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Recent developments in the diagnosis of metabolic bone disease

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Summary

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Methods for determining vitamin D and its metabolites. Threshold value of bone manifestations

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Summary

The vitamin D endocrine system (VDES), through the mediation of calcitriol, regulates more than 3% of all the genes of the organism, with multiple effects both at the bone and extra-osseous level. The total concentration of circulating 25OHD, (expression of the sum of the concentrations of 25OHD3 and 25OHD2), constitutes a robust and reliable biomarker of the nutritional status of the VDES, used by health authorities and Scientific Societies in America and Europe. The current methods to measure VDES metabolites are classified into two types: physical detection methods, which include high pressure liquid chromatography (HPLC) and tandem mass spectrometry liquid chromatography (LC-MS/MS) and immunoassay methods. Even today, there is no uniform international consensus that defines vitamin D deficiency and sufficiency for bone health. People at risk of 25OHD deficiency should always be tested for deficiency or insufficiency and intensity. However, there is no reported benefit of general population screening in healthy people.

Endocrine system metabolism of vitamin D

Since the time of its discovery a century ago, there have been advances into what was erroneously called "vitamin" D. It is now acknowledged that it is not a vitamin, though we continue to use that term out of custom and tacit consensus. In fact, it is an endocrine system, the vitamin D endocrine system (VDES), similar to that of other steroid hormones. Cholecalciferol or "vitamin" D_3, is the threshold (physiological) nutrient of the system, synthesized from 7-dehydrocholesterol, which is produced, and found, from single-celled organisms to the skin of higher animals, including human. This route represents around 90% of the physiological contribution to the body, the rest is obtained through diet. There is another isoform, of nutritional or pharmacological contribution, ergocalciferol, "vitamin" D_2 or produced by ultraviolet irradiation of ergosterol contained in fungi, yeasts, etc...[1].

To be hormonally active, "vitamin" D_3 requires sequential metabolic activations through the action of the enzyme 25 hydroxylase (*CYP2R1* and others) mainly in the liver, not hormonally regulated, but subject to various influences, it becomes calcifediol (or 25OHD3) which has a long half-life of two to three weeks. 25OHD3 is a substrate for, through the action of the enzyme 1 alpha hydroxylase (*CYP27B1*), synthesizing 1,25 dihydroxyvitamin D_3 (calcitriol; 1,25(OH)2D3), a system hormone, in the kidney for its systemic endocrine action and in multiple cells and tissues of the organism for its local auto/paracrine action. 1,25(OH)2D3 has a short half-life and is hormonally regulated to maintain a constant concentration within a narrow range. 1,25(OH)2D stimulates 24 hydroxylase (*CYP24A1*) to form 24.25 hydroxyvitamin D_3 or 1.24.25 trihydroxyvitamin[1].

Calcitriol or 1,25(OH)2D3 binds with high affinity to its receptor (VDR), whereas 25OHD3, 24.25 hydroxyvitamin D_3 or 1.24.25 trihydroxyvitamin D and other metabolites have a much lower affinity. VDR belongs to the superfamily of steroid nuclear receptors that use the same heterodimeric partner (RXR) and co-activators or repressors, and bind to similar hexanucleotide sequences in DNA (elements that respond to repeated direct hormone) separated by three or four nucleotides, respectively[1]. The metabolites of VDES, which are poorly soluble in water, need to bind for their transport to their transporter protein "vitamin D-binding protein" (or DBP), with different degrees of affinity, higher for calcitriol and lower for calcifediol, 24.25 hydroxyvitamin D_3 or cholecalciferol or albumin[1].

The VDES, through the mediation of calcitriol, regulates more than 3% of all the genes of the organism, with multiple effects, interacting not only on bone health, and phospho-calcium homeostasis, but on multiple physiological processes in muscle, innate immune system and adaptive, cardiovascular system; controlling cell growth and differentiation, hormonal secretion, xenobiotic metabolism and numerous biological processes throughout the organism[1] (Figure 1).

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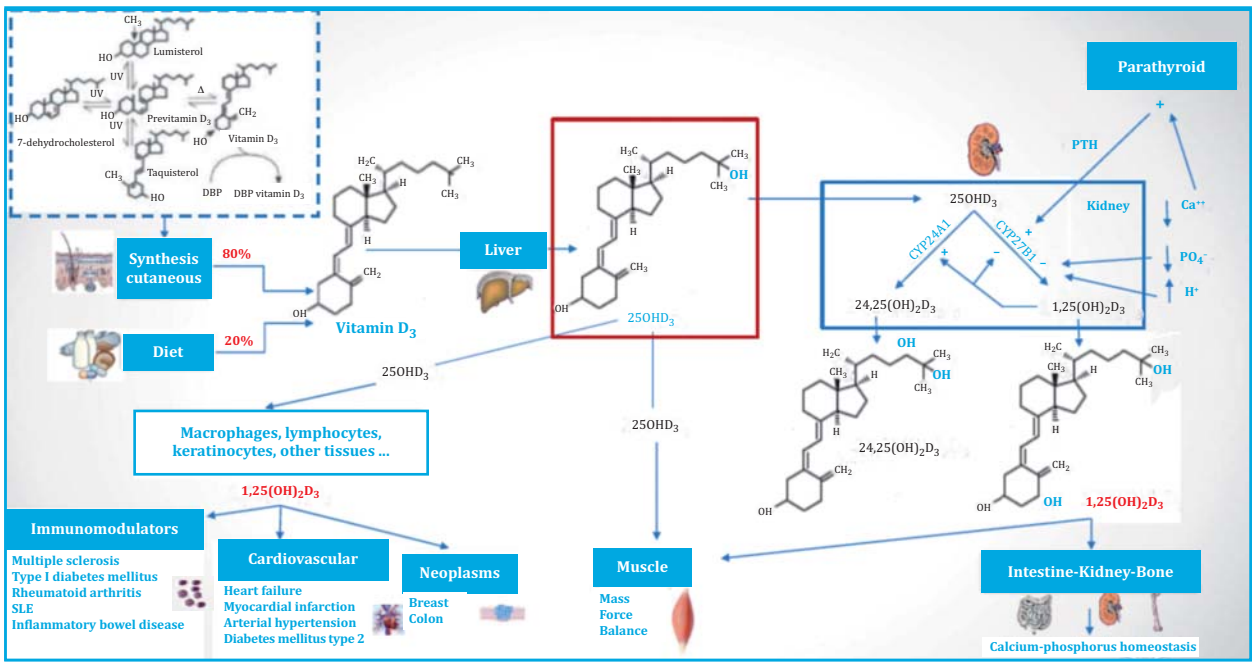
Figure 1. Endocrine system of vitamin D



Therefore, nowadays, the functional deficiency of the system should be related not only to rickets or osteomalacia and osteoporosis, but also to a greater potential risk of suffering cardiovascular, autoimmune, diabetes, oncological, and infectious diseases, among others[1,2]. Currently, we know that "vitamin D" deficiency is very prevalent, even in developed countries or with great potential for acquiring it, due to sun exposure, or due to the ease of accessing supplementation, as is the case in Spain[3].

Therefore, the demand for measurement of vitamin D metabolites used for clinical diagnosis and research of the role of VDES in human health has increased significantly in the past twenty years[4].

Measurement of endocrine metabolites of vitamin D

Serum is the usual matrix used for the measurement of the metabolites of VDES. It has the advantage of not being contaminated with anticoagulants used to obtain plasma, such as heparin, EDTA, or citrate. The assays of the metabolites of the system are very sensitive to the interferences generated by these substances and an appropriate validation must be carried out when considering using plasma for the determinations[4].

Although vitamin D_3 is the threshold nutrient of the VDES, the direct measurement of circulating vitamin D_3 (and/or D_2), strictly speaking, does not constitute a good marker of its nutritional status. Immediately after its cutaneous synthesis or intestinal absorption, it rapidly disappears from the circulation. From then on, it reappears as 25OHD, intensely linked to DBP, which has a long half-life and a higher concentration and is also the essential substrate for the synthesis of 1,25(OH)2D3, the hormone of the system[1,4].

Therefore, the measurement of the total concentration of circulating 25OHD, (expression of the sum of the concentrations of 25OHD3 and 25OHD2), constitutes a robust and reliable biomarker of the nutritional status of the VDES, used by health authorities and scientific societies in America and Europe to establish the status of normality, the definition of deficiency of "vitamin" D and the degrees of insufficiency of the same, on which to establish values of dietary reference intake for "vitamin" D, as well as the control in the population of the deficiency, insufficiency or excess of "vitamin"[5-7]. It should be noted that, in Spain, where, with very specific exceptions, vitamin D_2 is not taken, when 25OHD results are given in practice, 25OHD3 levels are being indicated.

Sometimes in routine clinical practice the quantification of serum levels of 1,25(OH)2D3 is requested to evaluate the nutritional status of the VDES. This constitutes an erroneous and inappropriate practice. The 1,25(OH)2D measurement is not a reliable marker for that goal. By regulating its circulating levels, strictly, in an endocrine way, the organism tends to maintain its values within a very narrow range of normality (20-50 pg/mL, more than a thousand times lower than the serum concentration of 25OHD), even in situations of intense substrate deficiency (25OHD) essential for its synthesis. Therefore, it should never be used to assess the nutritional status of the VDES. The determination of 25OHD is the marker of the nutritional status of the system, or what we colloquially call vitamin D.

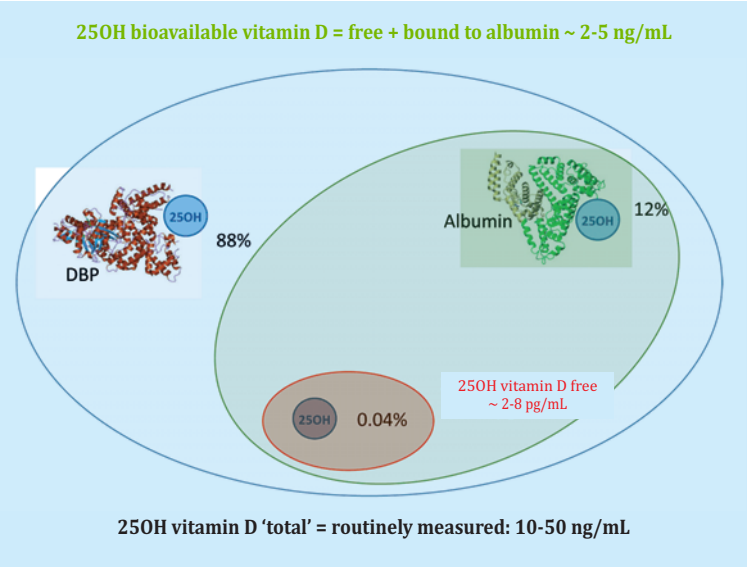
However, 1,25(OH)2D quantification may be useful as a second-level test in the evaluation of VDES, especially in patients with severe kidney disease[8], and allows us to identify a series of conditions, including 1α-hydroxylase deficiency or vitamin D-dependent rickets type 1, due to 1α-hydroxylase enzyme defect, vitamin D-dependent rickets type 2, or VDR defect, and in a series of granulomatous or lymphoproliferative diseases accompanied by hypercalcemia. Also in the diagnosis of hypo and pseudohypoparathyroidism. Measurement of 1,25(OH)2D also helps to distinguish between hypophosphatemic syndromes mediated and non-mediated by FGF23[9].

Quantification of the free 25OHD3 fraction, which represents about 0.04% of the total 25OHD concentration, is not routine clinical practice. The free and albumin-

bound fraction is called 25OHD bioavailable[10]. The set of three fractions is called total 25OHD, although the term "total" is often referred to in the literature as the sum of the 25OHD2 and 25OHD3 forms. Directly measured free 25OHD concentrations generally range from 1.2 to 7.9 pg/mL and are strongly correlated with total 25OHD concentrations and have been reported to account for 0.02% to 0.09% of total concentrations of 25OHD[10] (Figure 2).

In the so-called free hormone hypothesis, it is posited that only the free hormone crosses the cell membrane. To the extent that this is valid, it raises the question whether it is the free concentration that should be measured rather than the total 25OHD, especially in circumstances where the levels and/or affinities of the binding proteins are physiologically altered (eg, pregnancy), or pathophysiologically (liver disease, nephrotic syndrome, acute disease), or by genetic mutations of DBP[10,11].

Figure 2. Bioavailability of vitamin D



Pregnancy or taking contraceptives leads to an increase in DBP levels by approximately 50%, while, for example, liver failure and chronic kidney disease lead to a decrease in DBP concentration, also around 50%. In the case of a high concentration of binding proteins, the free 25OHD fraction is lower, and vice versa in the case of a low concentration of binding proteins. Under these conditions, the measurement of free 25OHD could be a better marker of vitamin D nutritional status than the classic measurement of total 25OHD[10,11]. Direct measurement of free 25OHD has been available since 2013 using a super sensitive ELISA[12]. The method has a detection limit of <3 pg/mL and a measurement range that covers 0.2-35 pg/mL. Repeatability and reproducibility are representative of ELISA technology.

