)] treatment in postmenopausal women with osteoporosis. Osteoporos Int. 2008; 19(1): 87-94.

37. Niimi R, Kono T, Nishihara A, Hasegawa M, Kono T, Sudo A. Efficacy of switsching from teriparatide to bisphosphonate or denosumab: A prospective, randomized, open-label trial. JBMR Plus. 2018; 2(5): 289-94.

38. McClung MR, Brown JP, Diez-Perez A, Resch H, Caminis J, Meisner P, et al. Effects of 24 months of treatment with romosozumab followed by 12 months of denosumab or placebo in postmenopausal women with low bone mineral density: A randomized, double-blind, phase 2, parallel group study. J Bone Miner Res. 2018; 33(8): 1397-1406.

39. Kendler DL, Roux C, Benhamou CL, Brown JP, Lillestol M, Siddhanti S, et al. Effects of denosumab on bone mineral density and bone turnover in postmenopausal women transitioning from alendronate therapy. J Bone Miner Res. 2010; 25(1): 72-81.

40. Miller PD, Pannacciulli N, Brown JP, Czerwinski E, Nedergaard BS, Bolognese MA, et al. Denosumab or zoledronic acid in postmenopausal women with osteoporosis previously treated with oral bisphosphonates. J Clin Endocrinol Metab. 2016; 101(8): 3163-70.

41. Boonen S, Marin F, Obermayer-Pietsch B, Simões ME, Barker C, Glass EV, et al. Effects of previous antiresorptive therapy on the bone mineral density response to two years of teriparatide treatment in postmenopausal women with osteoporosis. J Clin Endocrinol Metab. 2008; 93(3): 852-60.

42. Ettinger B, San Martin J, Crans G, Pavo I. Differential effects of teriparatide on BMD after treatment with raloxifene or alendronate. J Bone Miner Res. 2004; 19(5): 745-51.

43. Geusens P, Marin F, Kendler DL, Russo LA, Zerbini CA, Minisola S, et al. Effects of teriparatide compared with risedronate on the risk of fractures in subgroups of postmenopausal women with severe osteoporosis: The VERO trial. J Bone Miner Res. 2018; 33(5): 783-94.

44. Leder BZ, Tsai JN, Uihlein AV, Wallace PM, Lee H, Neer RM, et al. Denosumab and teriparatide transitions in postmenopausal osteoporosis (the DATA-Switch study): extension of a randomised controlled trial. Lancet. 2015; 386(9999): 1147-55.

45. Langdahl B. Treatment of postmenopausal osteoporosis with bone-forming and antiresorptive treatments: Combined and sequential approaches. Bone. 2020; 139: 115516.

46. Langdahl BL, Libanati C, Crittenden DB, Bolognese MA, Brown JP, Daizadeh NS, et al. Romosozumab (sclerostin monoclonal antibody) versus teriparatide in postmenopausal women with osteoporosis transitioning from oral bisphosphonate therapy: a randomised, open-label, phase 3 trial. Lancet. 2017; 390(10102): 1585-94.

47. Kendler DL, Bone HG, Massari F, Gielen E, Palacios S, Maddox J, et al. Bone mineral density gains with a second 12-month course of romosozumab therapy following placebo or denosumab. Osteoporos Int. 2019; 30(12): 2437-48.

48. Eastell R, Nickelsen T, Marin F, Barker C, Hadji P, Farrerons P, et al. Sequential treatment of severe postmenopausal osteoporosis after teriparatide: final results of the randomized, controlled European Study of Forsteo (EUROFORS). J Bone Min Res. 2009; 24(4): 726-36

49. Lindsay R, Scheele WH, Neer R, Pohl G, Adami S, Mautalen C, et al. Sustained vertebral fracture risk reduction after withdrawal of teriparatide in postmenopausal women with osteoporosis. Arch Intern Med. 2004; 164(18): 2024-30.

50. Cosman F, McMahon D, Dempster D, Nieves JW. Standard versus cyclic teriparatide and denosumab treatment for osteoporosis: A randomized trial. J Bone Miner Res. 2020; 35(2): 219-25.

51. Bone HG, Cosman F, Miller PD, Williams GC, Hattersley G, Hu MY, et al. ACTIVExtend: 24 months of alendronate after 18 months of abaloparatide or placebo for postmenopausal osteoporosis. J Clin Endocrinol Metab. 2018; 103(8): 2949-57.

52. Casado Burgos E. Nuevos datos sobre el tratamiento con bisfosfonatos: ¿son aconsejables unas vacaciones terapéuticas? [New data on biphosphonate therapy: are therapeutic holidays advisable?]. Reumatol Clin. 2011; 7 Suppl 2: S28-33.

53. Moro Álvarez MJ, Neyro JL, Castañeda S. Vacaciones terapéuticas en osteoporosis: estrategia en el tratamiento a largo plazo con bifosfonatos [Therapeutic holidays in osteoporosis: Long-term strategy of treatment with bisphosphonates]. Med Clin (Barc). 2016; 146(1): 24-9.

54. Wimalawansa SJ. Prevention and treatment of osteoporosis: efficacy of combination of hormone replacement therapy with other antiresorptive agents. J Clin Densitom. 2000; 3(2): 187-201.

55. Finkelstein JS, Wyland JJ, Lee H, Neer RM. Effects of teriparatide, alendronate, or both in women with postmenopausal osteoporosis. J Clin Endocrinol Metab. 2010; 95(4): 1838-45.

56. Leder BZ. Optimizing sequential and combined anabolic and antiresorptive osteoporosis therapy. JBMR Plus. 2018; 2(2): 62-8.

57. Cosman F, Eriksen EF, Recknor C, Miller PD, Guañabens N, Kasperk C, et al. Effects of intravenous zoledronic acid plus subcutaneous teriparatide [rhPTH(1-34)] in postmenopausal osteoporosis. J Bone Miner Res. 2011; 26(3): 503-11.

WNT16 rs2908004 missense variant acts as eQTL of FAM3C in human primary osteoblasts

Martínez-Gil N, Patiño J, Ugartondo N, Grinberg D, Balcells S
Department of Genetics, Microbiology and Statistics. Biology Faculty. University of Barcelona, CIBERER, IBUB, IRSJD. Barcelona (Spain)

Summary

Introduction: WNT16 is an important gene in bone homeostasis, found in a very complex locus that also includes neighboring genes: ING3, FAM3C, and CPED1. In addition to the clear role of WNT16 in determining bone mineral density (BMD), evidence has also been found for the importance of these three neighboring genes in bone metabolism. Therefore, it remains to be clarified whether the variants in WNT16 associated with BMD carry out their effect on WNT16 or if they do so by modifying the expression of these neighboring genes.

Material and methods: We have determined the expression levels of CPED1 and FAM3C in primary osteoblasts and we have verified whether WNT16 variants behave as loci of quantitative expression traits (expression quantitative trait loci; eQTL) of these genes.

Results: The amino acid change variant rs2908004 in WNT16 acts as the eQTL of FAM3C in primary osteoblasts under the dominant model hypothesis.

Discussion: It is possible that the effect of this variant on BMD is due to the modification of the expression levels of FAM3C in addition to or instead of a direct effect of the mutant WNT16 protein resulting from the amino acid change.

Key words: Bone mineral density, *WNT16*, osteoporosis, transcription.

INTRODUCTION

WNT16 is a ligand of the Wnt pathway that has been extensively studied for its importance in regulating bone homeostasis. This has been confirmed with the phenotype of knock-out (KO) mice and conditional KO mice in osteoblasts (cKO), which show spontaneous fractures due to low cortical bone mineral density (BMD), low bone strength and high cortical porosity, keeping the volume of trabecular bone unchanged[1-4]. On the contrary, Wnt16 overexpression in osteoblasts and osteocytes produces an increase in BMD and bone strength in both trabecular and cortical bone[5-7]. Despite this, the precise mechanism by which WNT16 acts is not known and different studies indicate that the effect on canonical and non-canonical Wnt pathways could be tissue specific[1,8-11]. In bone, WNT16 is expressed mainly by osteoblasts and carries out its function both by stimulating bone formation and by inhibiting its resorption indirectly through osteoprotegerin (OPG) or directly affecting the osteoclasts differentiation[1,12].

Several genome-wide association studies (GWAS) have shown an association between the locus containing *WNT16* and various skeletal phenotypes, including BMD and risk of fractures[2,3,13-26]. *WNT16* is found in a very complex locus, where several genes in the region show an important role in bone metabolism. The genes *ING3* and *CPED1* at 5 'and *FAM3C* at 3' of *WNT16* belong to this locus (figure 1).

ING3 (Inhibitor Of Growth Family Member 3) is responsible for the regulation of chromatin, since it is part of the NuA4 histone acetyltransferase (HAT) complex that recognizes the trimethylated form of lysine 4 of histone H3[27]. Other functions unrelated to chromatin regulation include apoptosis promotion, DNA repair, and modulation of cell mobility[27]. ING3 is expressed in a multitude of tissues, especially in those with a higher proportion of cell growth, bone being one of those with the highest expression of ING3[27]. The in vitro cellular model of ING3 KO mesenchymal cells shows involvement of osteoblastogenesis and stimulation of adipogenic differentiation[28].

Correspondence: Núria Martínez-Gil (nuriamartinez91@ub.edu)

Figure 1. Genes at the locus: ING3_1 (ENST00000315870.5), ING3_2 (ENST00000339121.5), CPED1_1 (ENST00000 310396.5), CPED1_7 (ENST00000450913.2), WNT16_1 (ENST00000222462.2), WNT16_2 (ENST00000361301.2), and FAM3C (ENST00000359943.3) by GRCh37/hg19
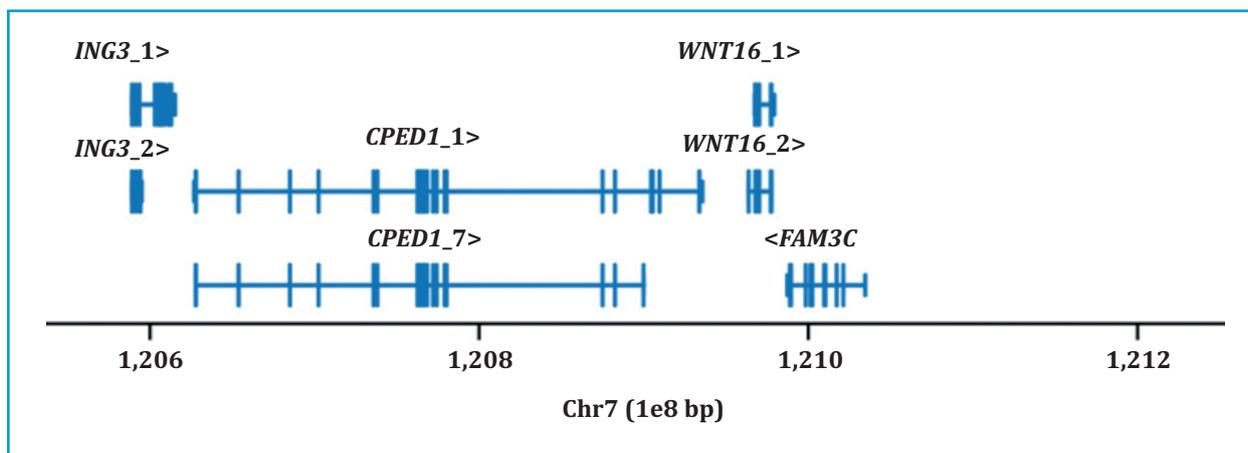
No specific function is known for CPED1 (Cadherin Like And PC-Esterase Domain Containing 1) in humans or mice. In the latter, it is uniformly expressed in a variety of solid tissues, including bone, although it is not detected in the RAW264.7 cell line or in circulating leukocytes[29]. Furthermore, CPED1 presents different isoforms due to alternative splicing and three active promoter regions during osteogenic differentiation, indicating a complex regulation during differentiation[29].

FAM3C (Family of sequence similarity 3c) is a cytokine-like growth factor expressed in a multitude of tissues[30], which plays a very important role in the epithelial-mesenchymal transition (EMT) and subsequent metastasis during cancer progression[31]. Its relationship with bone metabolism has been confirmed with the KO mouse model, which presents alterations in the cortical and trabecular structure, with an increase in cortical BMD, which generates a decrease in bone resistance[30]. In in vitro studies, it was found that the mesenchymal cells extracted from these KO mice showed accelerated osteoblastogenesis[31].

The relationship of the genes present at the *'ING3-CPED1-WNT16-FAM3C'* locus with bone metabolism and their repeated association with BMD raises the question of whether there is a single causative gene and, if so, which is it, or if instead, all genes are contributing to the phenotype. To this end, in the present work we have determined whether those *WNT16* variants associated with BMD in a previous work by our group[32] are found to modify the expression of neighboring genes *CPED1* and *FAM3C.*

MATERIAL AND METHODS

Cell culture

Human primary osteoblasts (hOB) were used for the loci assays that determine quantitative differences in gene expression (expression quantitative trait loci; eQTL). The hOBs were obtained from trabecular bone fragments discarded from knee replacement operations performed on women with osteoarthritis and who did not have any other disorder that could affect bone quality. The study was approved by the Clinical Research Ethics Committee of the Parc de Salut MAR (registration numbers: 2010/3882/I and 2013/5266/I) and was carried out in accordance with the Declaration of Helsinki, ob-

taining informed consent by written by all participants. The primary osteoblast culture protocol is described in Roca-Ayats et al.[33]. Briefly, bone samples were cut into small pieces and washed with phosphate buffered saline (PBS; Gibco, Life Technologies). These pieces were cultured in 140 mm plates with DMEM supplemented with 10% FBS, 1% w/s, 0.4% fungizone (Gibco, Life Technologies) and 100 µg/ml of ascorbic acid (Sigma-Aldrich). When the cells reached confluence, they were divided into three 75 cm² flasks, one for DNA extraction, one for RNA extraction, and the third for freezing and storage. Cells at passage 2 or lower were used for all extractions.

EQTL assay

DNA was extracted from cultured hOBs using the Wizard® Genomic DNA Purification Kit (Promega), according to the manufacturer's instructions. The concentration of the purified DNA and its quality was analyzed in a spectrophotometer (Nanodrop). The genotypes of the rs2908004, rs2707466, rs55710688 and rs142005327 variants were evaluated by Sanger sequencing using BigDye® Terminator v3.1 (Applied Biosystems) in the Genomics facilities of the CCiT of the University of Barcelona. The primers (Invitrogen, Thermo Fisher) were designed using Primer3 Input 0.4.0 (table 1). The total RNA of the cultured hOBs was extracted using the High Pure RNA Isolation kit (Roche), according to the manufacturer's instructions and the quantification and quality of the RNA were checked using a Nanodrop spectrophotometer. RNA was reverse transcribed to cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Thermo Fisher), according to the manufacturer's specifications. RT-qPCR was carried out using UPL probes (Roche) on a LightCycler 480 Instrument II (Roche). *HMBS* gene expression was used as a normalization control and the relative quantification (fold change) was calculated using the second derivative method. The number and sequence of the probe used, as well as the primers used for the amplification of the *CPED1, FAM3C* and *HMBS* genes are shown in table 1.

Statistic analysis

For the statistical analysis of the eQTL, the WGassociation[34] function was used in RStudio. This function performs an association analysis between a given SNP and

a dependent variable (in this case the expression levels of *CPED1* and *FAM3C*) in five different genetic inheritance models: codominant [homozygous for major allele vs. heterozygous vs. homozygous for minor allele], dominant [homozygous for major allele versus (heterozygous + homozygous for minor allele)], recessive [homozygous for minor allele versus (heterozygous + homozygous for major allele)], over-dominant [heterozygous versus (homozygous for allele major + homozygous for minor allele)] and log-additive [each allele modifies the risk by an additive amount] .

Results

Cis-eQTL analysis

The variants rs2908004, rs2707466, rs55710688 and rs142005327 of *WNT16* have been described as cis-eQTLs, according to the GTEx database in different human tissues (table 2). Unfortunately, this database does not have information on any bone tissue. This is why, using our own database of human primary osteoblasts (n=45), we have tested whether these variants act as cis-eQTL of the neighboring genes of *WNT16*: *CPED1* and *FAM3C*. Only the rs2908004 variant has shown a sig-

nificant association with *FAM3C* expression levels under the dominant hypothesis (p=0.03, table 3, figure 2). In addition, the rs2908004 and rs2707466 variants show a trend towards significance with *FAM3C* expression levels under the codominance hypothesis (p=0.05491) and under the dominant hypothesis (p=0.06954), respectively (table 3, figure 2). The presence of the G allele (rs2908004) and the C allele (rs2707466) are associated with an increase in the expression of *FAM3C* (table 3, figure 2). On the contrary, we have not found a significant association or trend between the *WNT16* variants analyzed and *CPED1* expression levels (table 3, figure 2).

Discussion

Over the past 20 years, many works have highlighted the importance of WNT16 in bone homeostasis. *WNT16* is found at position 7q31.31 along with 3 other genes also related to bone metabolism. In a previous study by our group, we found that the variants rs142005327, rs55710588, rs2908004, and rs2707466 were associated with BMD in a cohort of postmenopausal women from the Barcelona area (BARCOS)[32]. To determine whether these variants are related to an effect on *WNT16* or

Table 1. Primers used in the sequencing of four *WNT16* SNPs and for the RT-qPCR of *FAM3C*, *CPED1* and *HMBS*

Primer name	F (5'--> 3')	R (5'-->3')	Probe
WNT16-rs55710688	GGTAGCTCCAGTAAGAGATTC	CAGATTACCGTGTCTTTGGGT	
WNT16-rs2908004-rs142005327	ACTTTCAACTGAGGCTGGGG	CTGGAACTGGGGAGTCAGG	
WNT16-rs2707466	TGGGACAAAAACCAAAGGACG	TGACCACATGGGTGTTGTAAC	
FAM3C_qPCR	GGCAAATGGAAAAACAGGAG	TGTATGGCCTTCAGAAACTCAA	52
CPED1_qPCR	CCCAAGTCTGCCCTTGTTT	GAAGAAATAGGCTGTAACCCACA	6
HMBS_qPCR	TGCCCTGGAGAAGAATGAAG	CAGCATCATGAGGGTTTTCC	79

F: primer forward; R: primer reverse.