The determination of 24.25(OH)2D has aroused little clinical interest and not much in research areas. This metabolite is formed by hydroxylation of 25OHD by the CYP24A1 enzyme of the cytochrome P450 family, and has long been considered a pure catabolite of the VDES catabolic pathway (Figure 1).

The determination of 24.25(OH)2D is useful in the diagnosis of idiopathic infantile hypercalcemia where it is very high (80-100 ng/mL) and has the potential utility in the identification of other diseases, alone or as part of a ratio of 24.25(OH)2D/25OHD. This proportion is less than 0.09 in patients with vitamin D insufficiency and/or deficiency (serum levels of 25OHD <20 ng/mL)[13]. Historically, the ratio of PTH to 25OHD has been used to estimate the nutritional adequacy of VDES, recently it has been proposed that the molar ratio of 25OHD/24.25(OH)2D has greater potential. The decrease in 25OHD catabolism can also be measured by a lower concentration of 24.25(OH)2D and is associated with an increased risk of secondary hyperparathyroidism and possibly death[14].

C3-epi-25OHvitamin D, also called the C3 epimer, is a stereoisomer that is differentiated by a single chiral center. The hydroxyl function at position 3 of the molecule is reversed while the other chiral centers remain unchanged. C3-epi-25OHD is formed through an epimerization pathway, parallel to the conventional metabolic pathway[15]. C3-epi-25OHD is more abundant in infants under one year of age and is less extensive in adolescents and adults. For many years, it was questioned whether C3-epi-25OHD was as important as its analog 25OHD in the biological activity of vitamin D in the body. However, several groups have reported varying concentrations and prevalences, hindering an assessment of their true importance[16,17].

Techniques for determining the metabolites of VDES

The first measurements of 25OHD date back to the early 1970s, using competitive protein binding assays.

Current methods for measuring the metabolites of VDES are basically classified into two types:

1) Physical detection methods, including high pressure liquid chromatography (HPLC) and tandem mass spectrometry liquid chromatography (LC-MS/MS);

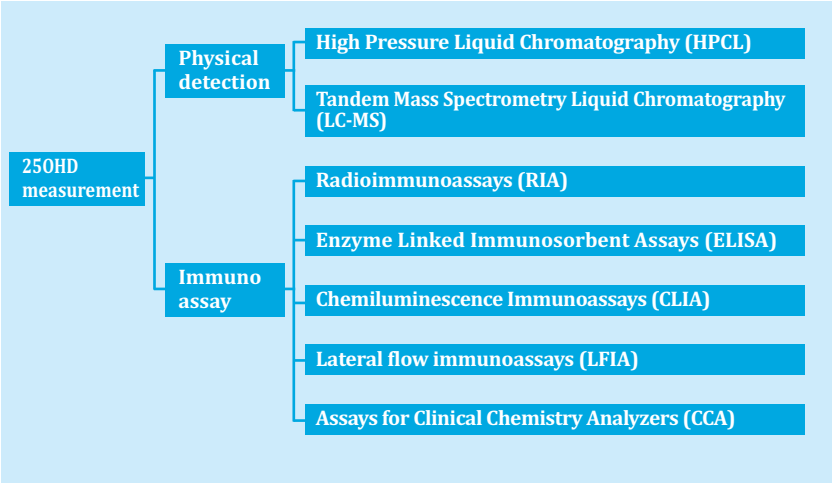
2) Immunoassay methods, encompassing radioimmunoassays (RIA), now obsolete, and enzyme-linked immunosorbent assays (ELISA), chemiluminescence immunoassays (CLIA), lateral flow immunoassays, and clinical chemistry analyzer assays (CCA).) (Figure 3).

Physical detection methods

High-pressure or high-performance liquid chromatography (HPLC) and LC-MS/MS are the physical detection methods used, each with their strengths and weaknesses[4]. In the former, ultraviolet (UV) detection thanks to its strong absorption at 264 nm is a powerful detection method for metabolites of VDES, but various metabolites of VDES exhibit similar UV patterns and need to be completely separated by the LC step to achieve success. be detected, be adequately quantified. Tandem mass spectrometry (LC-MS/MS) fragments molecules that have the same mass and similar affinities that make their chromatographic separation difficult, producing different fragmentation patterns for each individual compound that allow detection and quantification of metabolites separately. This is the case, among others, of 1,25(OH)2D and 24,25(OH)2D.

The benefits of using these measurement techniques of 25OHD as gold standard for determining the nutritional status of VDES are their high sensitivity (<1 ng/mL

Figure 3. Methods for measuring 25OH vitamin D



for LC-MS), high precision and excellent reproducibility profile (the coefficients of variation range from 2% to 7-8%). Thanks to its excellent results, the LC-MS is now recognized as the benchmark for 25OHD measurement.

HPLC and LC-MS also have drawbacks, mainly of a technical nature. Instrument cost and maintenance are also often an obstacle. Both require access to high-quality water, solvents, and chemicals, and serum or plasma needs to be cleaned before being tested[4].

The presence in serum of C3-epi-25OH remains a problem even for many HPLC and LC-MS methods[4,9]. Due to its similar UV pattern and identical monoisotopic mass and fragmentation patterns it cannot be adequately separated from 25OHD by UV or MS detection techniques. Therefore, the development of an LC protocol is required that completely separates the C3-epi metabolite from the desired 25OH vitamin D. Although technical solutions exist and are used by various laboratories, vitamin D C3-epi-25OH still interferes in many HPLC and LC-MS4 methods.

Competitive immunoassays for the measurement of 25OH vitamin D and other metabolites of the system

In competitive immunoassays, each metabolite, for example the 25OHD present in the sample, competes with a labeled 25OHD with a limited number of binding sites on an antibody. They differ in competitive 25OHD labeling and detection method. The RIA was based on radioactive iodine labeling 25OHD and used gamma-ray counters for detection. In the ELISA, the labeling is carried out with an enzyme, the detection is based on a colorimetric reaction and is quantified by measuring the absorbance in an ELISA reader. CLIA methods are also based on enzyme-labeled 25OHD, but detection is based on the emission of light by a specific substrate and is quantified using a photometer.

Technically, immunoassays require the release of 25OHD from its transporter proteins DBP and albumin, followed immediately by the binding of 25OHD by the antibody and its competition with the labeled 25OHD, being able to use polyclonal, monoclonal antibodies in immunoassays, even using different types of DBP.

After 25OH, vitamin D has been released from its binding proteins, different biological molecules can participate in immunoassays, including polyclonal antibodies, monoclonal antibodies and VDBP.

The specificity of the antibodies, that is, the cross-reactivity against the various metabolites of the VDES is one of the key aspects for the quantification of 25OHD and other metabolites in immunoassays because vitamin D3 coexists in serum, exceptionally in Spain 25(OH)D2; 24.25(OH)2D3/; 25.26(OH)2D and C3-epi-25OHD. Polyclonal antibodies generally lack the necessary specificity, limiting the quality of determinations.

For any type of method to be used in the assessment of vitamin D status, it should be an objective of state agencies, scientific societies and laboratories involved to participate in efficient standardization processes, such as DEQAS (Vitamin D External Quality Assessment Scheme), to obtain results with precision and accuracy, fundamental in research and assistance, that allow us to adequately define deficient and insufficient levels of vitamin D, in all age ranges, sex and for any health goal.

Currently the Vitamin D Standardization Program (VDSP) recognizes three Reference Measurement Procedures (RMP) (Ghent, NIST and CDC), which are all LC-MS methods[4].

Definitions of vitamin D deficiency and sufficiency for bone health

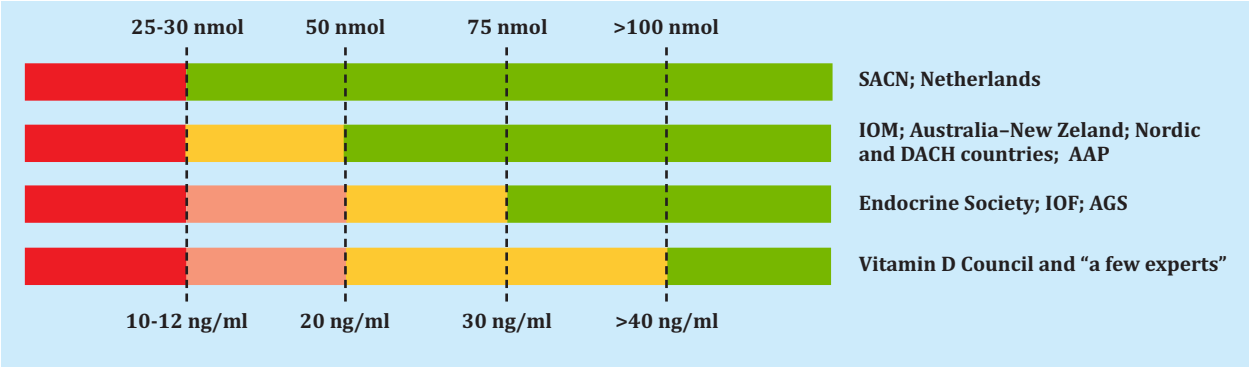
Even today, we do not have a uniform international consensus that defines vitamin D deficiency and sufficiency for bone health[18]. A serious problem in obtaining these definitions is that they depend to a great extent on the precision of the quantification of blood levels of 25OHD and the discussions on this aspect continue, without constructively advancing[19].

In 1998, the United Kingdom was the first country to adopt a serum cut-off point of 25OHD to define deficient status, Committee on Medical Aspects of Food and Nutrition Policy (COMA). More recently, the UK Scientific Advisory Committee on Nutrition (SACN), assessing musculoskeletal health goals (rickets, osteomalacia, falls, muscle strength and function), proposed that the risk of poor musculoskeletal health was higher with serum concentrations of 25OHD less than ~10-12 ng/ml.

On this basis, the SACN defined in 2016 that serum concentrations of 25OHD below 12 ng/ml were deficient for all age groups, and concluded that this threshold is the only one that has been shown to be beneficial for any related health outcomes. with 25OHD levels and that there was insufficient evidence to define that higher 25OHD levels were optimal for bone or even overall body health[20]. Although the Netherlands adopted these recommendations, this is not the opinion of the majority of societies or experts[18].

The US Institute of Medicine (IOM) (later renamed the National Academy of Medicine), selected in 2011 calcium absorption, bone mineral density (BMD) and rickets in children or osteomalacia in adults, to establish levels serum levels of 25OHD and facilitate the development of its recommendations for vitamin D intake, the so-called Dietary Reference Intakes (DRI)21. They defined a serum 25OHD concentration of 12 ng/ml (30 nmol/L) as the

Figure 4. Definitions of vitamin D deficiency and sufficiency for bone health



threshold below which a clinical vitamin D deficiency (severe deficiency) can occur, and established 25OHD levels of 12-20 ng/ml (30-50 nmol/l), to define an inadequate status, which represents an uncertain range that may or may not be sufficient for a given individual. It proposes a 25OHD concentration of 20 ng/mL (50 nmol/L) as the sufficiency threshold, in terms of bone health, for 97.5% of the population. It defines sufficiency for 25OHD levels between 20 and 30 ng/ml (50-75 nmol/l), and indicates that serum levels greater than 20 ng/ml of 25OHD would satisfy the physiological needs in vitamin D, without there being any added benefit. above 30 ng/ml (75 nmol/l), but warns that there could be potential harm with levels above 50 ng/ml (>125 nmol/l)[22]. Subsequently, the Nordic countries, the Swiss Federal Nutrition Commission and the European Food Safety Authority (EFSA) adopted the IOM 2011 guidelines for interpreting 25OHD concentrations[20]. Although it is important to note that the 20 ng/ml (50 nmol/L) suggested by the US National Academy of Medicine, were not proposed for the diagnosis of vitamin D deficiency, but were indicated to support the relationship between intake of vitamin D and the status of 25OHD, on which the dietary recommendations are established[21,22].

The US Endocrine Society (ES)[7] published another set of guidelines in 2011 which have become the focus of debate and controversy with the UK and IOM guidelines. The ES established through its Working Group on vitamin D in the United States a concentration of 25OHD to define deficiency as serum levels of 25OHD <20 ng/ml (50 nmol/l), insufficiency 21-29 ng/ml (52.5-72.5), sufficiency 30-100 ng/ml (75-250 nmol/l)) and possible damage> 100 ng/ml (> 250 nmol/l)), respectively. In summary, the ES has defined serum 25OHD levels of 20 ng/mL (50 nmol/L) as the deficiency threshold and 75 nmol/L (30 ng/mL) as the sufficiency threshold, for 97.5% of the population[7]. Several medical societies and non-governmental organizations have adopted the ES guidelines (Figure 4)[20].