Figure 2. Violin plots of the expression levels of FAM3C (top) and CPED1 (bottom) according to the three genotypes (homozygous for the majority allele, heterozygous, homozygous for the minority allele) of the 4 variants studied
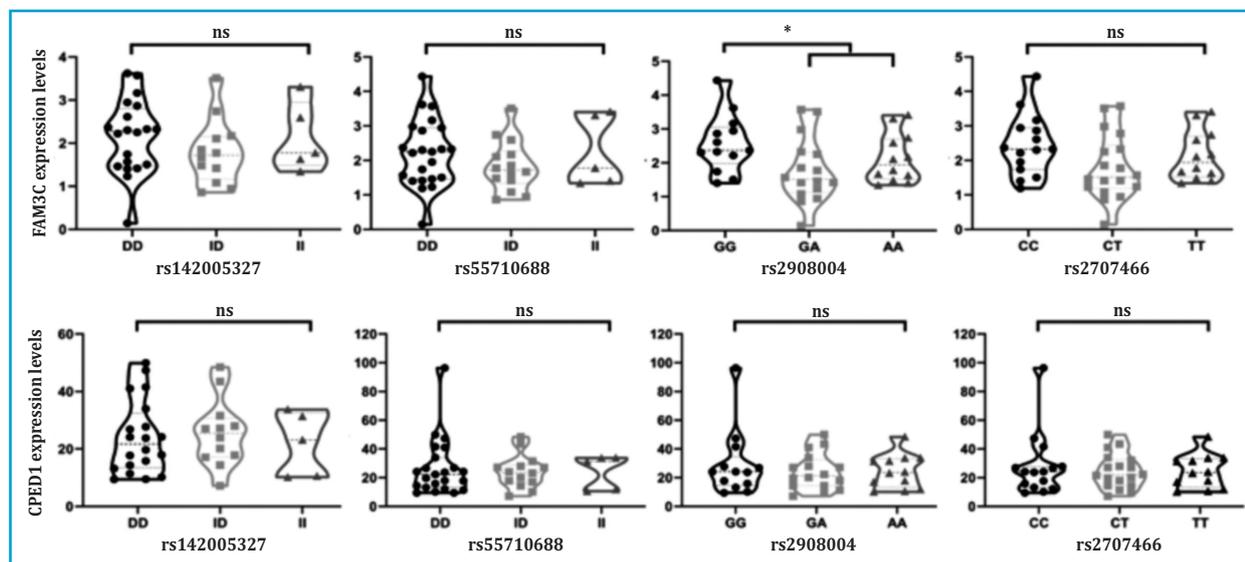
I: insertion; D: deletion.

to an effect on the expression of neighboring genes, we have verified whether they are acting as eQTL of *FAM3C* and *CPED1*. This work has allowed us to determine that the missense variant rs2908004 is acting as eQTL of the *FAM3C* gene under the hypothesis of a dominant model in human primary osteoblasts.

The missense variant rs2908004 (p.Gly72Arg/ p.Gly82Arg) has been associated with different bone parameters by us and others[2,32,35-38]. This amino acid change from glycine to arginine is considered tolerated and benign by the pathogenicity predictors SIFT and PolyPhen-2, so its effect on BMD could be due to its role as eQTL and not to a change in the resulting WNT16 protein.

It should be taken into account that to obtain a more robust statistical significance having a bank with a greater number of primary osteoblasts is required. Unfor-

tunately obtaining these samples is difficult and we have only managed to enter 45 samples into our bank.

In addition, it would be interesting to determine the expression levels of other neighboring genes that may be influencing BMD such as *ING3* or directly on the expression levels of *WNT16*, which have not been able to be quantified due to lack of RNA sample from primary osteoblasts.

CONCLUSION

Through this work we have determined that the variant rs2908004 of *WNT16* regulates the expression levels of the neighboring gene *FAM3C* under the hypothesis of a dominant model. If this association is confirmed in a larger primary osteoblast bank, it would indicate that the association of this variant with BMD could be due, at least in part, to the variation of *FAM3C* expression.

Table 2. Genes whose expression is modified by the variants rs2908004, rs2707466, rs55710688, rs142005327 in various human tissues (data extracted from the Genotype-Tissue Expression (GTEx) Portal)

SNP	Gen	Tissue
rs2908004	CPED1	Artery - Tibial
	CYCSP19	Testicle
	FAM3C	Skin - NE and E; Brain - Frontal Cortex (BA9); Esophagus - Muscularis
rs142005327	CPED1	Artery - Tibial
	FAM3C	Skin - NE and E; Muscle - skeletal; Heart - Left Ventricle; Chest - breast tissue; Heart - Atrial Appendix; Nerve - Tibial
	WNT16	Adipose - Subcutaneous
rs2707466	CPED1	Artery - Tibial
	CYCSP19	Testicle
	FAM3C	Skin - NE and E; Brain - Frontal Cortex (BA9)
rs55710688	CPED1	Artery - Tibial
	FAM3C	Skin - NE and E; Muscle - skeletal; Heart - Left Ventricle; Chest - breast tissue
	WNT16	Adipose - Subcutaneous

NE: not exposed to the sun (suprapubic); E: exposed to the sun (lower leg).

Table 3. Results of association of the 4 SNPs of *WNT16* and the expression of *CPED1* and *FAM3C*

				CPED1		*FAM3C*	
SNP	A may	A min	HWE	Codominant	Dominant	Codominant	Dominant
rs55710688	D	I	0.2597	0.90837	0.65906	0.44952	0.34621
rs2908004	G	A	0.2095	0.68815	0.38455	*0.05491*	**0.03061**
rs142005327	D	I	0.2325	0.84422	0.91336	0.49356	0.35142
rs2707466	C	T	0.2305	0.80454	0.50710	0.11531	*0.06954*

In bold, the significant associations (p<0.05) and in italics, those that show a trend.
A may: majority allele; A min: minor allele; HWE: Hardy-Weinberg equilibrium.

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

Bibliography

1. Movérare-Skrtic S, Henning P, Liu X, Nagano K, Saito H, Börjesson AE, et al. Osteoblast-derived WNT16 represses osteoclastogenesis and prevents cortical bone fragility fractures. Nat Med. 2014; 20(11): 1279-88.

2. Zheng H-F, Tobias JH, Duncan E, Evans DM, Eriksson J, Paternoster L, et al. WNT16 influences bone mineral density, cortical bone thickness, bone strength, and osteoporotic fracture risk. PLoS Genet. 2012; 8(7): e1002745.

3. Medina-Gomez C, Kemp JP, Estrada K, Eriksson J, Liu J, Reppe S, et al. Meta-analysis of genome-wide scans for total body BMD in children and adults reveals allelic heterogeneity and age-specific effects at the WNT16 locus. PLoS Genet. 2012; 8(7): e1002718.

4. Wergedal JE, Kesavan C, Brommage R, Das S, Mohan S. Role of WNT16 in the regulation of periosteal bone formation in female mice. Endocrinology. 2015; 156(3): 1023-32.

5. Alam I, Alkhouli M, Gerard-O'Riley RL, Wright WB, Acton D, Gray AK, et al. Osteoblast-Specific Overexpression of Human WNT16 Increases Both Cortical and Trabecular Bone Mass and Structure in Mice. Endocrinology. 2016; 157(2): 722-36.

6. Alam I, Reilly AM, Alkhouli M, Gerard-O'Riley RL, Kasipathi C, Oakes DK, et al. Bone Mass and Strength are Significantly Improved in Mice Overexpressing Human WNT16 in Osteocytes. Calcif Tissue Int. 2017; 100(4): 361-73.

7. Movérare-Skrtic S, Wu J, Henning P, Gustafsson KL, Sjögren K, Windahl SH, et al. The bone-sparing effects of estrogen and WNT16 are independent of each other. Proc Natl Acad Sci U S A. 2015; 112(48): 14972-7.

8. Jiang Z, Von den Hoff JW, Torensma R, Meng L, Bian Z. Wnt16 is involved in intramembranous ossification and suppresses osteoblast differentiation through the Wnt/β-catenin pathway. J Cell Physiol. 2014; 229(3): 384-92.

9. Hendrickx G, Boudin E, Verbeek M, Fransen E, Mortier G, Van Hul W. WNT16 Requires Gα Subunits as Intracellular Partners for Both Its Canonical and Non-Canonical WNT Signalling Activity in Osteoblasts. Calcified Tissue International. 2020; 106(3): 294-302.

10. Shen J, Chen X, Jia H, Meyers CA, Shrestha S, Asatrian G, et al. Effects of WNT3A and WNT16 on the Osteogenic and Adipogenic Differentiation of Perivascular Stem/Stromal Cells. Vol. 24, Tissue Engineering Part A. 2018. p. 68-80.

11. Sebastian A, Hum NR, Morfin C, Murugesh DK, Loots GG. Global gene expression analysis identifies Mef2c as a potential player in Wnt16-mediated transcriptional regulation. Vol. 675, Gene. 2018. p. 312-21.

12. Kobayashi Y, Thirukonda GJ, Nakamura Y, Koide M, Yamashita T, Uehara S, et al. Wnt16 regulates osteoclast differentiation in conjunction with Wnt5a. Biochem Biophys Res Commun. 2015; 463(4): 1278-83.

13. Moayyeri A, Hsu Y-H, Karasik D, Estrada K, Xiao S-M, Nielson C, et al. Genetic determinants of heel bone properties: genome-wide association meta-analysis and replication in the GEFOS/GENOMOS consortium. Hum Mol Genet. 2014; 23(11): 3054-68.

14. Kemp JP, Morris JA, Medina-Gomez C, Forgetta V, Warrington NM, Youlten SE, et al. Identification of 153 new loci associated with heel bone mineral density and functional involvement of GPC6 in osteoporosis. Nat Genet. 2017; 49(10): 1468-75.

15. Medina-Gomez C, Kemp JP, Trajanoska K, Luan J'an, Chesi A, Ahluwalia TS, et al. Life-Course Genome-wide Association Study Meta-analysis of Total Body BMD and Assessment of Age-Specific Effects. Am J Hum Genet. 2018; 102(1): 88-102.

16. Estrada K, Styrkarsdottir U, Evangelou E, Hsu Y-H, Duncan EL, Ntzani EE, et al. Genome-wide meta-analysis identifies 56 bone mineral density loci and reveals 14 loci associated with risk of fracture. Nat Genet. 2012; 44(5): 491-501.

17. Trajanoska K, Schoufour JD, de Jonge EAL, Kieboom BCT, Mulder M, Stricker BH, et al. Fracture incidence and secular trends between 1989 and 2013 in a population based cohort: The Rotterdam Study. Bone. 2018; 114: 116-24.

18. Morris JA, Kemp JP, Youlten SE, Laurent L, Logan JG, Chai RC, et al. An atlas of genetic influences on osteoporosis in humans and mice. Nat Genet. 2019; 51(2): 258-66.

19. Kichaev G, Bhatia G, Loh P-R, Gazal S, Burch K, Freund MK, et al. Leveraging Polygenic Functional Enrichment to Improve GWAS Power. Am J Hum Genet. 2019; 104(1): 65-75.

20. Chesi A, Mitchell JA, Kalkwarf HJ, Bradfield JP, Lappe JM, McCormack SE, et al. A trans-ethnic genome-wide association study identifies gender-specific loci influencing pediatric aBMD and BMC at the distal radius. Hum Mol Genet. 2015; 24(17): 5053-9.

21. Koller DL, Zheng H-F, Karasik D, Yerges-Armstrong L, Liu C-T, McGuigan F, et al. Meta-analysis of genome-wide studies identifies WNT16 and ESR1 SNPs associated with bone mineral density in premenopausal women. J Bone Miner Res. 2013; 28(3): 547-58.

22. Pei Y-F, Hu W-Z, Yan M-W, Li C-W, Liu L, Yang X-L, et al. Joint study of two genome-wide association meta-analyses identified 20p12.1 and 20q13.33 for bone mineral density. Bone. 2018; 110: 378-85.

23. Zhang L, Choi HJ, Estrada K, Leo PJ, Li J, Pei Y-F, et al. Multistage genome-wide association meta-analyses identified two new loci for bone mineral density. Hum Mol Genet. 2014; 23(7): 1923-33.

24. Wang H, Zhang F, Zeng J, Wu Y, Kemper KE, Xue A, et al. Genotype-by-environment interactions inferred from genetic effects on phenotypic variability in the UK Biobank. Sci Adv. 2019; 5(8): eaaw3538.

25. Zheng H-F, Forgetta V, Hsu Y-H, Estrada K, Rosello-Diez A, Leo PJ, et al. Whole-genome sequencing identifies EN1 as a determinant of bone density and fracture. Nature. 2015; 526(7571): 112-7.

26. Mullin BH, Zhao JH, Brown SJ, Perry JRB, Luan J 'an, Zheng H-F, et al. Genome-wide association study meta-analysis for quantitative ultrasound parameters of bone identifies five novel loci for broadband ultrasound attenuation. Hum Mol Genet. 2017; 26(14): 2791-802.

27. Nabbi A, Almami A, Thakur S, Suzuki K, Boland D, Bismar TA, et al. ING3 protein expression profiling in normal human tissues suggest its role in cellular growth and self-renewal. Vol. 94, European Journal of Cell Biology. 2015. p. 214-22.

28. Chesi A, Wagley Y, Johnson ME, Manduchi E, Su C, Lu S, et al. Genome-scale Capture C promoter interactions implicate effector genes at GWAS loci for bone mineral density. Nat Commun. 2019 19; 10(1): 1260.

29. Maynard RD, Godfrey DA, Medina-Gomez C, Ackert-Bicknell CL. Characterization of expression and alternative splicing of the gene cadherin-like and PC esterase domain containing 1 (Cped1). Gene. 2018 20; 674: 127-33.

30. Määttä JA, Bendre A, Laanti M, Büki KG, Rantakari P, Tervola P, et al. Fam3c modulates osteogenic cell differentiation and affects bone volume and cortical bone mineral density. Bonekey Rep. 2016; 5: 787.

31. Bendre A, Büki KG, Määttä JA. Fam3c modulates osteogenic differentiation by down-regulating Runx2. Differentiation. 2017; 93: 50-7.

32. Martínez-Gil N, Roca-Ayats N, Monistrol-Mula A, García-Giralt N, Díez-Pérez A, Nogués X, et al. Common and rare variants of WNT16, DKK1 and SOST and their relationship with bone mineral density. Sci Rep. 2018 19; 8(1): 10951.

33. Roca-Ayats N, Martínez-Gil N, Cozar M, Gerousi M, Garcia-Giralt N, Ovejero D, et al. Functional characterization of the C7ORF76 genomic region, a prominent GWAS signal for osteoporosis in 7q21.3. Bone. 2019; 123: 39-47.

34. Gonzalez JR, Armengol L, Sole X, Guino E, Mercader JM, Estivill X, et al. SNPassoc: an R package to perform whole genome association studies. Vol. 23, Bioinformatics. 2007. p. 654-5.

35. Mitek T, Nagraba Ł, Deszczyński J, Stolarczyk M, Kuchar E, Stolarczyk A. Genetic Predisposition for Osteoporosis and Fractures in Postmenopausal Women. Adv Exp Med Biol. 2019; 1211: 17-24.

36. García-Ibarbia C, Pérez-Núñez MI, Olmos JM, Valero C, Pérez-Aguilar MD, Hernández JL, et al. Missense polymorphisms of the WNT16 gene are associated with bone mass, hip geometry and fractures. Osteoporos Int. 2013; 24(9): 2449-54.

37. Correa-Rodríguez M, Schmidt Rio-Valle J, Rueda-Medina B. Polymorphisms of the WNT16 gene are associated with the heel ultrasound parameter in young adults. Osteoporos Int. 2016; 27(3): 1057-61.

38. Hendrickx G, Boudin E, Fijałkowski I, Nielsen TL, Andersen M, Brixen K, et al. Variation in the Kozak sequence of WNT16 results in an increased translation and is associated with osteoporosis related parameters. Bone. 2014; 59:57-65.

Diet as a risk factor for hypovitaminosis D in the Spanish pediatric population

Blarduni Cardón E[1], Arrospide A[2-4], Angulo Ugarte H[5], Urrutia Etxebarria I[6-7], Castaño González L[6-8], Etxebarria I[9], Mar J[2-4], Grupo Goivide[10]

1 Osakidetza. Zumárraga Hospital. Pediatric Service. Zumárraga (Spain)
2 Osakidetza. OSI Debagoiena. Research Unit. Arrasate-Mondragón (Spain)
3 Biodonostia Institute. Chronic Diseases Economic Evaluation Group. Donostia-San Sebastián (Spain)
4 Kronikgune Institute. Chronic Diseases Economic Evaluation Group. Barakaldo (Spain)
5 Osakidetza. Bizkaia Family and Community Medicine Teaching Unit. Galdakao Hospital. Galdakao (Spain)
6 IIS Biocruces Bizkaia. Cruces University Hospital. Barakaldo (Spain)
7 CIBERDEM, CIBERER. Carlos IIII Health Institute. Madrid (Spain)
8 University of the Basque Country. Department of Pediatrics. Barakaldo (Spain)
9 Osakidetza. OSI Debagoiena. Traumatology Service. Arrasate-Mondragón (Spain)
10 Osakidetza. OSI Goierri-Alto Urola. Primary Care Centers. Zumárraga (Spain)

Summary

Objetives: It is not clear whether diet in the Spanish general population is also a relevant determinant of hypovitaminosis D. The objective of the study was to analyze the impact of diet on the prevalence of hypovitaminosis D in healthy children.
Methods: Demographic, anthropometric, nutritional, analytical data and vitamin D (25 (OH) D) level were studied using an enzyme-immuno-analysis using an observational design in a sample of the pediatric population between 4 and 14 years old. The 24-hour reminder diet survey was evaluated with the DietSource 3.0 software. The probability of hypovitaminosis was analyzed using logistic regression.
Results: 280 healthy children with a mean age of 9.0 years were recruited. The prevalence of hypovitaminosis D (<20 ng/ml) was 18.15% and that of severe deficit (<10 ng/ml) 1.4%. Ethnicity, seasonality, skin phototype, and time of sun exposure were significantly associated with the presence of hypovitaminosis D. The distribution of nutrients did not show differences between the groups with and without hypovitaminosis except for Pyridoxine B6 and saturated fatty acids.
Conclusions: Diet plays a reduced role as a risk factor for hypovitaminosis D in healthy children and the relevant factors are those related to sun exposure. An adequate outdoor lifestyle, sun exposure free of sunscreens and dietary patterns that ensure a correct intake of vitamin D and calcium remain the ideal recommendations for the general population. Supplementation should be limited to risk groups.

Key words: Gipuzkoa, hypovitaminosis D, healthy population, risk factors, diet, sun exposure.

INTRODUCTION

Vitamin D is an essential micronutrient in bone and non-bone metabolism[1]. The high prevalence of its deficit has been documented in multiple studies[2-5]. However, the definition of its reference values continues to be controversial and consequently doubts are raised about its diagnosis and treatment[6]. Although rickets was eradicated in the mid 20th century with sun exposure and the enrichment of milk with vitamin D, in recent years there have been reports of rickets in different parts of the planet, the mainly affected black-skinned and exclusively breastfed babies[7-9]. While rickets is the most serious consequence of vitamin D deficiency, mere deficiency also has important health consequences[6].