The ES guidelines[7] were quite different from those proposed by the UK guidelines in 199120 or IOM in 2011[21,22] and thus sparked an intense debate, which has continued ever since. The Endocrine Society stated that its guidelines were designed for clinical practice and are directed primarily at patients with a wide variety of diseases and generally at increased risk of 25OHD deficiency, rather than the healthy population (the main group target of most government organizations). However, they do not offer consistent arguments to justify why the optimal vitamin D status in patients would be different from that of the healthy population[7]. To add to the confusion, in 2016, the SACN objected by stating that its guidelines were not for use in clinical practice, but rather public health guidelines for the healthy general population, not sick[20].

Both guidelines agree that the recommendations will require reconsideration in the future as additional standardization data for quantification of 25OHD levels and ongoing randomized trials become available. A minority of experts and grassroots organizations recommend even higher 25OHD levels (above 40 ng/ml (100 nmol/l)), based on the concept that "optimal vitamin D" status is best defined using the presumed Vitamin D status of early Homo sapiens living in equatorial Africa. These proposed target levels also imply that more than 90% of the current human population would be "vitamin D deficient or insufficient" and would require high-dose oral vitamin D supplementation. However, we must consider that it is very possible that the elevated serum concentrations of 25OHD found in primitive African tribes do not represent optimal serum concentrations, but rather the maximum tolerated in evolution to avoid chronic vitamin D toxicity[23].

In addition to the recommendations cited for the general population and with different conditions[24], the main randomized clinical trials of osteoactive anti-osteoporosis drugs used vitamin D and calcium supplements in both arms of these studies, indicating that vitamin supplements should be administered D/calcium to all patients receiving bisphosphonates or denosumab[25,26].

Quantification of 25OHD levels

People at risk of 25OHD deficiency should always be tested for deficiency, or insufficiency and intensity of these (Table 1). As in these patients, it is expected that the vitamin D replacement treatment will produce a rapid favorable health effect. From a public health perspective, determining 25OHD levels is absolutely cost-effective.

Although decreased serum levels of 25OHD in healthy people are frequently described worldwide, there is no evidence of a benefit in general population screening in healthy people for 25OHD deficiency, therefore screening studies for 25OHD are recommended. vitamin D deficiency in patients belonging to risk populations[7].

Therefore, measuring 25OHD is also recommended in osteoporotic patients with or without a history of non-traumatic fractures (particularly before starting treatment with osteoactive, anticatabolic or anabolic agents[25,26]), in the processes listed in table 1[27]. Patients with the clinical

Table 1. Cases that must always be analyzed to detect 25OHD deficiency

- Rickets-osteomalacia
- Osteoporosis
- Chronic kidney disease
- Liver failure
- Hyperparathyroidism
- Malabsorption syndromes:
  - Cystic fibrosis
  - Inflammatory bowel disease
  - Crohn's disease
  - Bariatric surgery
  - Radiation enteritis
- Drugs:
  - Anticonvulsants
  - Glucocorticoids
  - Anti-HIV
  - Antifungals, eg. Ketoconazole
  - Cholestyramine
- Dark skin color
- Pregnant and lactating women
- Elderly with a history of falls
- Elderly with a history of fractures non-traumatic
- Obese (children and adults BMI: >30 kg/m^2)
- Granulomas
- Sarcoidosis
- Tuberculosis
- Histoplasmosis
- Beriliosis
- Lymphomas

diagnosis of rickets or osteomalacia; elderly with a history of falls; pregnant and lactating women, obese (children and adults); people with insufficient sun exposure; patients with malabsorption syndromes (congenital or acquired); maldigestion and undergoing bariatric surgery; chronic kidney disease, liver failure, cystic fibrosis; primary and or secondary hyperparathyroidism. It is also worthwhile to evaluate 25OHD levels in patients undergoing treatments that interfere with the metabolism of the vitamin D endocrine system (anticonvulsant drugs, glucocorticoids, AIDS drugs, antifungals and cholestyramine, among others) and in granulomas and some lymphomas (in these cases, it is also advisable to evaluate serum levels of 1,25(OH)2D).

Conflict of interest: The author declares that he has no conflicts of interest.

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The role of new imaging techniques in predicting fracture risk

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Summary
The incorporation of dual-energy radiological absorptiometry (DXA) to the arsenal of diagnostic techniques unleashed a cascade of advances in the management of metabolic osteopathies. The extensive use of DXA has made it possible to recognize its indications and detect limitations in the evaluation of the risk of bone fracture. In the last decade new advances have been developed applicable to the original technique. These are the Trabecular Bone Score (TBS) and the 3D reconstruction of the DXA images. With different approaches, they allow to assess the microarchitecture of the trabecular bone (TBS) and the measurement with great accuracy of the trabecular and cortical bone, reaching the measurements of volumetric bone density, cortical thickness and geometric variables. This new information now accessible allows the calculation of subject-specific bone strength and opens the possibility of predicting biomechanical behavior in the face of trauma and overload, advancing the diagnosis of fragility before the appearance of fractures.

Introduction

In 1994, the WHO defined criteria for the diagnosis of osteoporosis using the measurement of bone mineral density (BMD). The DXA technique has established itself as the dominant technology for quantifying BMD due to:

a) strong correlation between BMD measured by DXA and bone strength in biomechanical studies,
b) Epidemiological studies that show a strong relationship between the risk of fracture and BMD,
c) For its use in clinical trials of treatments for the selection of subjects and monitoring based on its excellent precision and low radiation dose.

DXA is indicated to diagnose osteoporosis, assess fracture risk, and monitor changes in BMD over time. In recent years, there have been improvements to the initial DXA technology and it is used for other measurements beyond BMD (eg, femur geometry, vertebral fracture detection, body composition analysis).

One of the limitations in the diagnostic approach to bone mass measurement is the overlap of BMD values in subjects with and without fractures[1]. The predictive value of the BMD measurement is limited and the WHO diagnostic threshold of the T-score <-2.5 is taken as one more factor (albeit the most powerful), but not the only one, in taking of clinical decisions. The last definition of osteoporosis included the concept of alteration of the structural quality of bone.

Bone quality encompasses multiple factors not directly related to bone mass. Macro and microstructural factors have been identified due to their relationship with bone strength and therefore with fragility fractures. To satisfy the need for evaluation of these structural factors, new bone evaluation procedures have been developed using the most widely used technique, DXA. This document will briefly review the application of the TBS technique (Trabecular Bone Score, index or score of the trabecular bone) and the 3D reconstruction of the DXA image, which opens up new horizons such as the calculation of bone strength with a non-standard method simple and safe invasive.

TBS

TBS physical fundamentals

The TBS has been described by the developers as an image texture parameter that reflects the differences in the level of gray with which the cells (pixels) are represented in DXA images. The TBS is calculated using the raw data from the DXA acquisition but the calculation is done separately and by different methods than the BMD. The TBS is processed upon completion of the measurement and analysis of the BMD scan and applied to the same region of interest. The TBS calculation principle was published in 2008[2]. The variations in the gray scale with which the contiguous pixels are represented, in multiple random directions constitute the experimental variogram. A 3D image of a narrow network of trabeculae produces a 2D projection image with many small amplitude gray level variations and thus a steep slope of the variogram which offers a high TBS value (a conserved microarchitecture associated with good mechanical resistance). In contrast, a low TBS value indicates low-quality microarchitecture with few gray-level variations, of considerable amplitude, inherent in a gentle slope at the origin of the variogram. The TBS calculation software (TBS InSight®) is an application distributed by Medimaps (France) (Figure 1).

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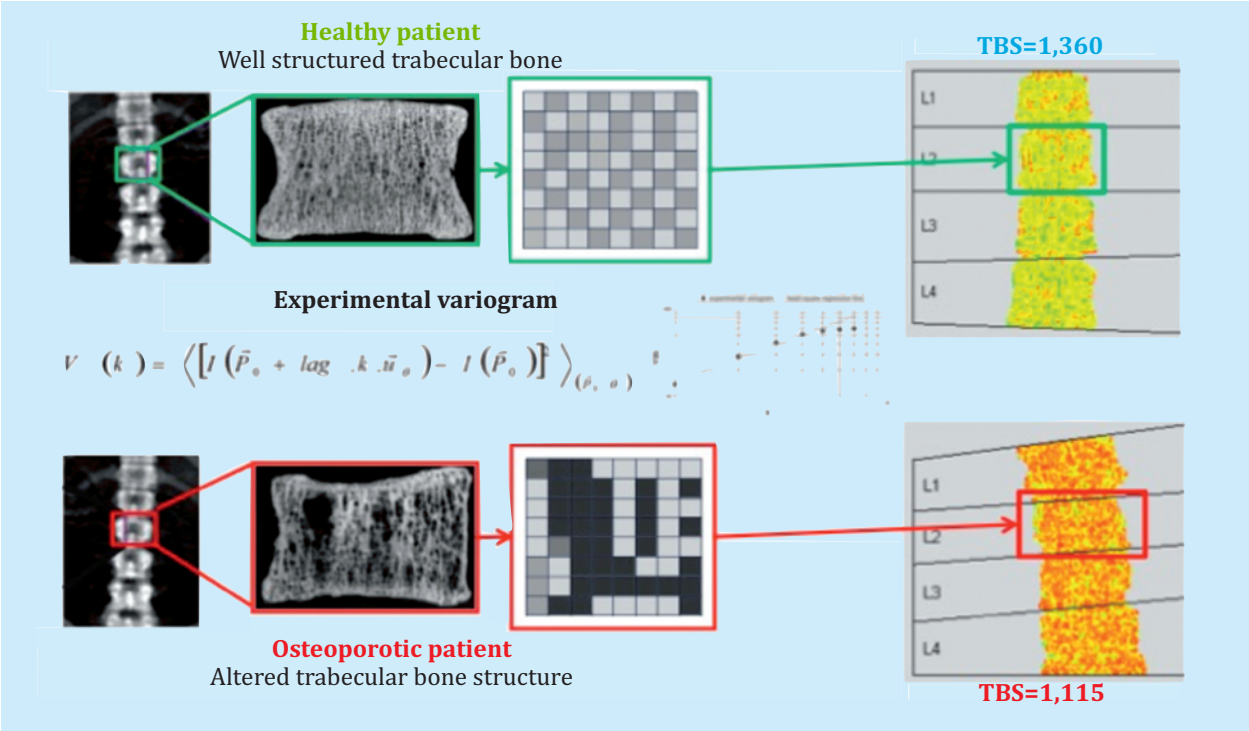
Figure 1. Synthesis of the TBS calculation method. Differences between TBS of a subject with healthy bone and another with osteoporosis



Correlation between TBS and bone microarchitecture parameters:
The correlations in the initial study[2] between TBS and the main 3D micro-architectural parameters measured in trabecular bone samples using micro-CT in different skeletal bones. The following microarchitecture parameters were determined: bone volume/total volume (BV/TV), trabecular thickness (TbTh), trabecular spacing (TbSp), number of trabeculae (TBN), and their connectivity (Conn.) (Table 1)

TBS in aging:
The first TBS development curves in relation to age were established on the results of 5,942 French women[3]. At present, normative studies have been carried out in several countries, including in Spain (SEIOMM-TBS Project) confirming a great similarity in TBS values in both sexes[4-7]. In the Spanish population (SEIOMM-TBS Project), TBS values in adult women and men in the 20-30 year age bracket were very similar and reached their highest value. TBS decreases with age in both sexes. The decline in TBS and BMD is similar in the 40-50 years. In women the average decrease in TBS between 20-80 years was -18% and in men it was -14%. TBS values showed poor correlation with body mass index (r=-0.1), weight (r=-0.1) and L1-L4 BMD (r=0.2) (Figure 2).

Reproducibility:
The coefficient of variation is similar to the BMD measurements for DXA, being 1.5% for TBS (1.2% for BMD)[8]. The in vivo reproducibility of TBS using the ISCD protocol in 30 unselected patients (26 women and four men) who presented no detectable vertebral fractures. The mean TBS was 1.239±0.082, the coefficient of variation was 1.9%, and the least significant difference was 0.065. For the same patients, the coefficient of variation was 1.2% for BMD.

Table 1. Correlation (r) between TBS value and micro-architecture parameters using computed micro-tomography

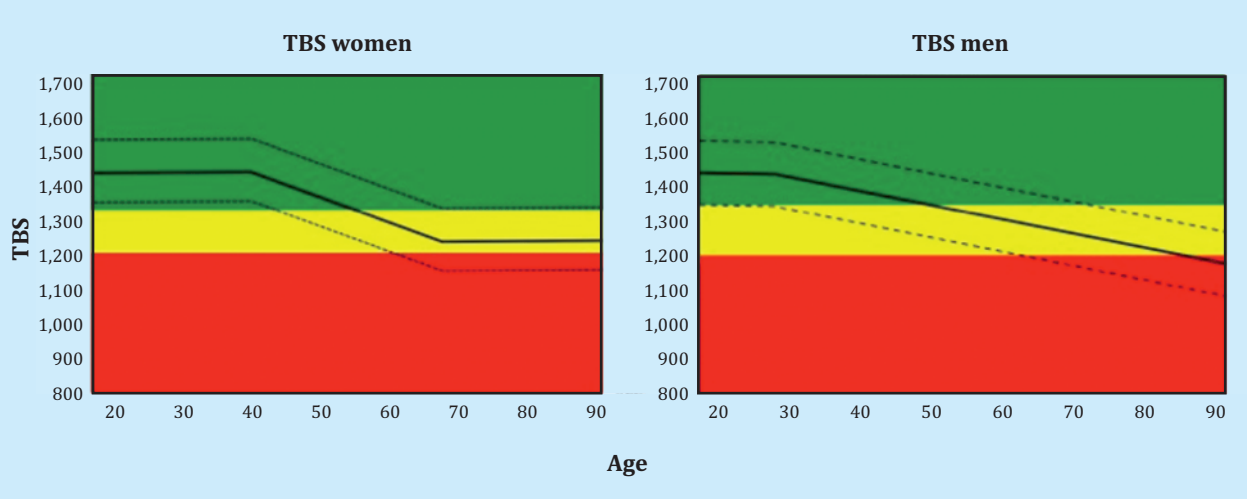
Origin sample	Number	BV/TV	TbTh	TbSp	TbN	Conn
Vertebra	20	-0.63	0.23	0.73	-0.84**	-0.85**
Neck Fem.	17	-0.25	0.57	0.62*	-0.52	-0.53
Radio Ultr.	20	0.00	0.83**	0.34	-0.46	-0.60

*p<0.01; p<0.0001.

Studies on the fracture-discrimination capacity of TBS:
Several studies have evaluated the ability of TBS to differentiate patients with fragility fractures from those without fractures. The TBS in all of them was significantly lower in patients with fractures than in controls. These cross-sectional studies indicate that TBS can discriminate individuals with fractures from controls. This discriminatory power of TBS is similar or greater than that of BMD and that the combination of TBS and BMD provide better discrimination than that of BMD. Solo BMD[8-11].

The most cited population study with TBS is the one carried out in the Manitoba Cohort. Lumbar spine TBS and BMD were prospectively compared in a large female population of 29,407 women older than 50 years[12]. For a given lumbar BMD range (normal or osteopenia or osteoporosis), the annual number of incident fractures was always higher in the lowest tertile TBS. Lumbar spine BMD and TBS were weakly correlated (r=0.32).

Figure 2. Evolution of TBS in relation to age in both sexes represented by solid line. Limits of ± 1 standard deviation



The results were similar for the prediction of hip fractures or any of the four main types of fracture considered. For the four types of fracture together, the predictive ability improved significantly when BMD and TBS were combined.

In the OFELY[13] and OPUS[14] cohort studies, TBS performance was significantly better than lumbar spine BMD for predicting clinical osteoporotic fractures. In radiological vertebral fractures, TBS and BMD of the spine had a similar predictive power. The combination of TBS and BMD increased performance, however, with a predictive capacity similar to the BMD of the total area of the femur and femoral neck. In non-osteoporotic women, TBS predicted incident fragility fractures similarly to BMD. The combination of TBS and BMD improved prediction in all scenarios compared to the use of BMD alone.

Impact of Osteoarthritis on TBS:
Osteoarthritis-related bone sclerosis generates more or less marked contrasts with adjacent healthy bone, and the method used to calculate TBS detects these interfaces and appears only minimally affected by large masses of osteoarthritic bone. In a retrospective cross-sectional study of 141 densitometries in patients with osteoarthritis, only in L4 (using the ISCD criterion, that is, a difference of more than 1 SD between the BMD values between L4 and L3) and 249 controls (defined using ISCD criteria such as BMD of L1 <L2 <L3, and> L4), showed that osteoarthritis had no significant effect on TBS values, as long as the resulting increase in BMD was less than 3.5 standard deviations in L3[3].

TBS as a new risk factor for FRAX®:
The developers of TBS and the University of Sheffield research group determined the impact of TBS on the probability of fracture, beyond that provided by the clinical risk factors used in the FRAX tool. The Manitoba, Canada cohort[15] was used in a retrospective study applying the TBS to the initial DXA scan and the rest of the risk variables already used in the FRAX. When fully adjusted for FRAX risk variables, TBS remained a statistically significant predictor of major osteoporotic fractures. Fit models have been derived for the main fractures and hip fractures, taking into account TBS and age. TBS has been found to be a predictor of the risk of osteoporotic fracture, independent of BMD of the femoral neck and clinical risk factors, being a risk factor for mortality.

Application in clinical practice:
The assessment of bone microarchitecture allows the identification of patients at high risk of fracture who have not been adequately classified only by bone mineral density. The application of TBS facilitates the management of patients by recognizing subjects with low BMD and altered bone structure[16]. In this sense, the population sector that can benefit most from the application of the TBS are those who have a BMD T-score <-1.0 and >-2.5 (Figure 3).

In the routine clinical practice, TBS should be considered as an additional "risk factor" that will help in the orientation and management of the patient at risk of osteoporosis. Since TBS is less affected by the artifacts that most influence BMD measurements, whether intrinsic, such as degenerative alterations, extra-skeletal calcifications, or extrinsic, such as orthopedic elements, they increase the diagnostic performance of the DXA technique.

Diagnostic thresholds:
The reference values used have been re-validated after the review of several cohorts of the European female population, which also included women from Spain. With the current data, a threshold of significant degradation of the microarchitecture of the trabecular bone is considered, a TBS result lower than 1,200.

Conclusions

The TBS is a new method of application of the DXA diagnostic technique that allows the evaluation of bone microarchitecture, a key determinant of bone strength. The TBS can be calculated in a simple way, using the widely available DXA technology and following the same conventional procedure for the measurement of BMD. The TBS is a reproducible quantitative value and therefore can be monitored. The clinical results obtained in large population groups confirm that the combination of BMD and TBS is capable of predicting fragility fractures and thus substantially improves the predictive capacity of fracture risk.

3D DXA

Although DXA accurately measures BMD, it is limited by its two-dimensionality and does not represent the spatial distribution of BMD in the bone structures examined. To overcome this limitation, quantitative computed tomography (QCT) allows 3D reconstruction and assessment of the distribution of BMD in bone. Several parameters evaluated in 3D are strongly correlated with the strength of the femur, such as trabecular and cortical BMD, volumetric BMD in specific regions, or geometric parameters such as the length of the neck axis and the cortical thickness, and justify the variation in force. necessary to achieve mechanical failure, improving the estimation of the risk of fracture when it complements the BMD[17]. However, QCT exposes a significantly higher radiation dose than DXA and has a higher cost, reasons that limit its application to research areas.

In recent years, it has been possible to reconstruct a statistical model that combines 3D bone shape and BMD distribution from an in vivo database of QCT scans of the proximal femur[18,19]. Reconstruction is performed using an intensity-based 3D-2D registration process and the similarity between your QCT projection and the DXA image is maximized. The methodology places emphasis on achieving a 3D reconstruction of the DXA image with a bone density model that resembles, through iterative updating, the information that a QCT would have, resolving the relationship with other bone structures (Figure 4).

This method has been specified in commercial software known as 3D-Shaper® (Galgo Medical, Barcelona, Spain), available for application in most of the currently existing densitometers (GE-Lunar, Hologic, DMS). One of the most interesting aspects of this technique is that it uses the DXA image obtained in a conventional way, without conditioning a different procedure or longer or higher radiation dose. It is currently available for 3D reconstruction of the proximal femur and lumbar spine.

Variables that it calculates:

The software allows 3D reconstruction and volumetric bone density (cm^3) measurements in isolation from the trabecular component, from the cortical bone or by integrating both compartments (integrated bone): trabecular volumetric BMD (trabecular vBMD, in mg/cm^3), volumetric BMD cortical (cortical vBMD, in mg/cm^3), volumetric bone integrated BMD (global vBMD, in mg/cm^3). The measurement of cortical thickness (Cth) in mm and the cortical surface BMD (cortical sBMD) is calculated, at each vertex of the femoral surface mesh, as the multiplication of the Cth (in cm) by the cortical vBMD (in g/cm^3) observed throughout this thickness. Cortical sBMD is expressed in grams per square centimeter. Cortical sBMD has been used in studies using QCT in the literature[20]. In the context of patient monitoring, any increase in Cth, cortical vBMD, or both will result in an increase in cortical sBMD. On the other hand, if Cth and cortical vBMD vary similarly in opposite ways (eg, increased Cth and decreased cortical vBMD), the cortical sBMD will remain unchanged.

If the region in question is the femur, the mean values of the aforementioned variables are calculated on the total region of interest of the femur (neck, trochanter, intertrochanteric region, diaphysis and total area), or in the lumbar spine (in the vertebrae L1 to L4 and their averages)[21].

Figure 3. Example interpretation of a TBS result

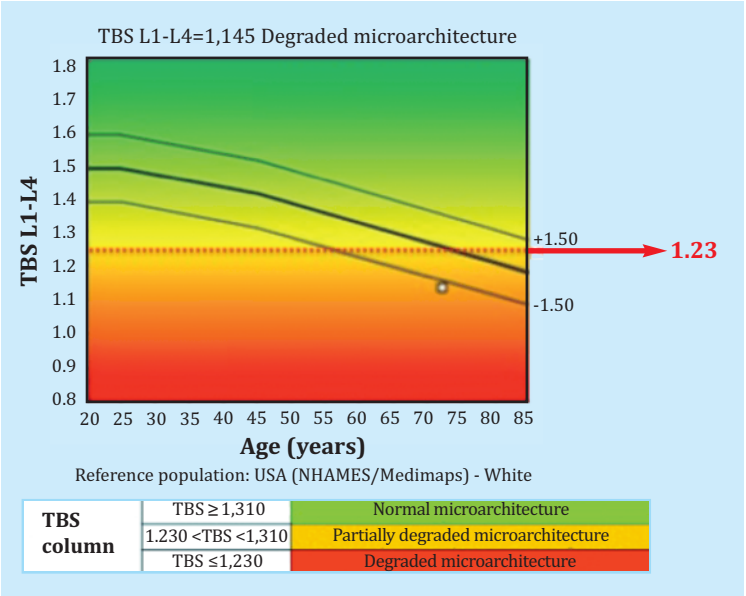


Figure 4. Synthesis of the 3D reconstruction procedure from 2D DXA image. The interaction of the process maximizes the similarity between the simulated projection and DXA optimizing in: orientation and size, bone shape and BMD distribution

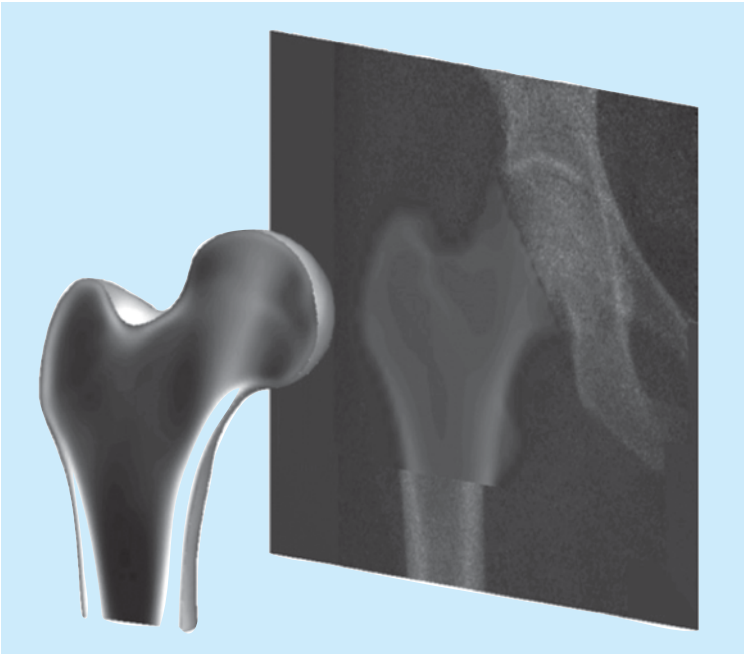
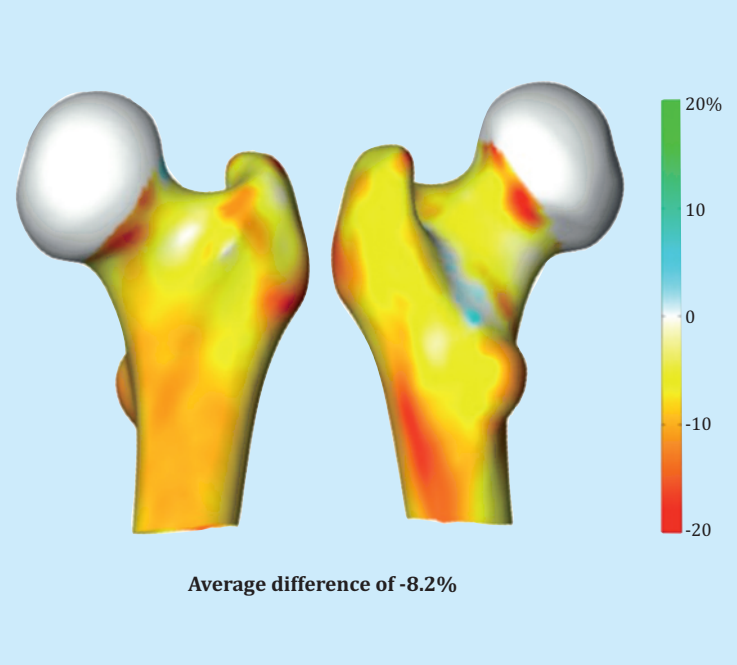