Studies that measure the level of vitamin D show great geographic variability due to the great regional differences in climate, sun exposure and diet. For this reason, there is a need to specifically investigate the role of the different determinants of hypovitaminosis D[10] in each country. The diet meets only 10% of the human body's vitamin D requirements, the remaining 90% being obtained through the photosynthesis process that occurs in the skin by the direct action of the sun's rays[11]. Risk factors repeatedly identified in the literature as causes of hypovitaminosis (dark skin phototype, low sun exposure, lack of physical exercise, latitude >40º north, and winter and spring seasons) act by interfering in the second mechanism[2,12]. Other factors associated with hypovitaminosis such as maternal deficit, obesity or advanced age could be related to both mechanisms of obtaining vitamin D[12-14].

In Spain there are numerous studies that measure hypovitaminosis D prevalence in children[2-4,15,16]. However, most focus on the risk factors related to sun exposure. Rodríguez-Sangrador et al. include dietary measurement of vitamin D intake[16]. Recently, the debate on the need to supplement with vitamin D was described as a puzzle in which the pieces begin to fit together and it was recommended to give vitamin D in adults to those who need it to maintain serum levels of 25 (OH) D above 20 ng/ml[17,18]. Clarifying whether schoolchildren with hypovitaminosis D have a deficit of intake in the diet would help to fit this piece to also supplement the child population. For this, it is necessary to jointly address both the factors associated with the exposure and synthesis and with the intake of vitamin D.

In this study, we analyze the impact of diet on the prevalence of hypovitaminosis D in healthy children in the Goierri-Alto Urola region to provide recommendations for the prevention of hypovitaminosis D in the school population.

METHODS

A cross-sectional observational design was used, with cluster sampling during a calendar year (September 1, 2012 to September 30, 2013). The study was carried out with the financing of a Research Grant from the Department of Health of the Basque Government (Project No. 2011111107) and was approved by the Ethics Committee of Gipuzkoa. The children participated after signing the informed consent form by their parents. The reference population was the one served by the Integrated Health Organization (OSI) Goierri-Alto Urola in Gipuzkoa and the sample was recruited among girls, boys and adolescents between 4 and 14 years old, who attended Primary Care Services, being their The main populations Beasain, with latitude 43.05 N, and Zumárraga, with latitude 43.11 N. During the study period they had an average solar irradiation of 10.4–11.8 MJ/m^2 (Euskalmet, Basque Meteorology Agency)[19]. It was estimated that a sample of 298 schoolchildren would be sufficient to achieve a precision of 4% in a universe of 891, with a 95% significance, losses of 10% and an estimated prevalence of 20%. The schoolchildren were recruited successively, coinciding with the reviews carried out by the Primary Care pediatricians on healthy children until the sample provided for each month was completed in order to collect measurements throughout the year. The study exclusion criteria were: lack of informed consent, nephrotic syndrome, chronic renal failure, cancer patients, prolonged use of ketoconazole or anticonvulsants (phenobarbital-phenytoin), treatment for tuberculosis, and severe liver injury.

The variables collected were: age, sex, ethnicity (Caucasian and non-Caucasian), weight, height, BMI, skin phototype with the Fitzpatrick scale (1: least pigmentation to 6: maximum pigmentation)[20], sports activity in the last month (in three categories: less time than school sports, only school sports and two days more than school sports), time of sun exposure in the last month (nothing, less than 30 minutes a day, more than 30 minutes a day, more than 1 hour daily), use of sunscreen and vitamin supplements in the last 3 months. The phospho-calcium metabolism (calcium, phosphorus, magnesium, alkaline phosphatase, parathyroid hormone (PTH) and 25(OH) D).

An individual survey on feeding was conducted using a 24-hour recall survey (ER24hs). After conducting the analysis, the Primary Care pediatrician who recruited the student was in charge of reviewing and re-interviewing the parents on the standardized and self-completed questionnaire with the food eaten in the previous 24 hours. For the conversion of food into nutrients, the DietSource 3.0 program was used, which has a food composition table from Nestlé Healthcare Nutrition S. A. (A. Jiménez, P. Cervera and M. Bacardi). By managing the dishes and food eaten, this program breaks down the daily menu by estimating the amounts of immediate principles, nutrients and caloric distribution in each child's diet. Although the method applied is less valid than records based on the weight of food consumed for three or more days, the literature that has compared them supports its use[21,22].

Analytical determinations of phosphocalcic metabolism (calcium, phosphorus, magnesium, alkaline phosphatase, PTH, 25 (OH) D) were carried out in the OSI Goierri-Alto Urola laboratory. To measure the level of 25 (OH) D in serum, the Elecsys-Chemiluminescent Immunoanalysis kit was used. The cut-off point of 20 ng/ml was used to determine the levels of vitamin D that are considered deficient and 10 ng/ml for the cases with severe deficiency. The results were presented according to whether the values were within the normal reference ranges that were 10-65 pg/ml for PTH, 8.8-10.8 mg/dl for calcium, 3.0-6.5 mg/dl for phosphorus, 1.5-2.6 mg/dl for magnesium and for alkaline phosphatase <269 IU/L from 4 to 6 years and <300 IU/L from 7 to 12 years.

Statistic analysis

Univariate analysis was carried out using the Chi-square statistic or Fisher's exact statistic for categorical variables and the Student's t-test or the non-parametric Mann-Whitney U test was applied in the case of continuous variables as a function of its distribution. The level of significance used throughout the study was 5% and the analyzes were performed with the statistical software Stata version 13.0. Multivariate analyzes were carried out using logistic regression models in which the dependent variable was the probability of having hypovitaminosis and the independent variables were risk factors associated with exposure and intake of vitamin D. Variables to be included in the model were selected based on clinical interest and the level of significance in the univariate analysis. The goodness of fit of the models was evaluated using the well classified percentage, the area under the ROC curve and the Hosmer and Lemeshow test.

RESULTS

281 schoolchildren (140 boys and 141 girls) were recruited for the study. The global prevalence of hypovitaminosis D in this population, understood as serum 25 (OH) D values lower than 20 ng/ml, was 18.1%. Cases of severe deficiency (25 (OH) D <10ng / ml) represented 1.4%.

Table 1 shows the distribution of demographic data and other previously known risk factors in the sample, classifying schoolchildren according to a higher or lower vitamin D level (hypovitaminosis) of 20 ng/ml. Statistically significant associations were observed for season of the year, ethnicity, skin phototype, time of exposure to the sun, and age of the child. The mean vitamin D level in schoolchildren of Caucasian ethnicity was 29.83 ± 9.45 ng/ml, while among non-Caucasians it was 19.25 ± 9.71 ng/ml.

Table 1. Description of categorical variables of the sample according to vitamin D levels

Total		Vitamin D deficiency		Normal vitamin D		
		n 51	% 18.1%	n 230	% 81.9%	p-value
Season						
	Winter	20	29.4%	48	70.6%	
	Spring	17	31.5%	37	68.5%	
	Summer	6	9.1%	60	90.9%	
	Autumn	8	8.3%	88	91.7%	<0.001
Sex						
	Man	22	15.7%	118	84.3%	
	Woman	29	20.6%	112	79.4%	0.353
Age						
	<10 years	21	14.3%	126	85.7%	
	>=10 years	30	22.4%	104	77.6%	0.089
Ethnicity						
	Caucasian	37	14.5%	218	85.5%	
	Other	14	53.8%	12	46.2%	<0.001
Tanner						
	Prepubertal	27	14.8%	156	85.2%	
	Puberalanner	24	24.5%	74	75.5%	0.052
Personal history						
	Yes	6	17.1%	29	82.9%	
	No	45	18.3%	201	81.7%	1.000
Sport						
	Less than school	2	6.9%	27	93.1%	
	School only	29	23.2%	96	76.8%	
	School + two days	20	15.7%	107	84.3%	0.078
Skin phototype						
	Red/blond/brown	33	14.5%	195	85.5%	
	Brown/black	18	34.0%	35	66.0%	0.002
Exhibithion time						
	<30 minutes	34	28.3%	86	71.7%	
	>30 minutes	17	10.6%	144	89.4%	<0.001
Sunscreen	No	38	21.7%	137	78.3%	
	Yes	13	12.3%	93	87.7%	0.055
Use of filter	No	41	20.6%	158	79.4%	
	Yes	10	12.2%	72	87.8%	0.125
Medication	No	41	17.5%	193	82.5%	
	Yes	10	21.3%	37	78.7%	0.537
Supplementation	No	50	18.5%	221	81.5%	
	Yes	1	10.0%	9	90.0%	0.696

Figure 1. Distribution of the percentages of the vitamin D level according to the ethnic group of the schoolchild
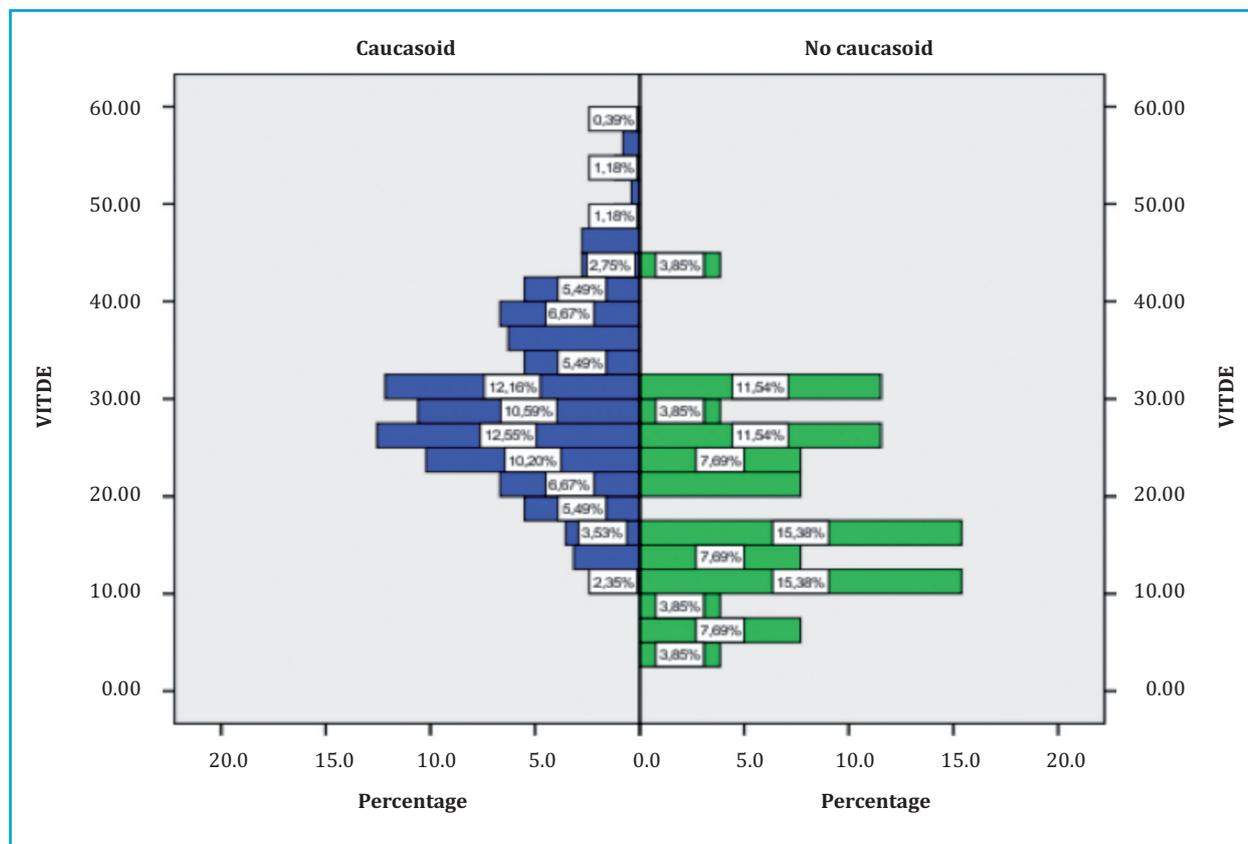
Correspondingly, only 14.5% of Caucasians were in the group with vitamin D deficiency, compared to 53.8% of non-Caucasians, with the difference in the distribution by groups also significant (p <0.001). Figure 1 shows the frequency of the percentages of vitamin D levels between children of Caucasian origin and those of non-Caucasian origin. The resulting distributions are clearly different. Laboratory tests were within normal ranges in all cases except PTH, which in 5 cases was above the cut-off point of 65 pg/ml.

Table 2 shows the comparison of the means for each of the micronutrients in the diet in the subsamples with levels greater or less than 20 ng/ml in vitamin D measured in serum. Statistically significant differences were observed in pyridoxine B6, saturated and monounsaturated fatty acids. The mean mg of vitamin D ingested was not statistically significant despite the fact that individuals with hypovitaminosis reported a 40% lower vitamin D intake.

The results of the logistic regression adjusted for season, sex, age (older or younger than 10 years), ethnicity and time of exposure to the sun show a statistically significant effect of pyridoxine B6 and saturated fatty acids (table 3). Increasing the intake of pyridoxine B6 by one milligram decreases 1.85 times the possibility of presenting vitamin D deficiency in our sample. Similarly, the intake of 1 gram more of saturated fatty acids the possibility decreases 1.04 times.

DISCUSSION

The main contribution of our work is that diet plays a reduced role as a risk factor for hypovitaminosis D in healthy children. On the contrary, and according to the li-

terature[2,3,5,23], factors related to sun exposure are the determinants of the presence of insufficient levels of vitamin D. From a clinical point of view, it has two consequences. The first is that a correct diet should not be a reason not to assess the possible risk of hypovitaminosis in a schoolchild. Second, it highlights that known risk factors such as non-Caucasian ethnicity and seasons with less sun exposure continue to be factors that determine a vitamin D assessment in schoolchildren. The strengths of our study should be highlighted as the size of the sample, its representativeness of the healthy population, and the obtaining of 25 (OH) D determinations throughout a year. A limitation of our work is the type of nutritional survey used since the diet with a 24-hour ER at a single moment is a method with limited precision. In addition, ER24hs requires adequate recent memory, it is not recommended for children under 12 years of age and in these cases the contribution of the parents was recorded. However, the work of Rodríguez Sangrador et al. that conducts a survey on the frequency of food consumption in 2 months of the year and measures the level of vitamin D but does so in a population of only 47 adolescents[16]. The difficulty of applying methods based on the weight of the food consumed over several days is the cause of the low number of published works. In the case of ER24hs we had trained interviewers, but we recognize the weakness of this type of survey in relation to recent memory and the low estimate of a person's nutritional and energy contributions. Despite its limitations, the literature that has compared the methods for measuring nutrient intake indicates that the ER24hs provides valid, although less precise, information[21,22].

Table 2. Nutrient content of the 24-hour intake

Total	Vitamin D deficiency		Normal vitamin D		
	n 43	% 84.3%	n 205	% 89.1%	
	Mean	SD	Mean	SD	p-value
Protein (g)	87.41	25.01	93.51	25.10	0.149
Lipids (g)	86.86	25.50	96.06	29.26	0.057
Carbohydrates (g)	227.19	62.15	232.29	59.07	0.611
Energy (kcal)	2040.16	437.83	2169.47	472.11	0.100
Proteins %	17.21	4.11	17.34	3.35	0.844
Lipids %	38,09	7.43	39.44	6.70	0.239
Carbohydrates %	44.63	8.00	43.15	7.48	0.545
Phosphorus (mg)	1294.27	347.95	1341.90	377.92	0.447
Magnesium (mg)	235.31	77.57	238.44	73.60	0.802
Calcium (mg)	1002.79	338.96	1054.87	359.35	0.384
Iron (mg)	13.04	4.31	13.44	4.54	0.590
Zinc (mg)	10.37	4.32	11.00	4.07	0.366
Sodium (mg)	1691.45	763.64	1806.33	677.01	0.324
Potassium (mg)	2590.07	828.46	2591.71	843.90	0.991
Iodine (mg)	44.90	30.04	48.06	33.50	0.569
Selenium (mg)	65.80	33.69	70.41	59.32	0.623
Copper (mg)	958.35	620.54	975.99	741.93	0.884
Fluorine (mg)	403.93	278.13	364.40	214.89	0.300
Chlorine (mg)	0.00	0.00	1.56	15.75	0.517
Manganese (mg)	0.00	0.00	0.01	0.05	0.517
Chromium (mg)	0.00	0.00	0.49	4.95	0.517
Molybdenum (mg)	0.29	2.98	0.00	0.00	0.517
Vitamin C mg	104.56	92.29	109.91	109.46	0.739
Thiamine B1 mg	1.50	0.67	1.32	0.59	0.098
Riboflavin B2 mg	1,80	0.61	1.70	0.51	0,330
Nicotinic Ac mg	20.19	9.43	19.45	9.50	0.640
Pyridoxine B6 mg	1.92	0.97	1.63	0.68	**0.018**
Vitamin A µg	1568.10	1443.63	1692.35	1444.86	0.608
Vitamin D µg	3.92	9.49	6.52	15.20	0.286
Vitamin E mg	9.22	3.83	8.36	4.55	0.192
Free folic acid µg	84.34	50.44	91.30	69.81	0.445
Total folic acid µg	203.41	110.43	200.85	109.06	0.890
Cyanocobalamin B12 µg	4.99	8.83	4.71	3.81	0.839
Biotin µg	0.34	3.62	0.00	0.00	0.538
Saturated AG g	29.22	11.59	24.87	8.45	**0.021**
Monounsaturated FA g	41.40	13.83	36.84	13.35	**0.049**
Polyunsaturated FA g	8.52	4.87	7.83	4.45	0.393
EPA g	0.06	0.16	0.08	0.28	0.408
DHA g	0.09	0.29	0.16	0.54	0.419
Cholesterol mg	412.54	229.78	407.56	254.68	0.899
MCT g	0.01	0.13	0.00	0.00	0.517
Dietary fiber g	14.77	6.69	14.90	6.47	0.904

SD: standard deviation; g: grams; FA: fatty acids; EPA: icosapentaenoic acid; DHA: docosahexaenoic acid; MCT: medium chain triglycerides.