Figure 5. Average differences in cortical thickness in case-control study of hip fractures



Accuracy:
The accuracy of the measurements and the potential need for a single or multiple bone region scan (different angles) was evaluated in vivo by comparing 3D reconstructions obtained from simulated DXA images using repeated DXA scans with patient repositioning and different inclinations, with 3D QCT reconstructions. The comparison showed that the use of a single DXA provides highly accurate 3D reconstructions (mean shape precision of 1.0 mm and BMD distribution errors of 7.0%)[22]. In another study, high-resolution micro-CT data from 23 proximal cadaver femurs were analyzed to determine a relationship between cortical thickness and density[23] and was complemented with a case-control study that included patients with osteoporosis. and age-matched controls with normal bone density to evaluate the method in a clinical setting. Cortical thickness (density) estimation errors were 0.07±0.19 mm (-18±92 mg/cm^3) using simulated clinical CT volumes with the smallest voxel size (0.33×0,33×0.5 mm^3), and 0.10±0.24 mm (-10±115 mg/cm^3) using the volumes with the largest voxel size (1.0×1.0×3.0 mm^3). The case-control study showed that osteoporotic patients had a thinner cortex and lower cortical density, with mean differences of -0.8 mm and -58.6 mg/cm^3 in the proximal femur compared to controls of the same age (p value <0.001).

In the lumbar spine, the accuracy error of the anatomical shape was 1.5 mm in the total vertebra and 0.6 mm in the vertebral body. The correlation coefficients between the measurements derived from DXA and QCT ranged from 0.8 to 0.9[21].

Precision:
The short-term in vivo precision of 3D measurements made with DXA acquisitions made with HOLOGIC and GE systems in patient follow-up at an 18-month interval between baseline examination and monitoring DXA[24]. Considering the minimum significant change for a 95% confidence interval, the recommended time intervals for the evaluation of trend in postmenopausal women were 2.9 years (integral volumetric BMD), 2.6 years (trabecular volumetric BMD) and 3.5 years (cortical surface BMD), using the Lunar iDXA densitometer. The trend assessment intervals for area BMD were 2.8 years in the neck and 2.7 years in the total femur. The time intervals in postmenopausal women were similar to those measured for 2D αMD measurements in the femur region.

Relationship of 3D parameters with bone strength
In vitro studies:
The method evaluated, in an experiment with bone samples ex vivo, the predictive capacity of bone strength in biomechanical examinations of 90 femurs from cadavers that were previously explored with DXA, obtaining a correlation coefficient of 0.85 between the load of predicted and measured fracture, while a regression using BMDa (DXA) measurements resulted in a correlation coefficient of 0.77[25].

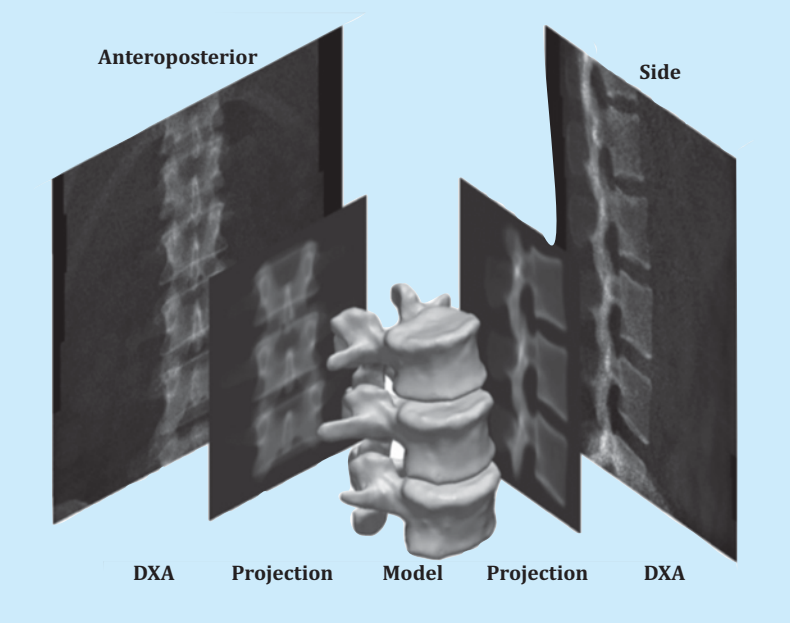
In vivo studies:
In a retrospective case-control study[26], 3D-DXA measurements were evaluated in a cohort of postmenopausal women with hip fracture. The total BMD of the total hip area of the fracture group was 10% lower compared to the control group. The differences in volumetric BMD (total hip) measured by 3D-Shaper were more pronounced in the trabecular compartment (-23%) than in the cortical compartment (-8%). The area under the curve (ROC curves) was 0.742 for trabecular volumetric BMD, 0.706 for cortical volumetric BMD, and 0.712 for total hip area BMD. Differences in the cortex were locally more pronounced on the median aspect of the shaft, the lateral aspect of the greater trochanter and the superolateral aspect of the neck. Marked differences in volumetric BMD were observed in the greater trochanter (Figure 5).

In a case study (61 hip fractures) and controls, the predictive capacity of fractures was assessed from 3D measurements of the lumbar spine, evaluating the association of 3D-DXA measurements of the lumbar spine in subjects who had suffered osteoporotic femur fractures. A stronger association was found between femoral neck fractures and lumbar spine cortical bone variables compared to trabecular bone measurements[27].

The association of 3D-DXA measurements with vertebral fractures was evaluated in a retrospective case-control study[28]. Lumbar spine DXA scans were acquired at baseline (ie, before the fracture event for fractured subjects). The BMD of the fracture group was 9.3% lower compared to the control group (p<0.01). However, a greater difference was found for trabecular BMD in the vertebral body (-16.1%, p<0.001), better discriminating the fracture and control groups, with an AUC of 0.733, compared to 0.682 for BMD. This study showed the ability of 3D-DXA measurements to discriminate patients with vertebral fractures and patients who have not suffered them (Figure 6).

Future projection:
The 3D reconstruction of bone regions with complex geometry can benefit from biomechanical analyzes based on finite elements (FE) that help improve the prediction of the risk of fracture by integrating definitive information on the biomechanical behavior when the bone is subjected to physical loads. In this sense, it is worth highlighting the study in which patients with a recent femur fracture and controls were included[29]. A lateral fall was simulated using a maximum static load that depended on the patient's mass and height. The results showed that the main maximum stress biomechanical variable was a better discriminator (AUC >0.80) than the volumetric BMD (AUC ≤0.70). A high discrimination capacity was achieved when the analysis was carried out by bone type, fracture zone and gender/sex (AUC of 0.91 for women, trabecular bone and trochanter area). The results suggested that the trabecular bone is essential for the discrimination of femur fractures. The application of finite element analysis to models derived from DXA scans can significantly improve the prediction of the risk of fracture of complex sectors such as the femur; providing a new perspective for clinicians to use this new technology.

Figure 6. 3D reconstruction procedure of the lumbar spine from 2D DXA images. Initial approach in which integration of two PA and lateral views was assessed ***(Tristan Whitmarsh, Ludovic Humbert, Luis M. Del Río Barquero, Silvana Di Gregorio, Alejandro F. Frangi. Medical Image Analysis 17 (2013) 475–487).***



Conflict of interest: The author declares that he has no conflicts of interest.

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Genetic studies in the diagnosing of osteoporosis and other metabolic bone diseases

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Summary
The study of the genetic cause of a disorder depends on the clinical characteristics. If a specific genetic alteration is suspected, sequencing can focus on one gene, a panel of related genes, or the entire exome, depending on whether or not a gene is clearly suspected. The specific strategies depend on the condition under study and the diagnostic protocols implemented in each center. In the coming years, however, when costs tend to be lowered and analysis procedures are streamlined, sequencing of the entire exome will progressively replace gene panels. On the contrary, in case of suspicion of alterations of a broader chromosomal region, procedures that allow the detection of structural variants, in general some type of "array", are indicated. Interpretation of results, especially in the case of "variants of uncertain significance" often requires the judicious integration of genetic, bioinformatics and clinical data.

DNA AND MUTATIONS

The nucleus contains most of the genetic information, distributed throughout the approximately 3 billion nucleotides of human haploid DNA. The approximately 21,000 genes that encode the proteins necessary for the various organic functions are represented there, as well as an indeterminate number of genes that are transcribed into RNAs that do not encode proteins, but have regulatory functions[1].

Mitochondrial DNA is smaller, having about 16,000 nucleotides, with genes to encode 13 proteins and 24 non-coding RNAs (transfer and ribosomal)[2].

DNA changes that lead to disease can be classified according to various criteria, including:

According to frequency

- *Polymorphisms.* They are relatively frequent variations in the population, present in more than 1% of people. Its functional impact is generally limited. So individually they are not usually disease-causing. However, there are polymorphisms that are associated with a greater or lesser response to certain drugs. In addition, when several harmful polymorphisms occur in combination, or when adverse environmental circumstances coexist, they can determine the risk of suffering some processes, such as osteoporosis and other prevalent complex diseases, that have a "polygenic" inheritance.
- *Mutations.* They are rare. Depending on the specific region of DNA affected, they can induce very important changes in the activity of genes or the proteins encoded by them. Hence, a single mutation may be enough to cause disease. This is the usual case of classic, monogenic or Mendelian hereditary diseases.

According to the transmission

- *Inherited.* They are the genetic variants that are already present in the cells of the progenitors, including the germ cells, whether or not they have the disease. Logically, the parents carrying these mutations can transmit them to several descendants.

As is well known, depending on whether the presence of 1 or 2 mutated alleles is necessary for the disease to develop, they will show a dominant, recessive or codominant inheritance pattern. Careful analysis of the family tree will help to establish which of these patterns occurs, and whether the mutation affects the autosomes (in which case the disease appears equally in males and females), the sex chromosomes (in the most frequent cases, of recessive inheritance linked to the X chromosome, it preferentially affects males, but is transmitted by women) or mitochondrial DNA (both sexes are affected, but it is only transmitted through the maternal line).

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• *De novo*. These mutations are not generally present in the tissues of the parents, but appear during the formation of germ cells and persist in the embryo if they affect the ovum or sperm involved in fertilization. Other times, these mutations are not present in the germ cells of the parents, but appear during embryonic development.

According to the affected tissues

• *Germ mutations*. Whether inherited or de novo mutations, they are present in the germ cells of the parents, so that they are also present in all tissues of the embryo and later of the adult individual. They are, therefore, transmissible to the offspring

• *Postzygotic or somatic mutations*. They appear in some cells of the developing embryo, so that only tissues derived from these cells show the mutation. Depending on the moment of appearance, they can affect the whole of a certain tissue, or only a part of it, causing mosaicism, that is, the coexistence in the same individual of normal cells and cells with mutated DNA. Depending on whether or not the germ cells carry the mutation, they may be transmissible to the offspring.

According to the number of nucleotides involved

• *Point mutations*. The mutation affects only one nucleotide, in which the usual base changes for another.

• *Small group of nucleotides*. In these cases, they are usually insertions or deletions of a few nucleotides. Other times it affects repetitive regions, so that there is a change in the number of repeats of groups of 3-5 nucleotides.

• *Large regions*. Sometimes deletions or duplications affect large regions of DNA, which can include thousands or millions of nucleotides. Extreme cases are those in which an entire chromosome is lost (such as Turner syndrome, in which the Y chromosome is missing) or an extra extra chromosome is acquired (as in the trisomy 21 of Down syndrome).

It should be taken into account that during DNA replication some mutations often occur, most of which do not have a negative impact. Thus, an estimated 50 or so de novo mutations occur in each individual, of which 1 or 2 are located in the exome. The exome is the set of coding regions. Although it only represents 1% of the DNA (about 30Mb), it is assumed that it is the seat of more than 80% of the mutations that cause monogenic diseases. The accumulation over the generations, makes the exome of an average individual present some 20,000 point variants compared to the reference genome. Many more variants accumulate throughout the genome, including, on average in a given individual, about 1,000 "copy number variations" (extensive duplications and deletions), about 350,000 insertions and deletions of one or a few nucleotides, and more than 3 million mutations and point polymorphisms. This degree of variation, although it is a minimal percentage compared to the 3 x 10^9 nucleotides of the genome, represents a very important complexity when interpreting the results of genetic tests.

Sequencing and other genetic tests

There are different types of genetic tests, with different objectives and procedures. We will only comment on some of the most frequently used tests, highlighting the aspects of interest to the clinician in the field of skeletal alterations.

Karyotype

It is one of the classic procedures, especially useful when an abnormal number of chromosomes or other extensive structural abnormalities (large deletions or duplications) are suspected.

Genotyping using arrays

The matrices or "arrays" explore some specific nucleotides. In general, it is about 0.1-1 x 10^6 nucleotides distributed, either throughout the entire genome, or preferably in coding regions or more frequently causing disease. They can be useful for detecting some specific mutations, but clinically their main utility is the detection of variations in copy number and other extensive chromosomal abnormalities, as they have higher resolution than other techniques, such as karyotyping.

Sequencing

In this case, the sequence of a more or less large region of DNA is exhaustively investigated, so that the complete sequence of the region studied is obtained. Traditional sequencing methods (Sanger sequencing) were expensive and time consuming, so they could only be applied to relatively small regions. For this reason, today they have been largely displaced by the so-called "next generation sequencing" (NGS) massive sequencing techniques. These techniques can be used to sequence a gene, multiple genes, the entire exome, or even the entire genome.

Currently, the most common approach to the use of massive sequencing in the clinic is based on the following criteria:

- Although it is not the most frequent, in some disorders (for example, hemochromatosis) most of the patients present the same type of mutation. In these cases, as a first approximation, only one or a few nucleotides can be analyzed, by Sanger sequencing or by other simple and inexpensive procedures, such as allele-specific PCR.
- If there is a strong suspicion of which gene is involved, but there is allelic heterogeneity (that is, there are many mutations that can cause the disease), that gene can be sequenced (using classical or NGS procedures), or only the area coding (which includes the exons and the adjacent part of the introns) or the entire gene. An example of this situation is hypophosphatasia, whose characteristic biomarker is low levels of alkaline phosphatase, and which is due to mutations in the ALPL gene.
- If the clinical picture has characteristics that allow it to be grouped within a set of processes, but the specific gene is not easily predictable, a "panel" of genes can be sequenced using NGS that includes the genes usually involved in this type of process. Depending on the case, those panels may include just a few genes, or several hundred. It is the approach often used, for example, in case of suspected osteopetrosis.
- If the clinical picture is difficult to classify or it is a high genetic heterogeneity situation (for example, mental retardation) or if previous studies do not allow to find the genetic basis of the picture, the entire exome can be sequenced, the "clinical exome"(reduced version that includes only the genes that are known as associated with diseases, about 7,000) or even the complete genome.

Table 1. Analysis techniques frequently used in clinical genetics

Test	Typical resolution	Detected anomalies	Genes explored	Applicable without definite suspicion of the genetic cause
Karyotype	5-10 Mb	Aneuplodia, large structural alterations	All	Yes
FISH	50-2000 Kb	Structural anomalies	1 o more	No (yes)*
CGH	10-1000 Kb	Structural anomalies	All	Yes
Array SNP	50-400 Kb	Structural abnormalities, genotyping	All	Yes
MLPA	50 b	Structural variations of intermediate length	1/more	No
MS-MLPA	50 b	Structural variations of intermediate length, alterations in methylation	1/more	No
Genome (NGS)	1 b	Point mutations, short insertions/deletions, Variations in copy number*** (expensive and complex analysis)	All	Yes
Exome (NGS)	1 b	Point mutations, short insertions/deletions, Variations in copy number*** (does not detect mutations in non-coding regulatory regions)	~ 21000	Yes
Clinical exome (NGS)	1b	Point mutations, short insertions/deletions, Variations in copy number*** (limited to genes 30 years most commonly associated with disease)	~ 6000	Yes
Disease-oriented gene panels** (NGS)	1b	Point mutations, short insertions/deletions, (does not scan non-coding regulatory regions)	2-400	No
Unique genes (NGS or Sanger)	1 kb	Point mutations, short insertions/deletions	1	No
Genotyping (various techniques)	1 kb	Point mutations	1 nucleotide	No
Genotyping (multiplex techniques)	1 kb	Point mutations	2-50 nucleotides in 1 or more genes	No

B: base or nucleotide; NGS: next generation sequencing; *: procedures based on fluorescent probe in situ hybridization (FISH) are used in principle to explore a specific locus. However, several probes can be mixed to explore multiple regions and even "paint" all the chromosomes, thus being able to detect structural abnormalities with greater sensitivity than the conventional karyotype; **: sequence interpretation today is often a more expensive and laborious step than sequencing itself. This is why “virtual dashboards” analysis is sometimes carried out. It is also known as a "directed exome." That is, the entire exome is sequenced, but only the variants in the genes potentially related to the phenotype are then analyzed; ***: it is not the most sensitive technique, some may not be detected.

These procedures often pose difficulties to interpret the results, since many differences with the reference genome are usually detected), but in many cases it is difficult to establish whether they are pathogenic mutations or not. For this, a combination of bioinformatic strategies is used, together with the judicious interpretation of clinical data. Thus, for example, it is usually valued:

- The population frequency of the variants (the very frequent ones are probably not pathogenic).
- If these variants have been previously described as a cause of disease.
- If, in light of "in silico" prediction systems, the variants produce important functional changes in the protein sequence. However, at present many of the variants that are found are classified as of uncertain significance (VUS, “variants of unknown significance”).
- Whether the zygosity conforms to the inheritance pattern or not. Thus, if family history suggests an autosomal recessive pattern of inheritance, heterozygous mutations may not be pathogenic. However, in these cases we must not forget the possibility that it is a composite heterozygous individual (that is, that it has two different heterozygous mutations in each of the alleles of the gene).

It is also very useful to sequence the genome of the parents (which is sometimes known as the "exome trio", since it includes the two parents and the patient under study) to facilitate the interpretation of the variants found. If the parents are healthy, the variants of the patient that are present in any of them must not be pathogenic.

To confirm pathogenicity, it is often necessary to carry out a “segregation” study, that is, to analyze the suspected variant in other relatives, to check whether this mutation is present in patients and absent in healthy ones.

Detection of structural variants and other sequencing limitations

Mass sequencing techniques have been a true revolution in genetic studies. They make it possible to determine extensive DNA sequences in a short time and at a relatively low and decreasing cost. However, it must be taken into account that these techniques allow us to know the nucleotide sequence in the analyzed DNA, but they present some limitations:

1. Although there are algorithms that make it possible to determine if there are alterations in the number of copies of the analyzed regions, these procedures are not completely effective in detecting **structural variants**. Therefore, in case of suspicion, carrying out procedures that are more sensitive is recommended to detect these types of alterations. Among them it is worth highlighting:

   a. *Comparative genomic hybridization or differential hybridization arrays (CGH arrays)*. They carry probes distributed throughout the genome. The results obtained in the patient are compared with those obtained in a healthy subject. They usually have a resolution between 40 and 400 kilobases.

   b. *Arrays of SNPs*. They analyze nucleotides scattered throughout the genome and make it possible to determine whether or not there are two copies of each of them, thus detecting the deletions and duplications that may exist. It should be noted that these techniques do not detect some structural variants that do not involve changes in the number of copies, such as rearrangements and inversions.

   c. *Amplification of probes after multiple ligation (MLPA, Multiplex Ligation-dependent Probe Amplification)*. It is a multiplex technique that allows a relative quantification of the number of copies of several dozen different regions. It is indicated when structural variants of one or a few genomic regions are suspected.

2. Cases of **mosaicism** represent an additional difficulty. Mosaicism can occur at the level of germ cells, somatic cells, or both. Genetic skeletal disorders that may be related to germinal mosaicism include osteogenesis imperfecta and Down syndrome[3]. The usual sequencing techniques (classical or NGS) are usually unable to detect mosaicisms in which less than 5-10% of the analyzed cell population present the mutation. Sometimes, in these cases, it is useful to repeat the studies that have been carried out with DNA extracted from blood cells in other samples, such as cells from the oral mucosa or from the skin.

3. Detection of epigenetic alterations (especially cytosine methylation) using sequencing techniques requires prior treatment of DNA with bisulfite, which converts cytosines to uracils, while methylated cytosines remain unchanged. To detect the methylation status of specific genomic sequences, a variant of MLPA known as MS-MLPA (Methylation-Specific MLPA) is also used, which combines the use of MLPA with that of restriction enzymes that allow detecting whether the DNA sequence is methylated or not.

4. The aforementioned techniques (except for the sequencing of the whole genome) do not usually allow the identification of mutations that affect **regulatory regions** that are outside the coding region, nor some of those that cause alterations in the splicing (process of cutting and elimination of intronic regions in RNA and exon regions joining to form mature messenger RNA). If the latter are suspected, not genomic DNA should be sequenced, but rather that synthesized in vitro from RNA (cDNA).

5. Most sequencing and genotyping procedures are aimed at examining genomic DNA. In case of suspicion of an alteration of the **mitochondrial DNA**, procedures specifically directed to this end are needed[4].

6. Genetic tests are usually carried out on blood samples, which are easily accessible and useful for detecting germ-line mutations, that is, those present in all tissues of the body. However, as mentioned, some processes are due to **somatic or postzygotic mutations**, so that only some cells carry the mutation. In these cases, it is necessary to carry out the study in the affected tissue, since the results will be normal in the blood and other non-involved tissues.

Osteoporosis and other processes with decreased bone mass

In the vast majority of patients with osteoporosis, the disease appears in older adults or the elderly. It is the result of the interaction between genetic predisposing factors and environmental factors, together with skeletal deterioration induced by the decrease in sex hormones and other phenomena associated with it. aging. In general, susceptibility has a polygenic basis, determined by several tens or hundreds of genetic variants that, although with limited functional influence in isolation, together have a notable influence on bone mass. Thus, genome-wide association studies (GWAS) and some candidate gene association studies have identified more than 500 loci associated with bone mineral density or risk of fracture[5-7]. Efforts are being made to try to combine these loci into indices (often referred to as polygenic risk indices) that help determine individual risk of osteoporosis[8]. However, its applicability to the clinic is still very limited.

Occasionally, osteoporosis is the result of a specific mutation that has a marked functional impact and alters a gene with an essential role in skeletal homeostasis. Among the "monogenic" forms of osteoporosis, cases due to mutations in the LRP5, WNT1, DKK1 or PLS3 genes have been described[5].

Cases of juvenile or childhood osteoporosis, as well as those with a particularly strong family history, especially if they appear at an early age, are more likely to be due to point mutations. Genetic studies in juvenile and young adult osteoporosis have not consistently established a genetic basis. However, in some patients mutations have been detected in genes involved in skeletal homeostasis, in particular, some related to the Wnt pathway, such as LRP5, WNT1 or DKK1, or in collagen synthesis[9]. Therefore, in these patients it may be interesting to analyze a panel of genes that includes those most frequently related to skeletal disorders. Of course, before the genetic study it is indicated to rule out that osteoporosis is secondary to other systemic disorders (malabsorption, hyperthyroidism, etc.).

If there is no secondary cause of osteoporosis, it is also worth making sure that there is no other genetic disorder associated with osteoporosis. Among them, the most important is osteogenesis imperfecta. It presents with fragility fractures and, in some cases, bone deformities and blue or gray scleras. In most cases it is due to mutations in the genes that encode the alpha and beta

chains of collagen type 1 (COL1A1 and COL1A2), so the study can begin by analyzing these genes. In a recent series of 364 patients with various clinical forms of OI, 50-66% had COL1A1 mutations and 18-37% had COL1A2 mutations. However, in 20% of the cases, no mutations were found in the type 1[10] collagen genes. These cases may be due to mutations in non-analyzed regulatory regions, but mutations in other genes, which are also associated with lesser forms, must be ruled out. frequent symptoms of osteogenesis imperfecta[11,12] (Table 2).