Table 3. Multivariate analysis of the probability of hypovitaminosis D, according to risk factors

	95% CI for AOR			
	AOR	**Lower**	**Higher**	**Sig.**
Summer-autumn season	0.17	0.06	0.42	0.000
Sex female	1.45	0.61	3.44	0.404
Age >= 10 years	1.21	0.40	3.62	0.736
Non-Caucasoid ethnic group	17.67	4.77	65.46	0.000
Exposure time >30 min	0.45	0.19	1.06	0.067
Pyridoxine B6 (mg)	0.54	0.31	0.95	0.031
Saturated AG (gr)	0.96	0.92	1.00	0.048
Vitamin D (µg)	1.02	0.99	1.06	0.126
Fluorine (mg)	1.00	1.00	1.00	0.106
Constant	0.38			0.312
Well ranked percentage	85.9%			
Area under the ROC curve	0.87	0.82	0.93	
Hosmer and Lemeshow test				0.382

AOR: adjusted Odds Ratio; AG: fatty acids; min: minutes.

Upon analyzing the different dietary micronutrients, statistically significant associations with hypovitaminosis D only appeared for pyridoxine B6 and saturated fatty acids in both the univariate and multivariate analyzes, with no pathophysiological explanation for this finding. Among the micronutrients, the frequencies of magnesium, calcium and potassium intakes were deficient regarding the recommendations for each sex and age. Regarding vitamins, the same happened with folic acid and vitamins C, A, D and E. The 2005 Basque Country Nutritional Survey showed similar nutritional habits to ours[24].

Our results indicate that hypovitaminosis D is associated with the same factors as in the literature[25,26]. The percentage with severe deficiency (<10 ng/ml of 25 (OH) D) was small (1.4%), as corresponds to a healthy population. Using 20 ng/ml as a cut-off point, the deficiency reached 18.15% prevalence. According to the American Institute of Medicine (USA), the level of 20 ng/ml covers the needs related to phosphocalcic metabolism of 97% of the population[25]. However, with a cut-off point of ≤30 ng/ml, as established by other studies[6], the level of hypovitaminosis D would reach 56.3%. Hypovitaminosis D was focused on risk groups such as the non-Caucasian population in which it was greater than 50%. In fact, a case of rickets in a non-Caucasian, Pakistani child was the trigger for this study. Other variables such as seasonality and weight were also relevant[3,5,23]. For this reason, the outdoor lifestyle, sun exposure free of sunscreen and dietary patterns that ensure a correct intake of vitamin D and calcium continue to be the recommendations to be followed by the general population. It is important to highlight that the population studied was healthy and that only 5 school-children found elevated levels of PTH (>65 pg/ml). In no case were there clinical manifestations or alterations in calcium, phosphorus and magnesium levels were found. The five children underwent a subsequent clinical evaluation and analysis of phosphocalcic metabolism (alkaline phosphatase, calcium, phosphorus, magnesium, PTH and vitamin D), with normal examination and analysis. For this reason, it was considered that the alterations in PTH levels corresponded more to physiological variations of adaptation than to a response to a vitamin deficiency[26].

Exposure to the sun for more than 30 minutes was statistically significant in the univariate analysis, but this relationship disappeared in the multivariate analysis with a significance level of 5% since the p was 0.67. This "anomaly" may be due to the sample size since, although the adjusted Odds Ratio was 0.45, its upper confidence interval exceeded one.

According to our results, population screening for vitamin D deficiency should not be performed and supplementing with it should be limited exclusively to risk groups[6,27]. The criteria for carrying out an analytical determination of 25 (OH) D and supplementing it, according to its results, have been described by expert committees[28-30]. However, these indications vary between different scientific societies. Thus, the American Pediatric Association (AAP)[30] and the European Association of Pediatrics (EAP)[29] reserve this indication for risk groups. A study by Saggese et al.[28] in a global consensus in Italy proposes supplementing with vitamin D in children and adolescents with the following risk factors for vitamin D deficiency: non-Caucasian ethnicity with dark skin pigmentation, reduced exposure to sunlight (due to lifestyle factors, chronic illness or hospitalization, institutionalization, complex disability, covering with clothing for religious or cultural reasons) or constant use of sunscreen, international adoption, obesity, chronic diseases (kidney, liver, Malabsorption syndromes, chronic therapies (anticonvulsants-antiretrovirals-glucocorticoids-systemic antifungals). In all these situations it is necessary to monitor vitamin D status at least once a year.

The vitamin D needs are covered by 90% with sun exposure and the remaining 10% is achieved through the diet, so both factors should be specifically valued in the Primary Care programs of the child and adolescent population, both for its prevention, diagnosis and treatment. Currently the best option to increase the dietary intake of vitamin D is food fortification. In the European Union, countries are divided into 3 categories; those with a policy of mandatory fortification (Norway, United Kingdom), voluntary (Spain, Portugal) or that there is no fortification. In Spain, there are exceptions to some products used for a long time as the sole source of nutrients by some population groups (infant formulas for

128 *Blarduni Cardón E, Arrospide A, Angulo Ugarte H, Urrutia Etxebarria I, Castaño González L, Etxebarria I, Mar J, Grupo Goivide* ORIGINALS

Rev Osteoporos Metab Miner. 2021;13(4):122-129

initiation, continuation and cereals – enteral and parenteral nutrition products for hospital use and low-energy diets for reduction of weight, whose fortification is mandatory for all member states of the European Union)[31]. In our country there is a tendency to fortify skimmed and semi-skimmed milk until reaching the vitamin D level of whole milk that is lost with the skimming process. In addition, there are other products on the market that are also fortified, such as cookies, yogurt, margarines, cheese, breakfast cereals, juices and beverages[32].

Children require less exposure to sunlight than adults to produce sufficient amounts of vitamin D, both because of their higher body surface area to volume ratio and because of the greater capacity of their metabolism to produce vitamin D[33]. In relation to sun exposure, it is known that the effective dose of UV radiation to produce 1000 IU of vitamin D, which guarantees sufficient levels of it in the blood, is achieved with 25% of the minimum erythromatogenic dose (MED), which is equivalent to 10-15 minutes in 25% of the body surface (face, arms, hands), without sunscreen and in the central hours of the day, from 10 to 15 hours[34].

A varied, balanced diet adapted to the needs of the different stages of its evolution is key for adequate physical and psychological growth, to prevent diseases and to obtain an optimal state of health. Their vigilance makes the risk of a short- and long-term nutritional deficit generally unlikely. Despite this, it should not be a reason to rule out vitamin D deficiency risk.

Promoting adequate levels of vitamin D in schoolchildren and adolescents is important because nutritional rickets can develop throughout the pediatric age and because its deficiency negatively affects bone health[35]. The comparison of the numerous studies that assess supplementing with vitamin D is very complex given the heterogeneity in the administration of vitamin D (interval, dose, duration) and the recruited population (sex, age, ethnicity, BMI, latitude of the country of residence, season of the year, baseline vitamin D status). In our region, an unfavorable latitude, there is little synthesis of vitamin D in late autumn, during the winter months and early spring. During this period, an adequate level of vitamin D is only maintained by endogenous stores accumulated during the previous summer or by exogenous supplements. The presence of hypovitaminosis D due to risk factors, the recommended doses are between 600 IU/day (reduced sun exposure) up to 1000 IU/day (multiple risk factors for vitamin D deficiency). The way to supplement is with intermittent doses (weekly-monthly) from 5-6 years and especially during adolescence; continuous doses should be reserved for children with permanent risk factors for hypovitaminosis D[28].

This work recognizes the importance of vitamin D during the pediatric stage and the challenge posed by an individualized assessment according to age, seasonality, skin phototype, adequate outdoor lifestyle, the controlled and prudent use of sunscreens and patterns nutrients that ensure a correct intake of vitamin D and calcium. The study of vitamin D also implies an effort to reduce health inequalities since it focuses on a social group with a low socioeconomic level and associated with immigration. Thus, pediatric control of hypovitaminosis D in schoolchildren is at the same time an exercise in the implementation of public health strategies aimed at promoting children's health.

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

Bibliography

1. Wolden-Kirk H, Gysemans C, Verstuyf A, Mathieu C. Extraskeletal effects of vitamin D. Endocrinol Metab Clin North Am. 2012; 41: 571-94.

2. Blarduni E, Arrospide A, Galar M, Castaño L, Mar J, Grupo GOIVIDE. Factors associated with the prevalence of hypovitaminosis D in pregnant women and their newborns. An Pediatr 2003. 2019; 91: 96-104.

3. González-Molero I, Morcillo S, Valdés S, Pérez-Valero V, Botas P, Delgado E, et al. Vitamin D deficiency in Spain: a population-based cohort study. Eur J Clin Nutr. 2011; 65: 321-8.

4. Rodríguez-Dehli AC, Riaño-Galán I, Fernández-Somoano A, Navarrete-Muñoz EM, Espada M, Vioque J, et al. [Hypovitaminosis D and associated factors in 4-year old children in northern Spain]. An Pediatr 2003. 2017; 86: 188-96.

5. Klingberg E, Oleröd G, Konar J, Petzold M, Hammarsten O. Seasonal variations in serum 25-hydroxy vitamin D levels in a Swedish cohort. Endocrine. 2015; 49: 800-8.

6. Holick MF, Binkley NC, Bischoff-Ferrari HA, Gordon CM, Hanley DA, Heaney RP, et al. Evaluation, treatment, and prevention of vitamin D deficiency: an Endocrine Society clinical practice guideline. J Clin Endocrinol Metab. 2011; 96: 1911-30.

7. Holick MF. Resurrection of vitamin D deficiency and rickets. J Clin Invest. 2006; 116: 2062-72.

8. Welch TR, Bergstrom WH, Tsang RC. Vitamin D-deficient rickets: the reemergence of a once-conquered disease. J Pediatr. 2000; 137: 143-5.

9. Prentice A. Vitamin D deficiency: a global perspective. Nutr Rev. 2008; 66: S153-64.

10. Holick MF. Sunlight and vitamin D for bone health and prevention of autoimmune diseases, cancers, and cardiovascular disease. Am J Clin Nutr. 2004; 80: 1678S-88S.

11. Reichrath J, Zouboulis CC, Vogt T, Holick MF. Targeting the vitamin D endocrine system (VDES) for the management of inflammatory and malignant skin diseases: An historical view and outlook. Rev Endocr Metab Disord. 2016; 17: 405-17.

12. Malouf-Sierra J. ¿Realmente es tan prevalente la deficiencia de vitamina D en España? Situación actual, causas y población de riesgo. Med Clin Barc. 2017; 18 Supl 1: 13-7.

13. Webb AR, Kline L, Holick MF. Influence of season and latitude on the cutaneous synthesis of vitamin D3: exposure to winter sunlight in Boston and Edmonton will not promote vitamin D3 synthesis in human skin. J Clin Endocrinol Metab. 1988; 67: 373-8.

14. Brinkmann K, Le Roy C, Iñiguez G, Borzutzky A. [Severe vitamin D deficiency in children from Punta Arenas, Chile: Influence of nutritional status on the response to supplementation]. Rev Chil Pediatr. 2015; 86: 182-8.

15. Martínez Redondo I, De Arriba Muñoz A, Rodríguez Martínez G, Labarta Aizpún J. Deficiencia de Vitamina D en niños aragoneses sanos. Nutr Hosp. 2018; 35: 782-8.

16. Rodríguez Sangrador M, Beltrán de Miguel B, Cuadrado Vives C, Moreiras Tuny O. [Influence of sun exposure and diet to the nutritional status of vitamin D in adolescent Spanish women: the five countries study (OPTIFORD Project)]. Nutr Hosp. 2010; 25: 755-62.

17. Orces CH, López Gavilánez E. Determinants of vitamin D supplementation among older adults and its effect on 25(OH)D levels according to bone mineral density status. Nutr Hosp. 2020; 37: 28-36.

18. Quesada Gómez JM, Calañas Continente A. [The pieces of the «vitamin» D puzzle begin to fit: give it to those who need it]. Nutr Hosp. 2020; 37: 4-5.

19. Euskalmet. Niveles de exposición solar en el País Vasco. [Internet]. Agencia vasca de Meteorología. [citado 17 de junio de 2017]. Disponible en: http://www.euskalmet.euskadi.eus/s075853x/es/meteorologia/meteodat/mapas.apl?e=5.

20. Eilers S, Bach DQ, Gaber R, Blatt H, Guevara Y, Nitsche K, et al. Accuracy of self-report in assessing Fitzpatrick skin phototypes I through VI. JAMA Dermatol. 2013; 149: 1289-94.

21. Bingham SA, Gill C, Welch A, Day K, Cassidy A, Khaw KT, et al. Comparison of dietary assessment methods in nutritional epidemiology: weighed records v. 24 h recalls, food-frequency questionnaires and estimated-diet records. Br J Nutr. 1994; 72: 619-43.

22. Straßburg A, Eisinger-Watzl M, Krems C, Roth A, Hoffmann I. Comparison of food consumption and nutrient intake assessed with three dietary assessment methods: results of the German National Nutrition Survey II. Eur J Nutr. 2019; 58: 193-210.

23. Durá-Travé T, Gallinas-Victoriano F, Chueca Guindulain MJ, Berrade-Zubiri S. [Vitamin d deficiency among children and adolescents with normal nutritional status]. Nutr Hosp. 2015; 32: 1061-6.

24. Departamento de Sanidad. Encuesta de nutrición, 2005: hábitos alimentarios y estado de salud de la población vasca de 4 a 18 años: primeros resultados. Vitoria-Gasteiz: Servicio Central de Publicaciones del Gobierno Vasco; 2006.

25. Ross AC, Manson JE, Abrams SA, Aloia JF, Brannon PM, Clinton SK, et al. The 2011 report on dietary reference intakes for calcium and vitamin D from the Institute of Medicine: what clinicians need to know. J Clin Endocrinol Metab. 2011; 96: 53-8.

26. Sahin ON, Serdar M, Serteser M, Unsal I, Ozpinar A. Vitamin D levels and parathyroid hormone variations of children living in a subtropical climate: a data mining study. Ital J Pediatr. 2018; 44: 40.

27. Valero Chávez F, Luengo Pérez L, Cubero Juánez J. Adecuación de las peticiones de los niveles de Vitamina D al laboratorio. Nutr Hosp. 2016; 33: 581.

28. Saggese G, Vierucci F, Prodam F, Cardinale F, Cetin I, Chiappini E, et al. Vitamin D in pediatric age: consensus of the Italian Pediatric Society and the Italian Society of Preventive and Social Pediatrics, jointly with the Italian Federation of Pediatricians. Ital J Pediatr. 2018; 44: 51.

29. Grossman Z, Hadjipanayis A, Stiris T, Del Torso S, Mercier J-C, Valiulis A, et al. Vitamin D in European children-statement from the European Academy of Paediatrics (EAP). Eur J Pediatr. 2017; 176: 829-31.

30. Golden NH, Abrams SA, Committee on Nutrition. Optimizing bone health in children and adolescents. Pediatrics. 2014; 134: e1229-43.

31. López-Sobaler AM, Aparicio A, González-Rodríguez LG, Cuadrado-Soto E, Rubio J, Marcos V, et al. Adequacy of Usual Vitamin and Mineral Intake in Spanish Children and Adolescents: ENALIA Study. Nutrients. 2017; 9: 131.

32. Ortega Anta RM, González-Rodríguez LG, Jiménez Ortega AI, Estaire Gómez P, Rodríguez-Rodríguez E, Perea Sánchez JM, et al. [Insufficient intake of vitamin D in spanish schoolchildren: determinants of the problem and basis for its improvement]. Nutr Hosp. 2012; 27: 1437-43.

33. Paller AS, Hawk JLM, Honig P, Giam YC, Hoath S, Mack MC, et al. New insights about infant and toddler skin: implications for sun protection. Pediatrics. 2011; 128: 92-102.

34. Gilaberte Y, Carrascosa JM. Sun protection in children: realities and challenges. Actas Dermosifiliogr. 2014; 105: 253-62.

35. Munns CF, Shaw N, Kiely M, Specker BL, Thacher TD, Ozono K, et al. Global Consensus Recommendations on Prevention and Management of Nutritional Rickets. J Clin Endocrinol Metab. 2016; 101: 394-415.

Genetic relationship between pulmonary diseases of environmental or occupational origin and osteoporosis: a bioinformatic approach

Andújar-Vera F[1], García-Fontana C[1,2,3], González-Salvatierra S[1,2,4], Martínez-Heredia L[4], Muñoz-Torres M[1,2,3,4], García-Fontana B[1,2,3]
1 Bio-health Research Institute of Granada (IBS.GRANADA). Granada (Spain)
2 Endocrinology and Nutrition Unit. San Cecilio University Clinical Hospital. Granada (Spain)
3 CYBERFES. Carlos III Health Institute. Madrid (Spain)
4 Department of Medicine. University of Granada. Granada (Spain)

Summary

Objetives: Identifying biomarkers that relate osteoporosis to occupational and environmental lung diseases.
Material and methods: Using integrated medical terminology databases, diseases related to lung diseases were obtained which, together with osteoporosis, were analyzed in DisGeNET to obtain the genes associated with each disease and form a protein-protein interaction network (PPI) through the Cytoscape StringApp. Applying different centrality algorithms using CythoHubba in Cytoscape, the 5 network proteins with the highest degree of centrality were selected.
Results: 9 diseases were included in the group of pulmonary diseases. 2,698 genes associated with lung diseases and osteoporosis were obtained. Genes associated with osteoporosis and with at least two of the included lung diseases resulted in a PPI network with 152 nodes and 1,378 axes. The proteins with the highest degree of network centrality were AKT1, ALB, IL6, TP53 and VEGFA.
Conclusions: There is a significant relationship between osteoporosis and the environmental lung diseases studied, through genes with dual involvement. We propose five important genes that link these diseases. This could provide a coherent basis for further research in this field.

Key words: osteoporosis, air quality, air pollution, lung disease, biomarkers, bioinformatics.

INTRODUCTION

Osteoporosis is currently defined as "a systemic skeletal disease characterized by decreased bone mass and a structural alteration of the bone tissue that determines a decrease in bone resistance resulting in a significant increase in fragility and susceptibility to fractures"[1]. The classic risk factors associated with the development of osteoporosis are age, a history of previous fracture or family history of osteoporosis, and prolonged estrogen deficiency[2].

On the other hand, one of the most important risk factors for mortality at the population level is air pollution. The consequences derived from air pollution have a high economic and social impact. In 2015, the costs derived from pollution-related morbidity and mortality reached 21 billion dollars worldwide and an estimated number of premature deaths between 6 and 9 million people in 2060 due to outdoor air pollution[3]. Air pollution has been shown to have a direct impact on health, causing various adverse effects[4]. The relationship between air pollution and environmental lung diseases has been reported in numerous studies[4-6]. Likewise, there is strong scientific evidence that relates poor air quality in different work environments with the development of different respiratory diseases[7,8].

In the 1980s, the first studies were published that showed an association between air quality and bone quality, observing a significantly higher incidence of fractures in city dwellers compared to those residing in rural areas[9-11]. Since then, studies in this line have increased notably over the last few years, indicating that prolonged exposure to air pollution is linked to a decrease in bone quality[12-14], and

Correspondence: Cristina García Fontana (cgfontana@ugr.es), Manuel Muñoz Torres (mmt@mamuto.es)

considered osteoporosis and fractures a modifiable risk factor[15]. However, a recently published systematic review points to the existence of inconsistent associations between air pollution and osteoporosis risk, which could be explained by the heterogeneity in the characteristics of the subjects participating in the related studies published[16].

Despite the fact that scientific evidence suggests the relationship between lung diseases and osteoporosis, currently no special attention is paid to air quality as a potential bone health problem. Considering the increase in air pollution in recent years, delving into this topic to understand the cross paths between the harmful effects of air pollution and bone health is urgent.

In this context, the identification of robust genetic markers that are present both in environmental and occupational lung diseases related to pollution and air quality, as well as in diseases related to bone quality, will allow the development of preventive and therapeutic strategies focused on the population at highest risk. For this type of study, bioinformatics plays an important role, allowing us to identify, through the use of different tools and algorithms, molecules that can act as common biomarkers between different diseases. In this sense, our study aimed to build and analyze a protein-protein interaction network relating genes involved in different environmental and occupational lung diseases related to pollution and air quality, with genes associated with the development of osteoporosis, to identify biomarkers common in both diseases.

MATERIAL AND METHODS

Data collection
First, a literature search was performed to obtain a selection of unified terms to determine pulmonary and occupational diseases. The unified medical terminology databases consulted were Unified Medical Language System (UMLS, https://www.nlm.nih.gov/research/umls/index.html) y Medical Subject Headings (MeSH, https://www.nlm. nih. gov/mesh/meshhome.html). Subsequently, each of the selected diseases was used to determine disease-associated genes (GDA) by using the platform DisGeNET (http:// www.disgenet.org/home/), which integrates information on the relationship between genes and diseases from various sources of public data and literature on gene expression, biomarkers, association between variants and diseases, single nucleotide polymorphisms and associations of clinical phenotypes with the corresponding diseases[17].

Selection of seed genes
Once the genes associated with the different lung diseases (of environmental or occupational origin) and osteoporosis had been obtained, an initial filtering was carried out selecting those genes common to osteoporosis and with at least one of the lung diseases framed within this study. In a second phase, those genes associated with osteoporosis and with two lung diseases were selected to generate the protein interaction network (PPI).

CONSTRUCTION OF PPI NETWORKS
To build the PPI network of genes associated with the diseases under study, the STRING application was used (1.6.0 version, released: 8 Sep 2020, http://apps. cytoscape.org/apps/stringapp)[18] within the Cytoscape platform (versión 3.8.2, https://cytoscape.org/)[19]. The

confidence cut-off point value was established at 0.7. Only those interactions with a value greater than or equal to 0.7 were considered significant. Proteins below this cut-off point and those unrelated to other proteins were discarded.

Study of centrality and identification of genes
Obtaining the central genes, that is, those important nodes with a high number of interactions towards other nodes, were selected using the CytoHubba complement in Cytoscape[20]. Five calculation methods were used: Degree, Betweennes, Maximal Clique Centrality (MCC), Bottleneck and Closeness[21]. The common genes derived from these six algorithms represent key candidate genes with important biological regulatory functions.

RESULTS

Data collection
After reviewing the unified medical terms, the diseases included in table 1 were selected. These were used to compare the genes associated with each of them with osteoporosis. Diseases in which genes associated with the disease had not been identified were not included.

Selection of seed genes
After applying the criteria established for the inclusion of genes in the PPI network, 157 genes associated with osteoporosis and at least two lung diseases were obtained. The complete list can be viewed in the supplementary material.

Construction of PPI networks
The generated PPI network included 152 proteins (nodes) and 1,378 relationships (axes). Of this set, 12 proteins were discarded because they were not linked to other proteins (figure 1).

Table 1. Unified terms for diseases of this study and unique identification code

*SLMU CUI	Unified Term/Pathology
C0003165	Anthracosis
C0003849	Asbestosis
C0006542	Byssinosis
C0024117	Chronic obstructive airway disease
C0025500	Mesothelioma
C0029456	Osteoporosis
C0037116	Silicosis
C0206062	Interstitial lung disease
C0264423	Occupational asthma
C0340092	Summer-type hypersensitivity pneumonitis

*SLMU: unified medical language system; CUI: unique identification code.

132 *Andújar-Vera F, García-Fontana C, González-Salvatierra S, Martínez-Heredia L, Muñoz-Torres M, García-Fontana B*   ORIGINALS

Rev Osteoporos Metab Miner. 2021;13(4):130-136

Study of centrality and identification of genes

10 central genes were determined by means of the Cyto-Hubba application from the centrality methods detailed in table 2. Next, the 5 genes that appeared most frequently in the different centrality algorithms were selected (table 3), which shared their presence in all the algorithms used, evidencing their importance within the constructed interaction network.

DISCUSSION

Any annual report on air quality reveals unflattering conclusions. The World Health Organization constantly warns about non-compliance with recommended standards for achieving healthy air quality. Poor air quality has a great health impact, causing 4.2 million premature deaths a year worldwide, mainly associated with exposure to small particles with a diameter less than or equal to 2.5 microns[22]. The main health effects of exposure to an environment with poor quality or polluted air are known and have been scientifically proven for many years. These include lung cancer, respiratory and cardiovascular diseases, stroke, etc. However, there are other less known effects or with little scientific evidence associated with the lack of studies in this area.

Due to the aging of the population, osteoporosis is considered one of the most prevalent diseases in the population worldwide. Currently, the association between poor air quality and osteoporosis is not well defined in the scientific literature, but it is beginning to be of great importance due to the health impact of the high percentage of the population with osteoporotic problems or low bone quality.

The results of this study show a set of genes associated with the presence of both lung diseases (derived from environmental contamination or associated with certain occupational activities) and osteoporosis. Pulmonary diseases are mainly caused by repeated exposure over time to chemical irritants, allergens or toxins, which can cause lasting effects on the individual.

The bibliographic search of pulmonary diseases of environmental or occupational origin, offered a set of diseases that, without being a priori related to osteoporosis, could share one or more genes linked to osteoporotic processes. Thus, we find anthracosis, a lung condition produced by exposure and inhalation of dust with a high concentration of carbon; asbestosis, in which asbestos is the main inhaled particle; byssinosis, produced by inhaling cotton dust among other particles of plant origin; chronic obstructive pulmonary disease (COPD), a consequence of long-term exposure to irritants such as polluted air, chemical fumes or dust, among others; mesothelioma, a specific form of lung mesothelial cancer that is usually related primarily to occupational exposure to asbestos; silicosis, caused by inhaling dust with silica particles; interstitial lung diseases, which encompass a group of lung diseases most of which are caused by progressive scarring of the lung tissue due to prolonged exposure to dangerous substances (such as certain organic substances, wood, metals, infectious agents such as viruses, drugs, etc.); occupational asthma, produced by inflammation of the pulmonary airways caused by inhalation of substances produced in the workplace such as wood dust, fungi, and/or chemical substances, among others, and hypersensitivity pneumonitis, caused by exposure to a large number of organic particles such as fungi or bacteria, causing a significant inflammatory response[23].

This set of diseases derived from poor air quality has been related to osteoporosis, reflecting a high genetic rela-

Figure 1. Protein-protein interaction network of genes associated with the lung diseases of this study and linked to osteoporosis
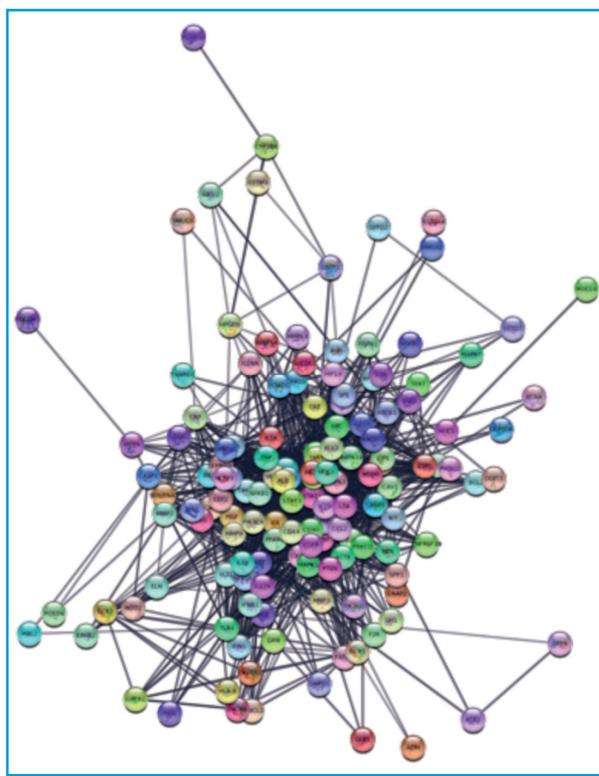
Table 2. Ranking of genes of the biological network according to the centrality algorithm used

Degree	Betweenness	*MCC	Bottleneck	Closeness
TNF	TNF	TNF	VEGFA	TNF
VEGFA	SRC	VEGFA	AKT1	VEGFA
AKT1	VEGFA	AKT1	HGF	AKT1
MAPK3	AKT1	MAPK3	SPP1	MAPK3
IL6	MAPK3	IL6	IL6	IL6
EGFR	IL6	MAPK8	TP53	MAPK8
TP53	EGFR	TP53	VCAM1	EGFR
CXCL8	TP53	CXCL8	CAT	TP53
ALB	ALB	CCL2	ALB	ALB
STAT3	MAPK1	ALB	MAPK1	STAT3

*MCC: maximal clique centrality.

tionship between the two types of diseases. The results of the present study are consistent with previous studies on the association of respiratory diseases with osteoporosis[12-14]. In addition, especially relevant genes have been obtained that could be considered as potential common markers between pulmonary and osteoporotic diseases.

Table 3. Ranking of Genes with more presence in the applied centrality algorithms

Genes	Unified name
AKT1	AKT Serine/Threonine Kinase 1
ALB	Albumin
IL6	Interleukin 6
TP53	P53 tumor protein
VEGFA	Vascular endothelial growth factor A

In systems biology, discovery of major proteins and their corresponding molecular pathways in complex diseases is booming thanks to PPI network analysis and enrichment analysis. The systems biology approach to investigating disease-associated biology is revolutionizing the understanding of cellular pathways and gene networks that underlie the onset of diseases, thus facilitating the discovery and development of new drugs and therapies thanks to the identification of markers in disease progression.

In biomolecular networks, where genes and/or proteins are nodes and the molecular interactions are the edges that interconnect the nodes, the importance of a node can be measured by the effect produced in the changes in the function of the interaction network. after deleting that node. These essential nodes are called hubs. In this study, 5 hubs have been determined that could consistently disturb the interaction network and that could cause important effects on the body's fitness. The hubs that have been selected and that relate pulmonary diseases of environmental or occupational origin with osteoporosis are AKT1, ALB, IL6, TP53 and VEGFA, table 4. However, future studies could suggest new hubs that present a lower score in the classification performed in this study.

The role that these hubs play in osteoporotic processes and lung diseases is well known and corroborates the importance of the genes selected in this work to relate the diseases under study.

In the case of the AKT1 gene, which encodes the protein called AKT1 kinase, it is found in various types of cells, playing important roles in many signaling pathways; in the case of osteoporosis, AKT1 acts as a negative regulator of osteoblast differentiation and as a positive mediator of osteoclastogenesis. It is therefore considered a potential target as a therapeutic target to improve osteoblast differentiation and bone mass formation while limiting osteoclast development and bone resorption[24,25]. This protein also has pulmonary involvement, participating in the appearance and progression of pulmonary fibrosis, by promoting the differentiation of myofibroblasts and the deposition of proteins in the extracellular space and on the other hand, through the regulation of TSP1. Due to this, AKT1 has been proposed as a biomarker of interstitial lung disease[26], so that the use of AKT1 inhibitors such as triciribine, is considered

as a potential therapeutic strategy for the treatment of these diseases. These data corroborate its dual role in both conditions.

Furthermore, the ALB gene encoding albumin was identified. Low levels of this protein have been associated with an increased risk of fractures due to low bone mineral density (BMD), with a positive correlation between serum albumin and BMD[27,28]. In relation to lung diseases, an association between albumin and interstitial lung disease has been shown, with significantly lower levels of albumin being observed in patients affected with interstitial lung disease[29].

Interleukin 6, encoded by the IL6 gene, is considered a key factor in postmenopausal osteoporosis due to its ability to activate osteoclasts and induce bone resorption[30]. Furthermore, it has been identified as a promising target for osteoporotic treatment due to its fundamental role as an inhibitor of osteogenesis and as a predictor of bone loss[31,32]. In relation to lung diseases, we can highlight studies that correlate the overexpression of IL6 with COPD[33,34], although there are others that point in the opposite direction[35]. With regard to silicosis, there are several studies that show increased levels of this cytokine from early stages of the disease, being considered as a potential biomarker for the early diagnosis and treatment of patients with silicosis[36]. Likewise, there are studies that relate the increase in IL6 levels with the development of anthracosis. In this sense, several studies show an association of certain IL6 polymorphisms with the development of anthracosis and/or anthrosilicosis as well as with associated genotoxic effects[37,38].

With regard to the tumor protein p53, encoded by the TP53 gene, there are several studies that relate it both to osteoporosis and to different lung diseases. In this context, several studies show that increased serum levels of p53 are associated with a decrease in bone mass, and the suppression of p53 partially reverses the decrease in BMD in vitro and in vivo[39]. In the case of lung diseases, it plays a leading role in the development of COPD, with increased levels of this protein being observed in affected patients[40]. Likewise, an association between p53 and mesothelioma has been observed[41]. In this sense, it has been reported that approximately 15% of patients affected with mesothelioma present some type of mutation in the TP53 gene. In the same way, in approximately 25% of human mesotheliomas there are co-deletions of the TP53 genes together with PTEN and/or CDKN2A/p14ARF, associating the cooperative losses of these genes with the development of a significant proportion of these aggressive neoplasms[42]. These findings may lead to the establishment of appropriate therapeutic strategies for the joint treatment of both types of diseases.

Lastly, the VEGFA gene encoding vascular endothelial growth factor A is known to play an important role in bone biology by participating in endochondral ossification. Furthermore, VEGFA is expressed at high levels in osteoblast precursors and can stimulate osteogenic differentiation in various types of cells[43]. On the other hand, decreased levels of VEGFA are reportedly associated with osteoporosis through an intracellular mechanism possibly mediated by the regulation of the transcription factor RUNX2, considered as a therapeutic target of the disease[44]. This gene has also shown relevant activity in lung diseases, being an autocrine growth factor in mesothelioma and a potent mitogen for mesothelial cells[45].

134 *Andújar-Vera F, García-Fontana C, González-Salvatierra S, Martínez-Heredia L, Muñoz-Torres M, García-Fontana B* ORIGINALS

Rev Osteoporos Metab Miner. 2021;13(4):130-136

VEGFA is involved in angiogenesis and stimulates neo-vascularization of tumors. In malignant mesothelioma, elevated levels of VEGFA and its receptor have been detected by immunohistochemistry[46,47] and have been correlated with microvessel density, increased tumor necrosis, and worse survival[48]. In this sense, drugs aimed at blocking VEGFA, such as bevacizumab, have recently shown their efficacy in the treatment of mesothelioma[49].

Despite the amount of information on the role of these genes in certain pulmonary and osteoporotic diseases, there are few studies that relate both types of diseases, therefore, it is necessary to delve into these aspects in order to identify possible common therapeutic targets. The association between lung diseases (triggered by air pollution or poor air quality in work environments) and bone quality could open the door to the design of behavioral strategies aimed at modifying the lifestyle that implies an improvement in bone health. On the other hand, it is important to consider the population with some type of lung disease, as a population at risk of bone fragility. Therefore, the knowledge provided by this type of study reveals the importance of studying BMD in people with lung diseases to establish early therapeutic and preventive measures in order to reduce the risk of fractures in this vulnerable population.

Table 4. Genes with a high score in centrality associated with the pulmonary pathologies used in this study

Genes	Unified name
AKT1	[a]COPD, Mesothelioma, Silicosis, [b]PID
ALB	COPD, PID
IL6	COPD, Mesothelioma, Silicosis, PID, Anthracosis
TP53	COPD, Mesothelioma, Silicosis, PID
VEGFA	COPD, Mesothelioma, PID

[a]COPD: chronic obstructive pulmonary disease; [b]PID: pulmonar interstitial disease.