In any case, one must always pay attention to the presence of other associated manifestations that suggest conditions in which osteoporosis is part of a systemic or syndromic process (for example, Turner syndrome, neurofibromatosis type 1, Marfan syndrome, etc.), which require a different diagnostic approach.

Osteopetrosis and other disorders with osteosclerosis

Disorders associated with osteosclerosis are much less common than those associated with decreased bone mass. Along with some acquired (osteoblastic metastases, myelofibrosis, fluorosis, etc.), others have a genetic origin[13]. They can be generalized or localized.

Among the forms of generalized osteosclerosis, osteopetrosis is the most common disorder. It may be due to mutations in several of the genes with an important role in osteoclastic activity, especially CLCN7 and TCIRG1 (which encodes the proton transporter ATPase). Other disorders that present with diffuse increase in bone mass are sclerosteosis and Van Buchem's disease, due to mutations in the SOST gene, which encodes sclerostin[14]. Probably the most effective approach to the genetic study of diffuse osteosclerosis includes the analysis of a panel of genes that includes those most frequently involved in these processes (Table 3). If the results are negative, the next step would be the sequencing of the entire exome.

The forms of localized osteosclerosis include various disorders, the diagnosis of which is generally based on the clinical and radiographic characteristics of the process, but whose genetic basis is generally not well established[15].

Mineral metabolism disorders

The approach to identifying the genetic basis for inherited disorders of mineral metabolism depends on the specific disorder in question.

Hypocalcemia

The most common genetic cause is pseudohypoparathyroidism, due to a loss of function of the GNAS gene[16,17]. This gene, which encodes a protein related to the G protein signaling pathway, is characterized by having a genetic imprint. That is, in most tissues the alleles transmitted by the two parents are expressed, but in some (such as the kidney, the pituitary, the gonads or the thyroid) only the maternal allele is expressed. Therefore, the inactivating mutations of the maternal allele cause resistance to PTH and other hormones, as well as a characteristic skeletal phenotype (short stature, rounded face, short metacarpals), which constitutes the so-called "hereditary Albright osteodystrophy". However, when the mutated allele is of paternal origin, osteodystrophy appears, but without hormonal alterations (pseudo-pseudohypoparathyroidism). The alterations of the GNAS gene that cause these conditions are of various types, including point mutations, structural abnormalities and alterations in the usual methylation patterns. Therefore, in suspicious cases, if the conventional analysis of the GNAS sequence does not reveal anomalies, the study should be deepened by resorting to other techniques (MLPA and MS-MLPA, above all)[18].

Table 2. Genes involved in monogenic forms of osteoporosis and osteogenesis imperfecta

Genes related to collagen synthesisand maturation	COL1A1 COL1A2 CRTAP PPIB P3H1 FKBP10 PLOD2 SERPINH1 BMP1
Genes related to other matrix proteins or osteoblastic activity	SPARC SERPINF1 IFITM5 PLS3 TMEM38B WNT1 SP7 (osterix) CREB3L1 MBTPS2 TENT5A (FAM46A) CCDC134

Table 3. Genes causing some processes that occur with increased bone density gen disease inheritance pattern

Gen	Disease	Pattern of inheritance
CLCN7	Osteopetrosis	AD/AR
TCIRG1	Osteopetrosis	AD/AR
CA2	Osteopetrosis	AR
OSTM1	Osteopetrosis	AR
SNX10	Osteopetrosis	AR
LRP4, LRP5	Various forms of hyperostosis	AD/AR
SOST	Sclerosteosis, Van Buchem disease	AD/AR
CTSK	Pycnodysostosis	AR
FAM20C	Raine syndrome	AR
GJA1	Oculodentodigital dysplasia	AD/AR
LEMD3	Osteopoikilosis	AD
TGFB1	Camurati-Engelmann disease	AD

Hypercalcemia

Genetic causes of hypercalcemia include familial hypocalciuric hypercalcemia, which is usually due to inactivating mutations in the gene that encodes the calcium

sensing channel (CASR). In case of suspicion, then, the study will begin with the analysis of this gene. If CASR mutations are not detected, the study will be extended to other genes (GNA11, AP2S1), which are responsible for a third of the cases. Interestingly, activating mutations of the CASR or GNA11 genes give rise to rare hypocalcemia of autosomal dominant inheritance[19].

Familial hyperparathyroidism may occur within a syndrome of multiple endocrine neoplasms (MEN1 or 2, often with abnormalities in the MEN1 or RET genes, respectively) or in isolation. The genetic basis of these latter cases is not always known, but some patients have mutations in the HRPT2 gene (CDC73) or in the CASR.

Hypophosphatasemia
The persistent decrease in alkaline phosphatase levels, in the absence of anti-resorptive treatment or other acquired causes that explain it, should lead to suspect an alteration of the ALPL gene, which encodes non-tissue-specific alkaline phosphatase (bone, liver). Therefore, in these cases the initial study should be aimed at sequencing said gene. Mutations in this gene give rise to hypophosphatasia, which can have serious phenotypic repercussions when it occurs in children, but is usually much milder if it occurs in adults. In fact, many cases in adults are asymptomatic or have only mild, nonspecific symptoms. However, detailed study may reveal subtle remodeling alterations that may be associated with an increased risk of adverse effects with anti-resorptive drugs[20,21].

Hypophosphatemia and other rickets
Family tree analysis usually gives very important information about the type of inheritance. The most common form of inherited rickets is X-linked hypophosphatemic rickets, due to mutations in the PHEX gene. Autosomal inherited rickets include, among others, those due to mutations in genes related to vitamin D (vitamin D-dependent rickets), such as those encoding renal hydroxylase involved in the synthesis of 1,25-dihydroxyvitamin D (CYP27B1) and the vitamin D receptor (VDR). Other autosomal inherited rickets are due to mutations in the FGF23, DMP1, ENPP1, and SCLC34A3[22] genes.

Developmental disorders

Many developmental disorders include abnormalities in the growth or shape of the bones. In some cases, they are part of complex syndromes, involving multiple organs and systems. The detailed study of the phenotype is essential to focus the genetic study. In many cases, these disorders are due to alterations in large chromosomal regions that affect several genes, in which case the first step may be aimed at identifying structural alterations, using karyotype, CGH arrays, or SNP arrays. Chondrodysplasias are a large group that includes more than 350 disorders in which the alteration of endochondral or endomembranous ossification causes often serious alterations of the skeleton[23,24]. They may or may not be accompanied by injuries at other levels. The phenotype can be very indicative in some cases with typical characteristics (for example, in achondroplasia) and the diagnosis can be confirmed by the targeted analysis of one or a few genes. However, in other cases with a less characteristic phenotype, it will be necessary to study, using massive sequencing procedures, a large panel of about 50-100 genes, or even to sequence the entire exome.

Localized lesions

Some single or multiple focal skeletal lesions may also have a genetic cause.

Paget's disease
Although Paget's disease can show a familial aggregation, in most cases the gene responsible for genetic susceptibility is not identified. However, in some cases it may be the result of point mutations in genes related to the sequestosome (SQSTM1/p62) or the RANKL pathway. Sequestosome mutations are identified in approximately 25-40% of familial Paget cases and 4-8% of sporadic cases[25,26]. On the other hand, there are forms of juvenile Paget's disease and other conditions with skeletal and extraskeletal involvement due to mutations in the TNFRSF11A and TNFRSF11B genes (encoding RANK and osteoprotegerin, respectively)[27].

Multiple exostoses
Patients with multiple exostoses develop excretory lesions (osteochondromas) at the level of the metaphyses from the first years of life. They can be asymptomatic, cause pain or affect growth, especially of the long bones of the extremities. It is generally due to mutations in the EXT1 gene, or, less often, the EXT2 gene, with autosomal dominant inheritance[28].

Multiple enchondromatosis
It also occurs with multiple skeletal lesions in children and young people, but, unlike exostoses (osteochondromas), enchondromas typically grow inside the bone. The cause is not firmly established, but somatic mutations of the PTHR1, IDH1, or IDH2[29] genes have been found in the enchondromas of some patients.

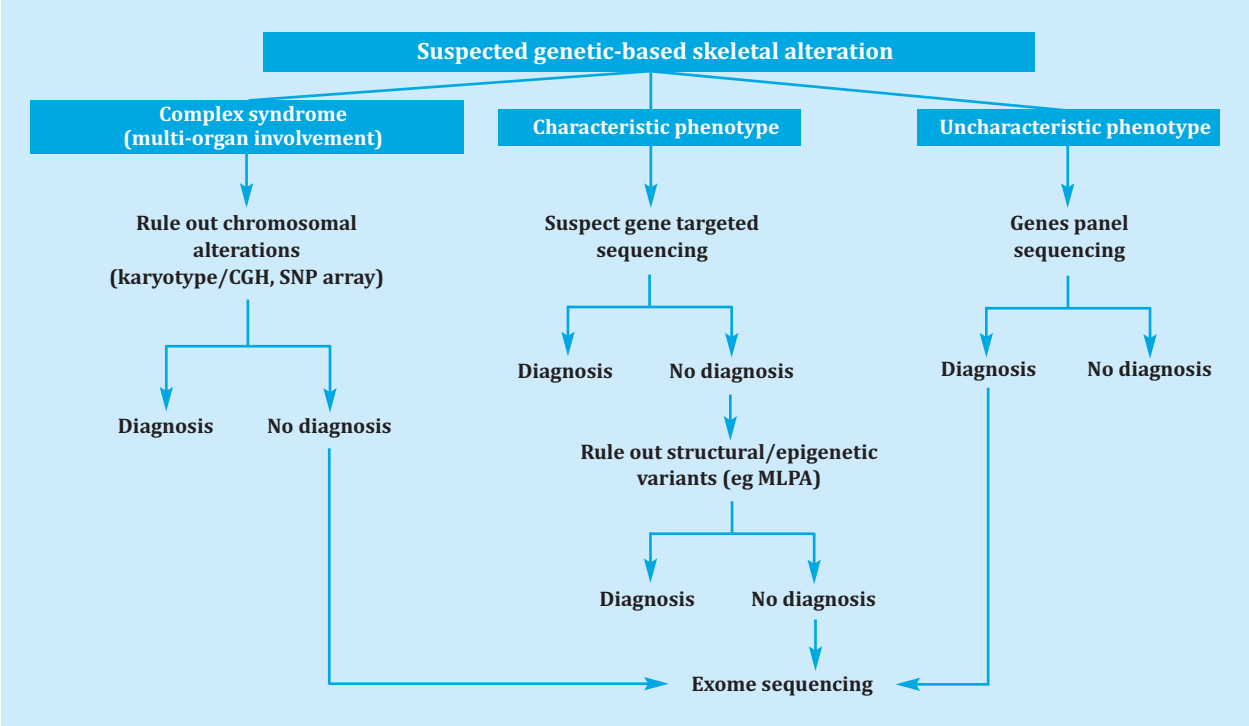
Fibrous bone dysplasia
Fibrous bone dysplasia can manifest as single or multiple bone lesions. Sometimes it is part of McCune-Albright syndrome, which also includes hyperpigmented skin macules and/or alterations due to an increase in the production of some hormones (precocious puberty, hyperthyroidism, hypercortisolism, excess GH). Excessive production of FGF23 in lesions makes hypophosphatemia a frequent manifestation. It is due to an activating somatic (postzygotic) mutation in the GNAS gene, which encodes a protein involved in the G protein signaling pathway[30]. Therefore, if a genetic study is to be carried out, it must be done with affected tissue, since the result in blood cells is usually normal.

Conclusion

The study of the genetic cause of a disorder depends on the suspected condition based on a detailed analysis of the phenotype. One of the first questions to answer is whether a disorder is suspected due to a mutation affecting a gene, or a broader chromosomal alteration. In the first case, the approach usually begins with the sequencing of the suspicious gene or genes, while in the second case, the procedures that allow the detection of structural variants, in general some type of array, are indicated.

If a specific genetic alteration is suspected, sequencing can focus on a gene, on a panel of related genes, or on the entire exome, depending on whether a gene is clearly suspicious, that is, a condition with genetic heterogeneity (Figure 1). The specific strategies depend not only on the condition under study, but also on the

Figure 1. Initial approach to skeletal disorders of genetic cause



availability of the diagnostic tests and protocols implemented in each center. However, it is likely that in the coming years, when costs are lowered and analytical procedures streamline, whole-exome sequencing will progressively replace gene panels. In fact, already at this moment, the realization of "virtual panels" can be more efficient in many cases[31]. In other words, the entire exome is sequenced, although at first only the existing variants in the genes potentially related to the phenotype are analyzed, thus reducing the number of mutations to be assessed by bioinformatics and literature analysis. However, since the entire exome was actually sequenced, if the initial analysis of the selected genes does not reveal pathogenic mutations, the study can be extended to other genes, without the need to sequence the sample again.