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

Bibliography

1. Consensus development conference: diagnosis, prophylaxis, and treatment of osteoporosis. Am J Med; 1993; 94: 646-650.
2. Guía práctica del manejo de la Osteoporosis y prevención de la fractura por fragilidad en At. Primaria. https://www.semg.es/index.php/manuales-y-libros-semg/114-guia-practica-del-manejo-de-la-osteoporosis-y-prevencion-de-la-fractura-por-fragilidad-en-at-primaria.
3. The Economic consequences of outdoor air pollution | READ online. OECD iLibrary https://read.oecd-ilibrary.org/environment/the-economic-consequences-of-outdoor-air-pollution_9789264257474-en.
4. Nakao M, Ishihara Y, Kim C-H, Hyun I-G. The impact of air pollution, including Asian sand dust, on respiratory symptoms and health-related quality of life in outpatients with chronic respiratory disease in Korea: A panel study. J Prev Med Public Health Yebang Uihakhoe Chi 2018; 51: 130-139.
5. Cazzolla Gatti R. Why we will continue to lose our battle with cancers if we do not stop their triggers from environmental pollution. Int J Environ Res Public Health 2021; 18: 6107.
6. Waidyatillake NT, Campbell PT, Vicendese D, Dharmage SC, Curto A, Stevenson M. Particulate matter and premature mortality: A bayesian meta-analysis. Int J Environ Res Public. Health 2021; 18: 7655.
7. Lemen RA, Landrigan PJ. Sailors and the Risk of Asbestos-Related Cancer. Int J Environ Res Public Health 2021; 18: 8417.
8. Moon J, Yoo H. Misdiagnosis in occupational and environmental medicine: a scoping review. J Occup Med Toxicol Lond Eng 2021; 16: 33.
9. Falch JA, Ilebekk A, Slungaard U. Epidemiology of hip fractures in Norway. Acta Orthop Scand 1985; 56: 12-16.
10. Jónsson B, Gärdsell P, Johnell O, Sernbo I, Gullberg B. Life-style and different fracture prevalence: A cross-sectional comparative population- based study. Calcif Tissue Int 1993; 52: 425-433.
11. Sernbo I, Johnell O, Andersson T. Differences in the incidence of hip fracture. Comparison of an urban and a rural population in southern Sweden. Acta Orthop Scand 1988; 59: 382-385.
12. Prada D, López G, Solleiro-Villavicencio H, Garcia-Cuellar C, Baccarelli AA. Molecular and cellular mechanisms linking air pollution and bone damage. Environ Res 2020; 185: 109465.
13. Ranzani OT, Milà C, Kulkarni B, Kinra S, Tonne C. Association of Ambient and Household Air Pollution With Bone Mineral Content Among Adults in Peri-urban South India. JAMA Netw Open 2020; 3: e1918504.
14. Chang KH , Chang MY, Muo CH, Wu TN, Hwang BF, Chen CY, et al. Exposure to air pollution increases the risk of osteoporosis. Medicine (Baltimore) 2015; 94: e733.
15. Chang KH , Chang MY, Muo CH, Wu TN, Hwang BF, Chen CY, et al. Exposure to air pollution increases the risk of osteoporosis. Medicine (Baltimore) 2015; 94: e733.
16. Pang KL, Ekeuku SO, Chin KY. Particulate air pollution and osteoporosis: A systematic review. Risk Manag Healthc Policy 2021: 14: 2715-2732.
17. Piñero J, Queralt-Rosinach N, Gutiérrez-Sacristán A, Deu-Pons J, Centeno E, García-García J, et al. DisGeNET: a comprehensive platform integrating information on human disease-associated genes and variants. Nucleic Acids Res 2017; 45: D833-D839.
18. Szklarczyk D, Gable AL, Lyon D, Junge A, Wyder S, Huerta-Cepas J et al. STRING v11: protein–protein association networks with increased coverage, supporting functional discovery in genome-wide experimental datasets. Nucleic Acids Res 2019; 47: D607-D613.
19. Shannon P, Markiel A, Ozier O, Baliga NS, Wang JT, Ramage D, et al. Cytoscape: A software environment for integrated models of biomolecular interaction networks. Genome Res 2003; 13: 2498-2504.
20. Chen SH, Chin CH, Wu HH, Ho CW, Ko MT, Lin CY. Cyto-Hubba: a cytoscape plug-in for hub object analysis in network biology. https://citeseerx.ist.psu.edu/viewdoc/download?doi=10.1.1.800.333&rep=rep1&type=pdf.
21. Chaudhary R, Balhara M, Jangir DK, Dangi M, Dangi M, Chhillar AK. In silico protein interaction network analysis of virulence proteins associated with invasive Aspergillosis for drug discovery. Curr Top Med Chem. 2019; 19: 146-155.
22. Calidad del aire ambiente (exterior) y salud. https://www.who.int/es/news-room/fact-sheets/detail/ambient-(outdoor)-air-quality-and-health.
23. MedlinePlus - Health Information from the National Library of Medicine. https://medlineplus.gov/.
24. Mukherjee A, Rotwein P. Selective signaling by Akt1 controls osteoblast differentiation and osteoblast-mediated osteoclast development. Mol Cell Biol 2012; 32: 490-500.
25. Zhang Y, Wang N, Ma J, Chen XC, Li Z, Zhao W. Expression profile analysis of new candidate genes for the therapy of primary osteoporosis. Eur Rev Med Pharmacol Sci 2016; 20: 433-440.
26. Abdalla, M, Sabbineni H, Prakash R, Ergul A, Fagan SC, Somanath PR. The Akt inhibitor, triciribine, ameliorates chronic hypoxia-induced vascular pruning and TGFβ-induced pulmonary fibrosis. Br J Pharmacol 2015; 172: 4173-4188.
27. Kunutsor SK, Voutilainen A, Whitehouse MR, Seidu S, Kauhanen J, Blom AW, et al. Serum albumin and future risk of hip, humeral, and wrist fractures in caucasian men: New findings from a prospective cohort study. Med Princ Pract 2019; 28: 401-409.
28. Kim NH, Oh HJ, Park EJ, Kim DH, Choi, JS. Serum albumin and bone mineral density in healthy men above 30 years old. Korean J Fam Pr 2013; 3: 331-337.
29. Zhao R, Wang Y, Zhou W, Guo J, He M, Li P, et al. Associated factors with interstitial lung disease and health-related quality of life in Chinese patients with primary Sjögren's syndrome. Clin Rheumatol 2020; 39: 483-489.
30. IL-6 contributes to the defective osteogenesis of bone marrow stromal cells from the vertebral body of the glucocorticoid-induced osteoporotic mouse - PubMed. https://pubmed.ncbi.nlm.nih.gov/27128729/.
31. Li, X, Zhou Z, Zhang Y, Yang H. IL-6 contributes to the defective osteogenesis of bone marrow stromal cells from the vertebral body of the glucocorticoid-induced osteoporotic mouse. PLoS ONE 2016; 11: e0154677.
32. Scheidt-Nave C, Bismar H, Leidig-Bruckner G, Woitge H, Seibel MJ, Ziegler R, et al. Serum interleukin 6 is a major predictor of bone loss in women specific to the first decade past menopause. J Clin Endocrinol Metab 2001; 86: 2032-2042.
33. Wedzicha JA, Seemungal TA, MacCallum PK, Paul EA, Donaldson GC, Bhowmik A, et al. Acute exacerbations of chronic obstructive pulmonary disease are accompanied by elevations of plasma fibrinogen and serum IL-6 levels. Thromb Haemost 2000; 84: 210-215.
34. Kleniewska A, Walusiak-Skorupa J, Piotrowski W, Nowakowska-Świrta E, Wiszniewska M. Comparison of biomarkers in serum and induced sputum of patients with occupational asthma and chronic obstructive pulmonary disease. J Occup Health 2016; 58: 333-339.
35. Nuñez B, Sauleda J, Garcia-Aymerich J, Noguera A, Monsó E, Gómez F, et al. Lack of correlation between pulmonary and systemic inflammation markers in patients with chronic obstructive pulmonary disease: A simultaneous, two-compartmental analysis. Arch Bronconeumol 2016; 52: 361-367.
36. Shao Q, Zhao J. [Analysis of systemic cytokine levels in silicosis complicated with pulmonary infection]. Zhonghua Lao Dong Wei Sheng Zhi Ye Bing Za Zhi Zhonghua Laodong Weisheng Zhiyebing Zazhi Chin. J Ind Hyg Occup Dis 2021; 39: 133-136.
37. Volobaev VP, Larionov AV, Kalyuzhnaya EE, Serdyukova ES, Yakovleva S, Druzhinin VG, et al. Associations of polymorphisms in the cytokine genes IL1β (rs16944), IL6 (rs1800795), IL12b (rs3212227) and growth factor VEGFA (rs2010963) with anthracosilicosis in coal miners in Russia and related genotoxic effects. Mutagenesis 2018: 33: 129-135.
38. Zhai R, Liu G, Yang C, Huang C, Wu C, Christiani DC. The G to C polymorphism at -174 of the interleukin-6 gene is rare in a Southern Chinese po-

136 *Andújar-Vera F, García-Fontana C, González-Salvatierra S, Martínez-Heredia L, Muñoz-Torres M, García-Fontana B* ORIGINALS

Rev Osteoporos Metab Miner. 2021;13(4):130-136

pulation. Pharmacogenetics 2001; 11: 699-701.

39. Yu T, You X, Zhou H, Kang A, He W, Li Z, et al. p53 plays a central role in the development of osteoporosis. Aging 2020; 12: 10473-10487.

40. Gu C, Li Y, Liu J, Ying X, Liu Y, Yan J, et al. LncRNA‑mediated SIRT1/FoxO3a and SIRT1/p53 signaling pathways regulate type II alveolar epithelial cell senescence in patients with chronic obstructive pulmonary disease. Mol Med Rep 2017; 15: 3129-3134.

41. Sementino E, Menges CW, Kadariya Y, Peri S, Xu J, Liu Z, et al. Inactivation of Tp53 and Pten drives rapid development of pleural and peritoneal malignant mesotheliomas. J Cell Physiol 2018; 233: 8952-8961.

42. Sementino E, Menges CW, Kadariya Y, Peri S, Xu J, Liu Z, et al. Inactivation of Tp53 and Pten drives rapid development of pleural and peritoneal malignant mesotheliomas. J Cell Physiol 2018; 233: 8952-8961.

43. Fuchs S, Dohle E, Kirkpatrick CJ. Chapter twenty-two - sonic hedgehog-mediated synergistic effects guiding angiogenesis and osteogenesis. In Vitamins & Hormones (ed. Litwack, G.) vol. 88 491–506. Academic Press, 2012.

44. Prockop DJ. New Targets for Osteoporosis. N Engl J Med 2012; 367: 2353-2354.

45. Scagliotti GV, Gaafar R, Nowak AK, Reck M, Tsao AS, van Meerbeeck J, et al. LUME-meso: Design and rationale of the phase III part of a placebo-controlled study of nintedanib and pemetrexed/cisplatin followed by maintenance nintedanib in patients with unresectable malignant pleural mesothelioma. Clin Lung Cancer 2017; 18: 589-593.

46. Demirag F, Ünsal E, Yilmaz A, Cağlar A. Prognostic significance of vascular endothelial growth factor, tumor necrosis, and mitotic activity index in malignant pleural mesothelioma. Chest 2005; 128: 3382-3387.

47. Ohta Y, Shridhar V, Bright RK, Kalemkerian GP, Du W, Carbone M, et al. VEGF and VEGF type C play an important role in angiogenesis and lymphangiogenesis in human malignant mesothelioma tumours. Br J Cancer 1999; 81: 54-61).

48. Edwards JG, Swinson DEB, Jones JL, Muller S, Waller DA, O'Byrne KJ. Tumor necrosis correlates with angiogenesis and is a predictor of poor prognosis in malignant mesothelioma. Chest 2003; 124: 1916-1923.

49. Chia PL, Russell P, Asadi K, Thapa B, Gebski V, Murone C, et al. Analysis of angiogenic and stromal biomarkers in a large malignant mesothelioma cohort. Lung Cancer Amst Neth 2020; 150: 1-8.

Muscle strength as a predictor of bone fragility in patients with type 2 diabetes mellitus

Hayón-Ponce M[1], García-Fontana B[1,2,3], Avilés-Pérez MD[1,2], González-Salvatierrra S[2,4], Moratalla-Aranda E[2,5], Muñoz-Torres M[1,2,3,6]
1 Unit of Bone Metabolism, Endocrinology and Nutrition. San Cecilio Clinical University Hospital. Granada (Spain)
2 Biosanitary Research Institute of Granada (IBS.GRANADA) (Spain)
3 CIBERFES, Carlos III Health Institute. Madrid Spain)
4 Foundation for Biosanitary Research of Eastern Andalusia (FIBAO). Granada (Spain)
5 Nuclear Medicine. San Cecilio Clinical University Hospital. Granada (Spain)
6 Department of Medicine. University of Granada (Spain)

Summary

Introduction: Most studies have shown a decrease in muscle function and strength in patients with type 2 diabetes mellitus (DM2). However, the relationship between muscle function and bone health in patients with DM2 is not well defined.
Objective: The objective of this study was to analyze the relationship between muscle strength and bone fragility in patients with DM2.
Material and methods: This observational cross-sectional study included 60 patients with DM2 (60% men and 40% postmenopausal women) ranging in age from 49 to 85 years. Demographic, anthropometric, clinical and biochemical variables were studied. Bone mineral density (BMD) in the lumbar spine (LS), femoral neck and total hip was determined using DXA (Hologic QDR 4500), and TBS values (TBS iNsight Software, version 3.0.2.0, Medimaps, Merignac, France). Hand grip (kg/cm^2) was measured with a Jamar® manual hydraulic dynamometer (5030j1; Jackson, MI). To assess the level of mobility and the risk of falls, the Time Up and Go test was carried out. Statistical analysis was performed using the SPSS program (SPSS, inc, v 25.0).
Results: The mean age of the patients was 66.3±8.3 years. The mean HbA1c was 7.7±1.1%, with inadequate glycemic control (HbA1c >7.5%) observed in 73.3% of the patients. 91.7% of the women and 77.8% of the men had low muscle strength. 41.7% of women and 25% of men presented a high risk of falls. Subjects with low hand grip strength and those with high risk of falls had significantly lower TBS values than those with greater hand grip strength (0.99±0.17 vs 1.12±0.15; p=0.03) and low risk of falls (0.94±0.13 vs 1.04±0.19; p=0.02). Patients with normal and partially degraded TBS had greater hand grip strength than subjects with degraded TBS (p=0.031). Hand grip strength was positively associated with TBS (p<0.05) regardless of age, waist circumference, 25OH vitamin D levels, and BMD in LS. There were no significant differences in hand grip strength as a function of BMD values.
Conclusion: Our study shows that the reduction in muscle strength may be related to bone microarchitecture deterioration determined by TBS in patients with DM2.

Key words: type 2 diabetes mellitus, hand strength, bone fragility, Trabecular bone score, bone densitometry.

INTRODUCTION

Type 2 diabetes mellitus (DM2) and osteoporosis are highly prevalent diseases due to the aging of the population that are associated with an increased risk of fragility fractures that substantially increase the morbidity and mortality of the population[1]. Recently, sarcopenia, defined as muscle weakness related to aging, has been recognized as a complication of DM2 that often increases these patients' frailty[2,3].

The musculoskeletal system is closely related, both by its physical connection and by its regulation through multiple common elements. Although muscle has been found to exert an influence on bone through the neuroendocrine system and mechanical forces, their relationship is complex and not entirely well known[4]. Many studies have shown a link between sarcopenia and osteoporosis, but the results are inconsistent due to variable diagnostic criteria and divergent assessment methods for sarcopenia and osteoporosis.

▶▶▶ **Correspondence:** Beatriz García Fontana (bgfontana@fibao.es)

138 Hayón-Ponce M, García-Fontana B, Avilés-Pérez MD, González-Salvatierrra S, Moratalla-Aranda E, Muñoz-Torres M   ORIGINALS

Rev Osteoporos Metab Miner. 2021;13(4):137-144

Most studies have focused on evaluating the relationship between muscle mass and bone mineral density (BMD), while only a few have assessed the effect of sarcopenia on bone quality[5,6]. However, around two thirds of patients with fractures do not present osteoporosis as defined by BMD values[7]. Patients with DM2 have an increased risk of fracture despite having preserved or even increased BMD[1]. These results suggested that BMD alone is insufficient to assess bone strength and estimate fracture risk.

Trabecular bone score (TBS) is a method for assessing the microarchitecture of the trabecular bone using TBS iNsight Software (Medimaps, Merignac, France). It is a simple application technique that permits microstructural analysis of the trabecular bone on DXA scan images of the lumbar spine with a new technological approach on the variation of image texture[8]. Studies carried out to date have shown that the determination of TBS can predict the risk of fracture independently and in addition to BMD both in the general population[9] and in patients with DM2[10].

Sarcopenia was first defined by Rosenberg IH in 1988 as an aging-related loss of muscle mass and function[11]. The state prior to sarcopenia is "dynapenia", defined by Clark BC and Manini TM as a decrease in muscle strength related to aging before the reduction of muscle mass[12], determined through the evaluation of the knee extension strength and hand grip strength.

Our objective was to investigate the relationship between the components of dynapenia determined by hand strength, and bone fragility determined by BMD and bone microarchitecture measured by DXA and TBS in subjects with DM2.

MATERIAL AND METHODS

Study population

A cross-sectional observational study was carried out in which 60 patients with DM2 (60% men and 40% postmenopausal women) between 49 and 85 years old, recruited consecutively from 2016 to 2018 in the reference area of the San Cecilio of Granada Clinical University Hospital.

The exclusion criteria included the presence of other conditions that alter bone metabolism such as the diagnosis of non-osteoporotic metabolic bone disease, chronic diseases, such as rheumatoid arthritis, chronic liver and kidney diseases and active neoplastic diseases, as well as hormone replacement therapy and glucocorticoid or antiosteoporotic treatment.

The study was carried out with the approval of the Ethics Committee of the San Cecilio Clinical University Hospital in accordance with the code of ethics of the World Medical Association (Declaration of Helsinki). Written informed consent was obtained from all study subjects.

Clinical evaluation

Height, weight, and waist circumference (WC) were measured in all patients. Body mass index (BMI) was calculated as weight (kg)/height (m²).

The percentage of total body fat was estimated using a linear anthropometric equation called relative fat mass (RFM) applying the following equation: 64 - (20 x height/ waist circumference) + (12 x sex); sex = 0 for men and 1 for women[13].

The manual hand grip force (kg/cm²) was measured with a Jamar® hydraulic manual dynamometer (5030j1;

Jackson, MI) three times for each hand with the patient seated and the arm resting on a table holding the dynamometer in a vertical position[14], using the average value of these measurements to represent the strength of the hand. Hand grip strength values <27 kg (men) and <16 kg (women) were defined as low muscle strength[15].

To assess the level of mobility and the risk of falls, the Time Up and Go (TUG) test was carried out, which determines the time needed to get up from a chair, walk to a mark located at 3 meters, turn around and sit down again in the chair. A score of less than 12 seconds was defined as low risk of falling and greater than 12 seconds as high risk of falling[16].

Participants completed specific health questionnaires, which included their medical history, drug treatment, tobacco and alcohol use and level of physical activity.

Biochemical determinations

Blood samples were collected from all patients in the morning after an 8 hour overnight fast.

Serum levels of albumin, calcium, phosphorus, creatinine, alkaline phosphatase, insulin, fasting plasma glucose, albumin, and lipid profile (triglyceride and cholesterol levels (high- and low-density lipoproteins)) were measured by standard biochemical methods.

Glycated hemoglobin (HbA1c) was determined by high performance liquid chromatography (ADAMS A1c, HA-8160; Menarini, Florence, Italy) and was expressed as a percentage.

Bone densitometry and TBS

BMD (grams/cm²) in the lumbar spine (LS), femoral neck (FN) and total hip (TH) was determined using a Hologic QDR 4500 densitometer (Whatman, MA) with a coefficient of variation of 1.70%, 1.80% and 1.50% for LS, FN and TH, respectively. The diagnosis of osteoporosis was made based on World Health Organization criteria[17].

TBS was measured in LS using TBS iNsight software version 3.0.2.0 (Medimaps, Merignac, France) with a coefficient of variation of 1.82%. The bone microarchitecture classification was based on the following TBS ranges: TBS greater than or equal to 1.31 corresponded to normal microarchitecture, TBS between 1.23 and 1.31 was defined as partially degraded microarchitecture and TBS equal to or less than 1.23 as degraded microarchitecture[18].

Statistic analysis

Continuous variables were expressed as mean ± standard deviation (SD), while categorical variables were expressed as absolute (n) and relative (%) frequencies. The normality of the variables was analyzed using the Kolmogorov-Smirnov test. The difference between continuous variables was determined by Student's t test. The Chi-square test was used to compare the categorical variables. Pearson's correlation coefficient was used to evaluate linear relationships. Values of $p<0.05$ were considered significant. Multiple linear regression analysis was used to test the association between TBS and variables that influence bone quality, adjusted for other possible confounding factors. P values <0.10 were considered significant.

Statistical analysis was performed using the SPSS statistical program (version 25.0; SPSS, Chicago, IL, USA).

RESULTS

Table 1 shows the characteristics of the study subjects according to sex. The consumption of tobacco and alcohol, as well as cardiovascular disease and the time of evolution of DM2 was greater in men than in women, while women presented higher levels of total cholesterol and LDL cholesterol. Men presented lower RFM values and higher hand grip strength and BMD values in LS and TBS than women.

91.7% of the women and 77.8% of the men had low muscle strength. The prevalence of low muscle strength was analyzed by age group, quartiles of BMI and WC (figure 1). When the prevalence of dynapenia was evaluated by age group, we observed a progressive increase with age (figure 1a). The prevalence of low hand grip strength was higher in the first and fourth quartiles of BMI. The prevalence of low muscle strength by quartiles of WC is shown in figure 1c, the group of the fourth quartile was the one that showed the highest prevalence of low hand grip strength.

According to the results obtained in the TUG test, 25% of men and 41.7% of women had a high risk of falls. Patients with high risk of falls showed significantly lower hand grip strength values than those with low risk of falls (13.8±7.4 vs 18.7±8.1; p=0.027).

81.7% of the patients with DM2 had a degraded microarchitecture (TBS ≤1.23), 13.3% had partially degraded microarchitecture (TBS between 1.23 and 1.31) and 5% had normal values of TBS (TBS ≥1.31). According to the BMD values in LS, 3.7% of the patients were classified in the range of osteoporosis (T-score ≤2.5), 37% in that of osteopenia and 59.3% had a BMD in LS normal. Subjects with normal and partially degraded TBS presented greater hand grip strength than subjects with degraded TBS (p=0.031). However, there were no significant differences in hand grip strength between subjects with osteopenia/osteoporosis compared to those with normal BMD.

Mean DM2 evolution was 14.9±8.7 years with inadequate glycemic control in 73.3% of the patients. Patients with a high risk of falls had longer term DM2 evolution than those with a low risk of falls (18.1±8.9 vs 13.1±8.1 p=0.037). Patients with adequate metabolic control (HbA1c <7.5%) showed greater hand strength, although no significant differences were observed with respect to the group with worse metabolic control. No significant association was observed between insulin treatment (66.7%) and the risk of falls or the presence of fractures.

Simple correlation analysis showed a significant positive association of weight with BMD in LS (r=0.372; p<0.01), FN (r=0.430; p<0.01) and TH (r=0.547; p<0.01) and negative with TBS (r=-0.369; p<0.01). Both WC and BMI were significantly associated with BMD in TH (positive) (r=0.464; p<0.01) and (r=0.418; p<0.01), respectively, and with TBS (negative) (r=-0.402; p<0.01) and (r=-0.624; p<0.001). RFM was negatively correlated with TBS in both men (r=-0.620; p<0.001) and women (r=-0.410; p<0.05) while hand strength was positively associated with TBS (r=0.326; p<0.05). Although we did not observe a significant correlation between BMI, WC and RFM with the manual grip strength, we did find a significant progressive decrease in the grip strength of the hand with the increase in the quartiles of RFM (p<0.001) (figure 2).

Given the significant relationship between bone quality measured by TBS and hand grip strength, a subgroup analysis of hand grip strength was carried out.

Subjects with low hand grip strength had significantly lower TBS values than those with greater hand strength (0.99±0.17 vs 1.12±0.15; p=0.03). Although there was no significant correlation between the TUG test and TBS, lower TBS values were also observed in subjects with high risk of falls versus those with low risk of falls (0.94±0.13 vs 1.04±0.19; p=0.02) (figure 3). There were no significant differences in BMD values in both groups. As shown in figure 4a, mean values of hand grip strength showed a progressive decrease according to the TBS categories. Furthermore, the percentage of patients presenting degraded TBS (TBS <1.23) showed a decreasing trend with increasing quartiles of hand grip strength (figure 4b).

To determine the variables that influence the TBS (dependent variable), a multiple linear regression analysis was performed adjusting for the effect of age, WC, manual grip strength, levels of 25 (OH) vitamin D and BMD in LS. Our results showed that WC (B=-0.491, [-0.013-/-0.004], p=0.001) negatively affect TBS values while hand grip strength (B=0.284, [0.000-0.013], p=0.038) exerts a positive effect on the bone microarchitecture determined by TBS.

DISCUSSION

Loss of muscle mass and strength, called sarcopenia and dynapenia respectively, has recently been recognized as a complication associated with diabetes mellitus[3]. Mori, et al. observed that the prevalence rate of dynapenia was higher than sarcopenia in patients with DM2[19]. The European Working Group on Sarcopenia in the Elderly (EWGSOP)[15] and the Asian Working Group on Sarcopenia (AWGS)[20] consider hand grip strength as a simple method that reliably predicts deterioration in muscular function. The prevalence of sarcopenia in DM2 varies between 5% and 50% in the different studies carried out to date[21]. In a recently published meta-analysis, it were patients with DM2 reportedly had lower muscle strength than non-diabetic patients, despite no difference in muscle mass[22]. In our study, the prevalence of low muscle strength was 83.3 %.

When analyzing the prevalence of low hand grip strength, we observed a progressive decrease with increasing age. Not surprisingly, increasing age represents a risk factor for low muscle strength in DM2, as well as in the general population, due to age-related decline in muscle mass and strength. However, it would be interesting to know if the age factor could be more decisive for the development of dynapenia and sarcopenia in individuals with DM2 compared to non-diabetic patients.

In this regard, Tamura, et al. did not show differences in the risk of sarcopenia according to age categories between individuals with and without DM2[23]. Similarly, Çeliker, et al. and Trierweiler, et al. observed a higher prevalence of sarcopenia in individuals with DM2 compared to those without DM2, while no significant differences were observed in terms of age[24,25].

In previous studies, too low or too high a BMI and a high percentage of body fat have been associated with higher mortality[26]. According to the results of the body composition analysis, the prevalence of low muscle strength was higher in the first and fourth quartiles of BMI. However, the evaluation of the relative fat mass showed a significant progressive decrease in the manual grip strength with the increase of the quartiles of RFM. This finding suggests that diabetic patients with a high

140   *Hayón-Ponce M, García-Fontana B, Avilés-Pérez MD, González-Salvatierrra S, Moratalla-Aranda E, Muñoz-Torres M*   ORIGINALS

Rev Osteoporos Metab Miner. 2021;13(4):137-144

Table 1. Demographic characteristics and clinical variables

	Total (n=60)	Man (n=36)	Woman (n=24)	p
Age (years)	66.35±8.09	66.19±7.62	66.58±8.92	0.857
Weight (Kg)	83.89±13.06	86.37±10.68	80.18±15.50	0.072
Size (cm)	165.09±8.25	169.54±6.32	158.42±6.03	<0.001*
BMI (kg/m^2)	30.80±4.60	30.11±3.95	31.86±5.36	0.152
WC (cm)	103.83±11.44	104.60±9.42	102.95±13.58	0.635
RFM (%)	69.28±6.06	63.67±0.03	75.69±0.04	<0.001*
Smoker (%)	56.7	75.0	29.2	<0.001*
Alcohol (%)	23.3	33.3	8.3	0.025*
Sedentary lifestyle (%)	11.8	13.9	4.5	0.163
Hypertension (%)	83.3	86.1	79.2	0.480
Dyslipidemia (%)	88.3	88.9	87.5	0.870
Overweight/Obesity (%)	86.7	88.9	83.4	0.535
Cardiovascular disease (%)	45.0	63.9	16.7	<0.001*
Microvascular complications (%)	33.3	41.7	20.8	0.094
Falls (%)	30.0	25.0	37.5	0.301
Fractures (%)	13.3	11.1	16.7	0.535
Basal glucose (mg/dl)	147.97±52.27	144.08±51.03	153.79±54.66	0.486
HbA1c (%)	7.70±1.13	7.64±1.24	7.79±0.96	0.612
Time evolution of DM2 (years)	14.93±8.68	16.81±9.21	12.13±7.10	0.031*
Total cholesterol (mg/dl)	162.65±44.06	150.33±39.06	181.13±45.44	0.007*
HDLc (mg/dl)	45.35±12.19	42.14±10.36	50.17±13.33	0.054
LDLc (mg/dl)	87.83±36.84	80.36±32.66	99.04±40.48	0.01*
Triglycerides (mg / dl)	155.02±74.55	146.61±71.84	167.63±78.26	0.289
Hand grip (Kg)	17.29±8.12	22.50±5.62	9.47±3.82	<0.001*
TUG (s)	11.74±4.35	10.39±2.75	13.70±5.45	0.01*
BMD_LS (gr/cm^2)	1.07±0.22	1.12±0.23	0.99±0.16	0.032*
BMD_FN (gr/cm^2)	0.82±0.15	0.84±0.14	0.78±0.17	0.157
BMD_TH (gr/cm^2)	1.04±0.17	1.07±0.17	0.99±0.18	0.082
TBS	1.01±0.18	1.06±0.17	0.94±0.17	0.012*

RFM: relative fat mass; BMI: body mass index; WC: waist circumference; HbA1c: glycated hemoglobin; HOMA2-IR: insulin resistance index; HDL: high-density lipoprotein; LDL: low-density lipoprotein; TUG: test Time Up and Go; TBS: Trabecular bone score; BMD: bone mineral density; LS: lumbar spine; FN: femoral neck; TH: total hip; SD: standard deviation. Continuous variables are expressed as mean ± SD. Categorical variables are expressed as percentages. P values were determined using Student's t test for continuous variables and the Chi-square test for categorical variables. * Significance level <0.05.

percentage of body fat and a low or too high BMI present an increased risk of developing dynapenia. Furthermore, our results showed an inverse association between WC and TBS, suggesting a predictive role for WC regardless of age, hand grip strength, 25(OH) vitamin D levels and BMD in LS. These results support the role of central fat mass in bone microarchitecture determined by TBS[27,28]. Therefore, the assessment of obesity in diabetic patients should not focus solely on BMI. Rather, it should be considered in combination with body fat mass.

Although the link between sarcopenia, risk of falls, and risk of fractures has been studied in the general population, there are few studies that have examined the clinical impact of sarcopenia and bone fragility in patients with DM2. Our study analyzed the relationship between hand strength and bone fragility measured by BMD determined by DXA and the bone microarchitecture estimated by TBS in patients with DM2.

There are inconsistent results regarding the relationship between hand grip strength and BMD. Aydin's group concluded that hand grip strength was not a good predictor of BMD in men[29] while Pereira's group did observe a significant correlation between hand grip strength and BMD[30]. In our study, we did not observe an association between hand grip strength and BMD.

Most of the studies that have evaluated the relationship between sarcopenia and bone quality have used data derived from invasive techniques such as quantitative computed tomography (QCT), presenting a limitation in routine clinical practice due to its low availability[31,32]. TBS is a non-invasive technique for evaluating bone quality that can predict the risk of fracture independently and in addition to BMD in patients with DM2[10]. In our study, we observed a positive relationship between hand grip strength and TBS values. Subjects with lower hand grip strength had lower TBS values and a decreasing trend was observed in the prevalence of degraded TBS (TBS <1.23) with increasing hand grip strength quartiles. In agreement, our results showed a positive association between TBS and manual grip strength independent of the effect of age, WC, levels of 25 (OH) vitamin D and BMD in LS in the multivariate analysis. These findings suggest that the measurement of manual grip strength could be an easily implemented strategy to estimate the state of bone microarchitecture in clinical practice.

Our results concur with the study carried out by Hanmei's group, which showed that TBS was positively correlated with hand grip strength in women[33] and with the STRAMBO study, which showed that bone size and not bone size BMD seemed to correlate mainly with muscle mass, while bone microarchitecture was mainly correlated with muscle strength[32]. Our results confirm previous data and suggest that muscle strength has a greater influence on bone quality than on BMD and may reflect a deterioration of bone microarchitecture more reliably than bone status measured by BMD. Therefore, low muscle strength could be a good predictor of bone fragility measured by TBS in patients with DM2.

Both sarcopenia and dynapenia increase the risk of falls in patients with DM2[19]. The TUG test assesses the level of mobility and the risk of falls and is an indicator of severe sarcopenia[15]. Lower TBS values and hand strength in patients with a higher risk of falls suggests that the higher risk of fragility fractures in these patients could be related to the coexistence of severe sarcopenia and deterioration of the microarchitecture trabecular

Figure 1. Prevalence of low hand grip strength: a) Evaluation of low hand grip strength by age; b) Evaluation of low hand grip strength by BMI quartiles; c) Evaluation of low manual grip strength by quartiles of WC
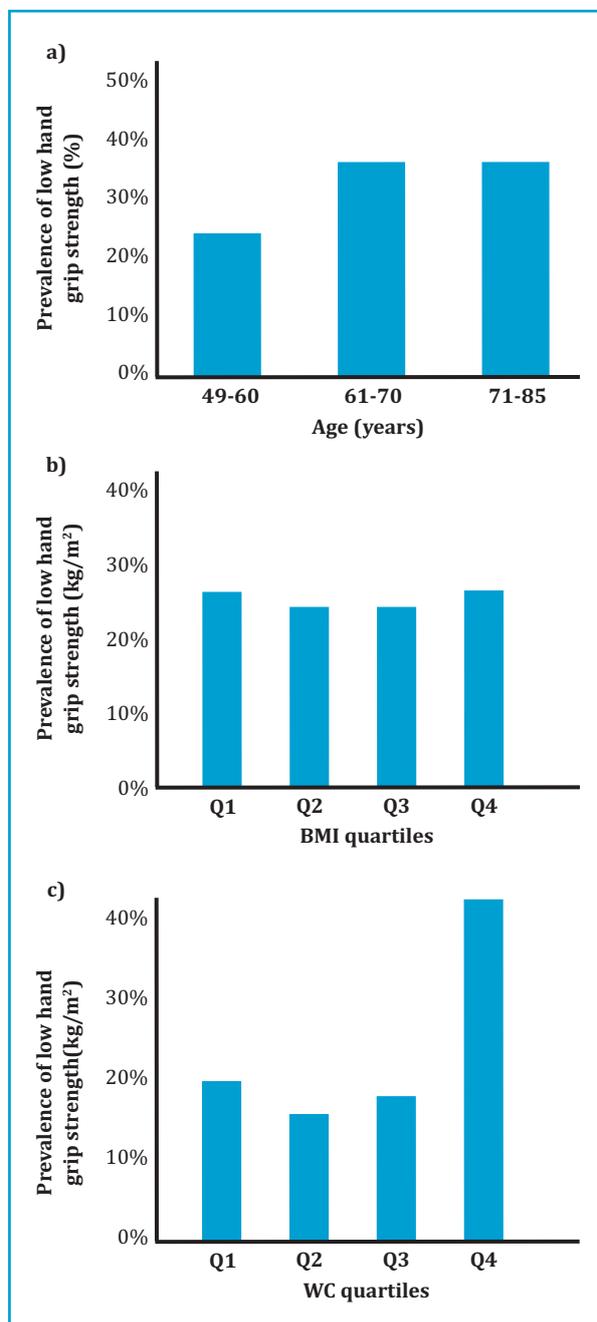
BMI: body mass index; Q1, first quartile, <27.9 kg/m^2; Q2, second quartile, 27.9-30.7 kg/m^2; Q3, third quartile, 30.8-33.9 kg/m^2: Q4, fourth quartile >33.9 kg/m^2. WC: waist circumference; Q1, first quartile, <96 cm; Q2, second quartile, 96-104 cm; Q3, third quartile, 104.1-111.5 cm; Q4, fourth quartile >111.5 cm.

bone despite increased BMD.

The etiology of the effect of DM2 on the musculoskeletal system is multifactorial and not entirely well known[34]. Previous studies suggest that a longer duration of diabetes and sustained hyperglycemia affect muscle weakness in patients with DM2[35]. In our study, patients with a high risk of falls had a greater evolution of DM2 than those with a low risk of falls. However, we did not observe significant differences in the time of evolution of

142 *Hayón-Ponce M, García-Fontana B, Avilés-Pérez MD, González-Salvatierrra S, Moratalla-Aranda E, Muñoz-Torres M* ORIGINALS

Rev Osteoporos Metab Miner. 2021;13(4):137-144

Figure 2. Hand strength and quartiles of RFM
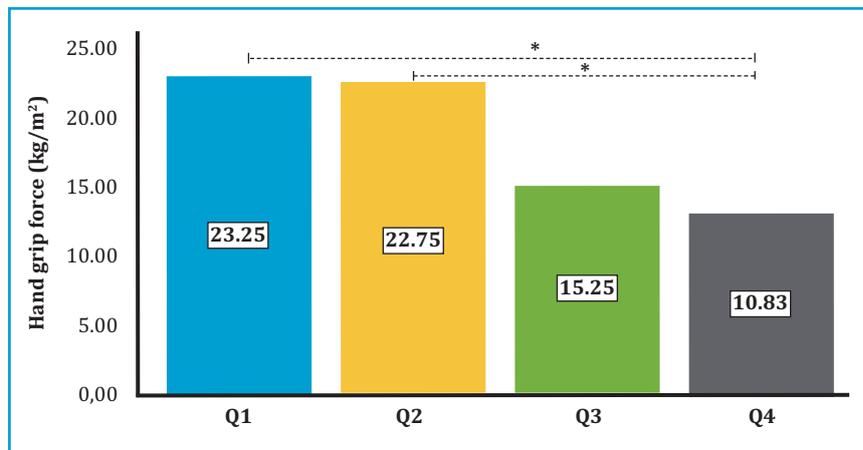
Hand grip force (kg/cm^2) was measured with a Jamar® RFM hydraulic hand dynamometer: relative fat mass. Q1, first quartile, <63.6; Q2, second quartile, 63.6-63.7; Q3, third quartile, 63.8-75.7; Q4, fourth quartile >75.7. * Significance level <0.01.

Figure 3. Effect of hand strength and risk of falls on TBS
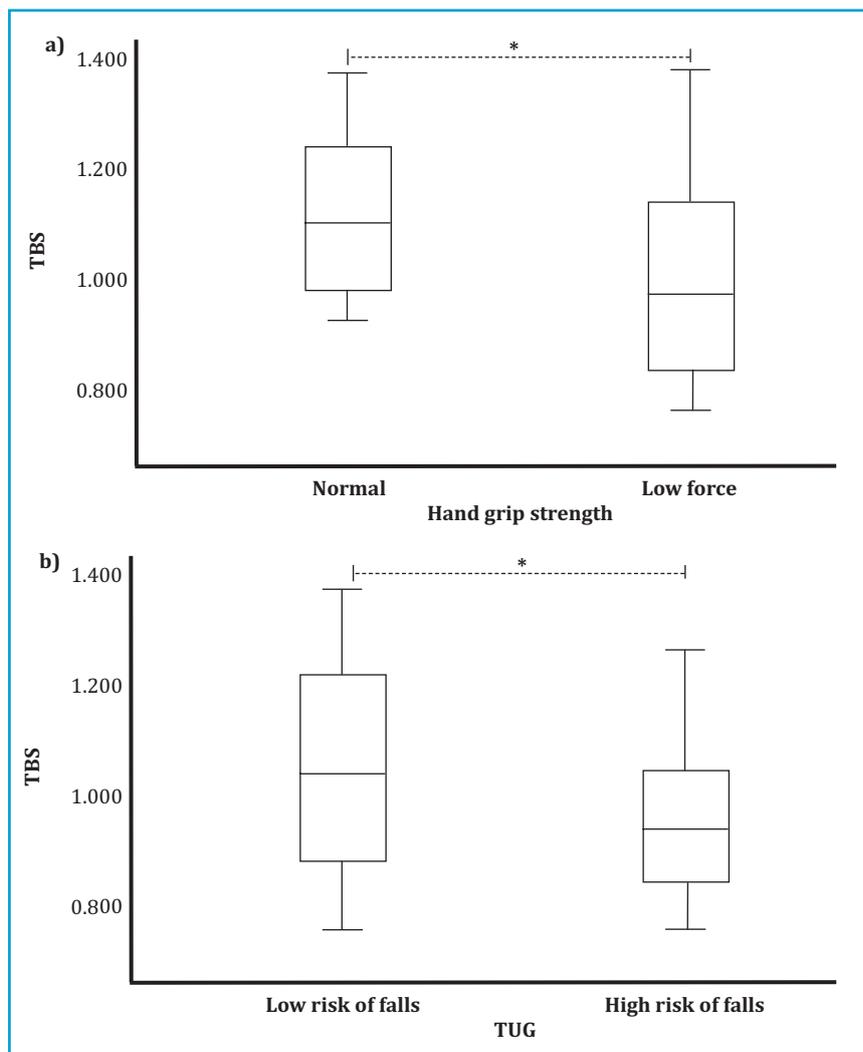
Hand grip force (kg/cm^2) was measured with a Jamar® hydraulic hand dynamometer. Hand grip strength values <27 kg (men) and <16 kg (women) were defined as low muscle strength. The risk of falls was assessed using the Time up and Go test. A score of less than 12 seconds was defined as low risk of falling and greater than or equal to 12 seconds, high risk of falling. TUG: test Time Up and Go; TBS: Trabecular bone score. P values were determined using Student's t test. * Significance level <0.05.

the disease between subjects with normal or decreased muscle strength. Some studies have reported a higher prevalence of sarcopenia associated with longer term diabetes[36]. However, other studies have not found a relationship between the prevalence of sarcopenia and the time of DM2 evolution[22].

A recent study has shown that hyperglycemia itself reduces muscle mass through increased KLF15 in myocytes[37]. On the other hand, Kalyani, et al. observed that HbA1c is associated with weakness in muscle strength independent of muscle mass[35]. In agreement, our results showed that patients with adequate metabolic control (HbA1c <7.5%) presented greater muscle strength in the hand, although no significant differences were observed between both groups, probably due to the limited number of patients included in the study. However, previous studies have not found a relationship between metabolic control and muscle strength[38]. Therefore the prevention of the development of dynapenia and sarcopenia cannot be exclusively focused on metabolic control, in older patients, especially in elderly patients. Other factors must be taken into account, such as the presence of micro and macrovascular complications, body composition, nutritional status, and life expectancy, which determine morbidity and mortality.

Our study has certain limitations. First, the cross-sectional design of the study allowed investigating the association between study variables, but not causality. Second, the sample size was relatively small; however, our participants are representative of patients with DM2 in daily clinical practice. Third, we did not assess muscle mass, which is a determinant of sarcopenia, although when the components of sarcopenia have been examined individually in other studies, only low muscle strength was associated with recurrent incidence of falls, independent of muscle mass or gait speed[39].

Despite these limitations, to our knowledge, the present study is the first to investigate

ORIGINALS   _Muscle strength as a predictor of bone fragility in patients with type 2 diabetes mellitus_
Rev Osteoporos Metab Miner. 2021;13(4):137-144

143

Figure 4. a) Mean values of manual pressure force according to the TBS categories. b) Prevalence of degraded bone microarchitecture (TBS ≤1.23) according to quartiles of hand grip strength
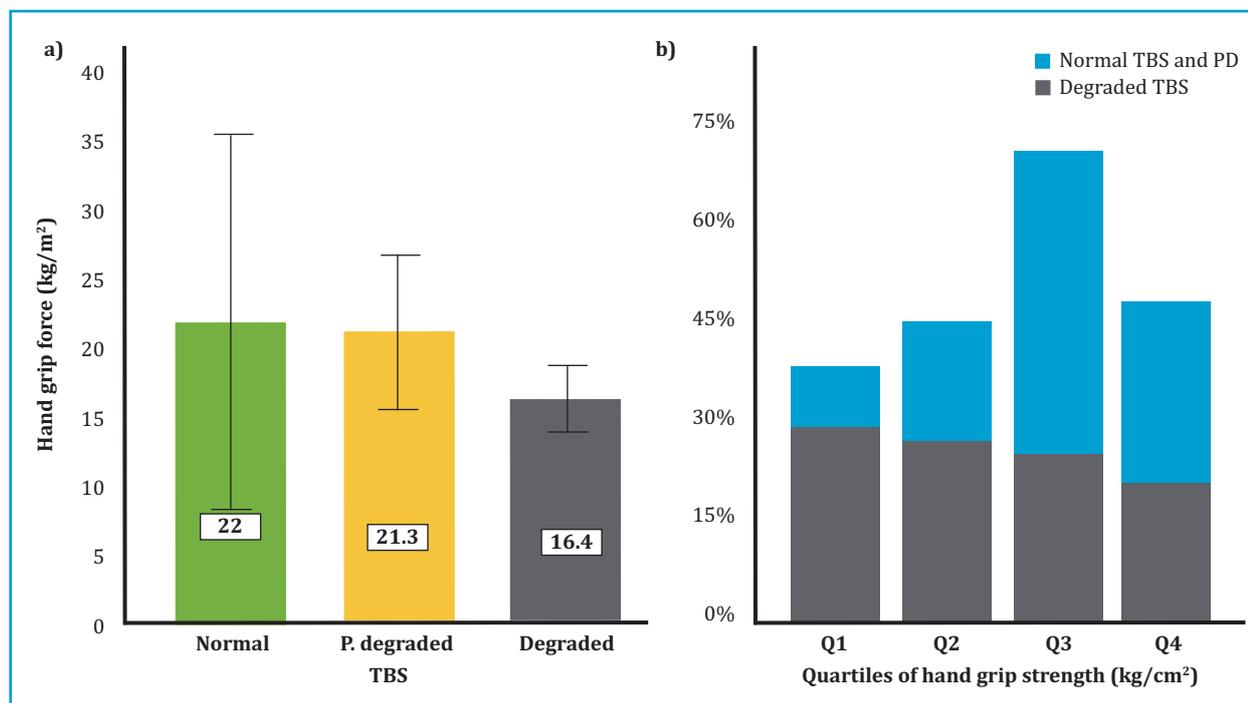
Hand grip force (kg/cm^2) was measured with a Jamar® hydraulic hand dynamometer. TBS: Trabecular bone score; PD: partially degraded. The classification of bone microarchitecture was based on the following TBS ranges: TBS ≥1.31 corresponded to normal microarchitecture, TBS between 1.23 and 1.31 was defined as partially degraded microarchitecture and TBS ≤1.23 as degraded microarchitecture. Q1, first quartile, <9.5 kg/cm^2; Q2, second quartile, 9.5-19.7 kg/cm^2; Q3, third quartile, 19.8-23.5 kg/cm^2; Q4, fourth quartile >23.5 kg/cm^2.

the association between manual grip strength and TBS in patients with DM2.

In conclusion, our study suggests that patients with DM2 present a high prevalence of age-related decrease in muscle strength. The results of the body composition analysis highlight the importance of assessing fat mass rather than assessing BMI in diabetic patients at risk of sarcopenia. A low or too high BMI and a high percentage of body fat tend to increase the risk of developing dynapenia. The TBS in patients with DM2 is usually low despite an increased BMD. Muscle strength was significantly associated with a deterioration in bone microarchitecture. The decrease in manual grip strength and the TUG test can be an easily applied tool in routine clinical practice to identify patients with DM2 at risk of falls and osteoporotic fractures.

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

144 Hayón-Ponce M, García-Fontana B, Avilés-Pérez MD, González-Salvatierrra S, Moratalla-Aranda E, Muñoz-Torres M   ORIGINALS

Rev Osteoporos Metab Miner. 2021;13(4):137-144

Bibliography

1. Leslie WD, Rubin MR, Schwartz AV, Kanis JA. Type 2 diabetes and bone. J Bone Miner Res. 2012;27(11):2231-7.

2. Park SW, Goodpaster BH, Strotmeyer ES, Kuller LH, Broudeau R, Kammerer C, et al. Accelerated loss of skeletal muscle strength in older adults with type 2 diabetes: the health, aging, and body composition study. Diabetes Care. 2007;30(6):1507-12.

3. Park SW, Goodpaster BH, Lee JS, Kuller LH, Boudreau R, de Rekeneire N, et al. Excessive loss of skeletal muscle mass in older adults with type 2 diabetes. Diabetes Care. 2009;32(11):1993-7.

4. Tagliaferri C, Wittrant Y, Davicco MJ, Walrand S, Coxam V. Muscle and bone, two interconnected tissues. Ageing Res Rev. 2015;21:55-70.

5. Patil R, Uusi-Rasi K, Pasanen M, Kannus P, Karinkanta S, Sievanen H. Sarcopenia and osteopenia among 70-80-year-old home-dwelling Finnish women: prevalence and association with functional performance. Osteoporos Int. 2013;24(3):787-96.

6. Tarantino U, Piccirilli E, Fantini M, Baldi J, Gasbarra E, Bei R. Sarcopenia and fragility fractures: molecular and clinical evidence of the bone-muscle interaction. J Bone Joint Surg Am. 2015;97(5):429-37.

7. Boutroy S, Hans D, Sornay-Rendu E, Vilayphiou N, Winzenrieth R, Chapurlat R. Trabecular bone score improves fracture risk prediction in non-osteoporotic women: the OFELY study. Osteoporos Int. 2013;24(1):77-85.

8. Silva BC, Leslie WD, Resch H, Lamy O, Lesnyak O, Binkley N, et al. Trabecular bone score: a noninvasive analytical method based upon the DXA image. J Bone Miner Res. 2014;29(3):518-30.

9. Leslie WD, Johansson H, Kanis JA, Lamy O, Oden A, McCloskey EV, et al. Lumbar spine texture enhances 10-year fracture probability assessment. Osteoporos Int. 2014;25(9):2271-7.

10. Leslie WD, Aubry-Rozier B, Lamy O, Hans D, Manitoba Bone Density P. TBS (trabecular bone score) and diabetes-related fracture risk. J Clin Endocrinol Metab. 2013;98(2):602-9.

11. Rosenberg IH. Sarcopenia: origins and clinical relevance. J Nutr. 1997;127(5 Suppl):990S-1S.

12. Manini TM, Clark BC. Dynapenia and aging: an update. J Gerontol A Biol Sci Med Sci. 2012;67(1):28-40.

13. Woolcott OO, Bergman RN. Relative fat mass (RFM) as a new estimator of whole-body fat percentage horizontal line A cross-sectional study in American adult individuals. Sci Rep. 2018;8(1):10980.

14. Watanabe T, Owashi K, Kanauchi Y, Mura N, Takahara M, Ogino T. The short-term reliability of grip strength measurement and the effects of posture and grip span. J Hand Surg Am. 2005;30(3):603-9.

15. Cruz-Jentoft AJ, Bahat G, Bauer J, Boirie Y, Bruyere O, Cederholm T, et al. Sarcopenia: revised European consensus on definition and diagnosis. Age Ageing. 2019;48(4):601.

16. Bischoff HA, Stahelin HB, Monsch AU, Iversen MD, Weyh A, von Dechend M, et al. Identifying a cut-off point for normal mobility: a comparison of the timed 'up and go' test in community-dwelling and institutionalised elderly women. Age Ageing. 2003;32(3):315-20.

17. Cosman F, de Beur SJ, LeBoff MS, Lewiecki EM, Tanner B, Randall S, et al. Clinician's Guide to Prevention and Treatment of Osteoporosis. Osteoporos Int. 2014;25(10):2359-81.

18. Harvey NC, Gluer CC, Binkley N, McCloskey EV, Brandi ML, Cooper C, et al. Trabecular bone score (TBS) as a new complementary approach for osteoporosis evaluation in clinical practice. Bone. 2015;78:216-24.

19. Mori H, Kuroda A, Ishizu M, Ohishi M, Takashi Y, Otsuka Y, et al. Association of accumulated advanced glycation end-products with a high prevalence of sarcopenia and dynapenia in patients with type 2 diabetes. J Diabetes Investig. 2019;10(5):1332-40.

20. Chen LK, Liu LK, Woo J, Assantachai P, Auyeung TW, Bahyah KS, et al. Sarcopenia in Asia: consensus report of the Asian Working Group for Sarcopenia. J Am Med Dir Assoc. 2014;15(2):95-101.

21. Izzo A, Massimino E, Riccardi G, Della Pepa G. A Narrative Review on Sarcopenia in Type 2 Diabetes Mellitus: Prevalence and Associated Factors. Nutrients. 2021;13(1).

22. Anagnostis P, Gkekas NK, Achilla C, Pananastasiou G, Taouxidou P, Mitsiou M, et al. Type 2 Diabetes Mellitus is Associated with Increased Risk of Sarcopenia: A Systematic Review and Meta-analysis. Calcif Tissue Int. 2020;107(5):453-63.

23. Tamura Y, Ishikawa J, Fujiwara Y, Tanaka M, Kanazawa N, Chiba Y, et al. Prevalence of frailty, cognitive impairment, and sarcopenia in outpatients with cardiometabolic disease in a frailty clinic. BMC Geriatr. 2018;18(1):264.

24. Celiker M, Selcuk MY, Olt S. Sarcopenia in diabetic nephropathy: a cross-sectional study. Rom J Intern Med. 2018;56(2):102-8.

25. Trierweiler H, Kisielewicz G, Hoffmann Jonasson T, Rasmussen Petterle R, Aguiar Moreira C, Zeghbi Cochenski Borba V. Sarcopenia: a chronic complication of type 2 diabetes mellitus. Diabetol Metab Syndr. 2018;10:25.

26. Padwal R, Leslie WD, Lix LM, Majumdar SR. Relationship Among Body Fat Percentage, Body Mass Index, and All-Cause Mortality: A Cohort Study. Ann Intern Med. 2016;164(8):532-41.

27. Moon HU, Lee N, Chung YS, Choi YJ. Reduction of visceral fat could be related to the improvement of TBS in diabetes mellitus. J Bone Miner Metab. 2020.

28. Hayon-Ponce M, Garcia-Fontana B, Aviles-Perez MD, Gonzalez-Salvatierra S, Andujar-Vera F, Moratalla-Aranda E, et al. Lower trabecular bone score in type 2 diabetes mellitus: A role for fat mass and insulin resistance beyond hyperglycaemia. Diabetes Metab. 2021;47(6):101276.

29. Aydin G, Atalar E, Keles I, Tosun A, Zog G, Keles H, et al. Predictive value of grip strength for bone mineral density in males: site specific or systemic? Rheumatol Int. 2006;27(2):125-9.

30. Pereira FB, Leite AF, de Paula AP. Relationship between pre-sarcopenia, sarcopenia and bone mineral density in elderly men. Arch Endocrinol Metab. 2015;59(1):59-65.

31. Blaizot S, Boutroy S, Vilayphiou N, Boonen S, Chapurlat R, Szulc P. Poor bone microarchitecture in older men with impaired physical performance--the STRAMBO study. Osteoporos Int. 2012;23(12):2785-96.

32. Szulc P, Blaizot S, Boutroy S, Vilayphiou N, Boonen S, Chapurlat R. Impaired bone microarchitecture at the distal radius in older men with low muscle mass and grip strength: the STRAMBO study. J Bone Miner Res. 2013;28(1):169-78.

33. Qi H, Sheng Y, Chen S, Wang S, Zhang A, Cai J, et al. Bone mineral density and trabecular bone score in Chinese subjects with sarcopenia. Aging Clin Exp Res. 2019;31(11):1549-56.

34. Botushanov NP, Orbetzova MM. Bone mineral density and fracture risk in patients with type 1 and type 2 diabetes mellitus. Folia Med (Plovdiv). 2009;51(4):12-7.

35. Kalyani RR, Metter EJ, Egan J, Golden SH, Ferrucci L. Hyperglycemia predicts persistently lower muscle strength with aging. Diabetes Care. 2015;38(1):82-90.

36. Fukuoka Y, Narita T, Fujita H, Morii T, Sato T, Sassa MH, et al. Importance of physical evaluation using skeletal muscle mass index and body fat percentage to prevent sarcopenia in elderly Japanese diabetes patients. J Diabetes Investig. 2019;10(2):322-30.

37. Hirata Y, Nomura K, Senga Y, Okada Y, Kobayashi K, Okamoto S, et al. Hyperglycemia induces skeletal muscle atrophy via a WWP1/KLF15 axis. JCI Insight. 2019;4(4).

38. Leenders M, Verdijk LB, van der Hoeven L, van Kranenburg J, Nilwik R, et al. Patients with type 2 diabetes show a greater decline in muscle mass, muscle strength, and functional capacity with aging. J Am Med Dir Assoc. 2013;14(8):585-92.

39. Schaap LA, van Schoor NM, Lips P, Visser M. Associations of Sarcopenia Definitions, and Their Components, With the Incidence of Recurrent Falling and Fractures: The Longitudinal Aging Study Amsterdam. J Gerontol A Biol Sci Med Sci. 2018;73(9):1199-204.

Sociedad Española de Investigación Ósea
y del Metabolismo Mineral

Fundación Española de Investigación Ósea
y del Metabolismo Mineral