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FGF-23 and PTH, mirror hormones. Their role in bone metabolism

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Summary
FGF-23 and PTH are two fundamental proteins in bone metabolism closely related since FGF-23 directly regulates both the expression and secretion of PTH.
PTH is the main regulator of the RANK/RANKL/OPG system considered essential for bone shaping and remodeling, but it is also an important regulator of the Wnt pathway in the bone, key to bone formation. Decrease of Wnt pathway inhibitors in the bone due to high levels of PTH could contribute to maintaining bone health, but also favor vascular calcification in the vessels. On the contrary, the action of FGF-23 would be opposite to that of PTH as by inhibiting the Wnt pathway in the bone, it will contribute to the loss of bone mass, while attenuating vascular calcification in the FGF-23 vessel.

Introduction

Conventionally, calcium, phosphorus, calcitriol and PTH were considered the only regulators of bone and mineral metabolism. In recent years, this axis of regulation has been complicated due to emergence of other factors with a crucial role in bone and mineral metabolism, such as fibroblast growth factor 23 (FGF-23) and the so-called klotho anti-aging protein.

Biological actions of FGF-23 and PTH

Biological action of FGF-23

FGF-23 is a 251 amino acid protein synthesized and secreted by bone cells, mainly osteoblast[1]. FGF-23 has been identified as the main regulatory factor of phosphorus metabolism, a critical element for maintaining skeletal integrity and for the development of multiple enzymatic processes[2]. In addition, in the last decade it has been attributed a notable role in the pathophysiology of vascular calcifications[3] and cardiovascular disease (CV), both in the general population[4-6] and in patients with chronic kidney disease[7].

The biological action of FGF-23 depends on the expression of a gene that acts as its co-receptor, called klotho[8]. Klotho is a 130-kDa transmembrane protein predominantly expressed in the renal distal tubule and to a lesser extent in the parathyroid gland and choroid plexus[9]. Klotho increases the affinity between FGF-23 and its FGFR receptors, forming a klotho/FGFR complex[10]. The final action of FGF-23 is carried out through its binding to the klotho/FGFR complex, although FGF-23 is capable to act independently of klotho through the FGFR4 receptor via the calcineurin pathway in cardiac and liver tissue[11-13].

The biological actions of FGF-23 take place in different organs: parathyroid gland, choroid plexus, pituitary and the kidney, being the later the main target organ. At a bone level, FGF-23 indirectly influences mineralization through the control of serum phosphorus and calcitriol levels. At the same time, serum calcitriol levels are one of the main regulators of FGF-23 production. In animal models, it has been observed that calcitriol stimulates in a direct and dose-dependent way the secretion of FGF-23 by the osteoblast[14]. This system makes it possible to maintain serum phosphorus levels within narrow margins[15]. In those situations in which there is an increase in the levels of calcitriol and, therefore, in the gastrointestinal absorption of phosphorus, the stimulation of the production of FGF-23 by the osteoblast will favor phosphaturia to avoid hyperphosphatemia.

The increase in serum phosphorus levels stimulates the production of FGF-23 by the bone and vice versa[16]. Although in murine models, increments of phosphorus in the diet influence the serum concentration of FGF-23[14,16], clinical trials assessing the effect of phosphorus ingestion on the levels of FGF-23 and phosphaturia have shown contradictory results. While some authors have not found an association between FGF-23 levels and phosphorus overload[17,18], others have described notable increases in circulating FGF-23 levels after several days of following a diet high in phosphorus[19-21].

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This discrepancy between studies has been attributed to differences in sample size, duration of phosphorus overload, to the time FGF-23 levels were identified, and to the patients' diet control. A possible explanation for these results may be the fact that acute phosphorus overload leads to a rapid response in PTH secretion, which increases phosphaturia within a few hours, while FGF-23 secretion would decrease with chronic and sustained phosphorus overload[18,22].

Biological action of PTH

Parathyroid hormone (PTH) is an 84 amino acid peptide hormone synthesized in the main cells of the parathyroid glands. It is essential for the maintenance of serum calcium concentration within narrow limits through direct actions on bone and kidney, and indirectly through actions on the gastrointestinal tract[23]. PTH also regulates phosphorus metabolism[24], decreasing its serum levels by inhibiting renal phosphate reabsorption in the distal and proximal tubules, although the effect in the later is quantitatively the most important[25].

PTH is released from parathyroid cells in a pulsatile, circadian fashion. The synthesis and secretion of PTH are controlled by the calcium sensing receptor (CaSR) expressed on the membrane of parathyroid cells[26]. The signal for starting the production and secretion of PTH is a drop in the concentration of extracellular ionic calcium, while the signal for decreasing its production and secretion is an increase in extracellular ionic calcium. To a lesser extent, PTH secretion can also be stimulated by increasing phosphorus levels, either directly or by reducing calcium levels[25].

One of the key mechanisms by which PTH regulates calcium homeostasis is related to the direct actions of PTH on osteoblasts and osteocytes and its indirect effects on osteoclasts. Although PTH stimulates both bone resorption and formation, the end result of the net bone balance will depend on the dose and periodicity of the PTH signal. Continuous exposure to PTH produces catabolic effects in the skeleton, while low and intermittent doses of PTH produce anabolic effects in the bone[27]. The best characterized catabolic effect of PTH excess occurs in primary hyperparathyroidism, with bone loss at both cortical and trabecular levels[28-32]. On the contrary, the PTH amino terminal peptide 1-34, teriparatide, and PTH intact molecule (PTH 1-84) have an anabolic action on the treatment of osteoporosis when administered in low doses in a pulsatile or intermittent manner[33,34].

The actions of PTH are mainly mediated by a receptor called PTH1R. The two forms of administration of PTH, continuous and intermittent, can regulate different genes at the bone level in different ways, thus promoting bone resorption or formation[35,36].

Interaction between FGF-23 and PTH

FGF-23 regulates PTH secretion. Several studies, both in vivo and in vitro, have shown that FGF-23 has a direct inhibitory effect on PTH, decreasing the expression and protein secretion of PTH, in a dose-dependent manner[37,38].

As with calcitriol, serum PTH levels regulate FGF-23 levels. PTH can stimulate the secretion of FGF-23 by the osteoblast[39]. Studies in murine models with primary hyperparathyroidism show increases in FGF-23 levels that are reversed after parathyroidectomy. These same results have been reported by Carrillo-López et al. in rat models with secondary hyperparathyroidism, where parathyroidectomy can reduce FGF-23[40] levels by three times. PTH would act as a stimulator of FGF-23 production in hypercalcemia caused by hypersecretion of PTH. The boost of FGF-23 would increase the renal elimination of phosphorus, avoiding tissue damage by preventing the potential appearance of extraosseous calcification caused by the dangerous association of hypercalcemia and hyperphosphatemia.

FGF-23 and PTH. Its regulation in bone metabolism

RANK/RANKL/OPG system

PTH is the primary regulator of the receptor activator of NFkappa beta (RANK)/RANK ligand (RANKL)/osteoprotegerin (OPG) system that controls bone remodeling by inducing RANKL synthesis by osteoblasts, and negatively regulating OPG production. Both mechanisms favor osteoclastogenesis and bone resorption through a mechanism driven by protein kinase A (PKA)[41-43], since PKA agonists mimic PTH regulation of RANKL and OPG gene expression[42,44].

The RANK/RANKL/OPG system was identified in the mid-1990s as an essential regulator of bone shaping and remodeling[45]. Its role in bone maintenance is well known, but recent studies give it an important role in the calcification of vascular smooth muscle cells.

In the bone, osteoblasts and osteocytes synthesize and secrete RANKL, which binds to its transmembrane receptor RANK on bone marrow-derived osteoclast progenitors, allowing osteoclast maturation, activation and survival in order to initiate bone resorption. In addition, osteoblasts secrete OPG, a soluble decoy receptor for RANKL, which prevents the binding of RANKL to RANK, thus attenuating osteoclastogenesis[46].

Wnt/β-catenin pathway

PTH is also an important regulator of the Wnt/β-catenin pathway in the bone[47]. The activation of the signaling of the Wnt/β-catenin pathway is essential for bone formation[48,49] and has also been involved in the vascular calcification process[40,50-52].

The action on the Wnt pathway inhibitors in the bone is one of the most promising therapeutic goals in the prevention and treatment of the bone mass reduction, since the activity of this pathway is essential for the optimal remodeling and mineralization of the skeleton[53].

The rosozumab´s proven efficacy (an antibody against the best known Wnt pathway inhibitor, sclerostin (SOST)) in reducing bone loss in postmenopausal women, represents another therapeutic option for the treatment of these disorders[54]. SOST actions could include those on the vascular system. It is important to highlight that, in addition to its direct control of the bone remodeling and mineralization, SOST influences serum concentrations of calcitriol and FGF-23, both involved in the mineralization process[55].

Serum SOST increases in parallel with phosphorus, PTH and FGF-23[49,56,57], possibly due to its reduced renal clearance[58], and the use of anti-SOST monoclonal antibodies has been effective in preventing bone loss in normal rats or with chronic renal failure and low PTH[59]. However, anti-SOST therapy could not prevent bone damage in rats with the same degree of kidney damage, but with elevated PTH. These results contradict the finding by Cejka et al.[60], who suggested that serum SOST, a Wnt pathway inhibitor, could be an even more sensitive and accurate marker for bone remodeling than circulating PTH.

Studies in humans[56], in a mouse model with slowly developing polycystic disease[56,57] and in a model of chronic kidney disease with hyperphosphatemia[49], have shown that increased SOST in bone precedes serum increases in phosphorus, PTH and FGF-23. Increases in serum phosphorus, PTH, and FGF-23 simultaneously with the Wnt pathway signaling bone inhibition coincide with decreases in bone SOST, but with increases in other Wnt pathway inhibitors[49,56,57]. In fact, bone biopsies from patients with chronic kidney disease have shown that a greater inhibition of the Wnt pathway links to low levels of SOST in osteocytes[56], suggesting the contribution of other Wnt pathway inhibitors.

Our recent studies, analyzing the direct effect of PTH and FGF-23 on osteoblasts, have revealed that elevated PTH inhibits not only SOST increases, but also other Wnt pathway inhibitors, and that FGF-23 may have a direct inhibitory effect on the Wnt pathway in osteoblasts through the induction of DKK1[49].

SOST inhibition and other Wnt pathway inhibitors of the bone due to high levels of PTH could contribute to maintaining bone health, but it is important to note that the reduction of PTH of the Wnt pathway inhibitors in the vessels could favor vascular calcification. In fact, as mentioned above, recent studies in rats with chronic kidney disease fed a diet high in phosphorus, with both elevated and normal PTH levels (parathyroidectomy with supplementation of PTH 1-34 to avoid hypocalcemia) suggest that an elevated PTH favors vascular calcification. In contrast, normal circulating PTH levels were protective against aortic calcification despite elevated serum phosphorus[40]. In vitro studies confirmed this fact, showing that high doses of PTH in vascular smooth muscle cells subjected to a calcifying stimulus aggravated the calcifying process, while low doses of PTH were able to inhibit the calcification process, showing calcium content and osteogenic gene expression similar to those of cells not subjected to the calcifying stimulus[40].

On the contrary, the action of FGF-23 would be opposite to that of PTH, since, by inducing increases in DKK1, FGF-23 would inhibit the Wnt pathway in the bone, contributing to the loss of bone mass, while it could attenuate vascular calcification in the FGF-23 vessel.

Conclusion

The role of the regulatory axis consisting of calcium, phosphorus, calcitriol, PTH, FGF-23 and klotho exert on the activation or inactivation of the Wnt pathway, as well as the precision of the serum levels of Wnt activators and inhibitors to reflect its changes in a bone and vascular level, could allow the design of therapeutic strategies to prevent the bone-vessel axis deterioration.

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Health and economic impact of the use of vitamin D/calcium for fracture prevention: literature review

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Summary

Objetives: Health policies regarding fracture prevention programs must consider the health and economic impact of strategies such as the intake of vitamin D/calcium-fortified foods or vitamin D/calcium supplements. We review the available evidence on these strategies in terms of health and cost-effectiveness benefits.

Material and methods: We searched PubMed/MedLine's data bases to identify published studies from the last 10 years (up to December, 2020) assessing the impact of vitamin D/calcium-fortified foods or vitamin D/calcium supplements intake for fracture prevention connected to health and cost-effectiveness benefits.

Results: 11 articles were included in total. On one side, the identified studies suggest substantial benefit regarding fracture prevention, mortality, and life years and quality-adjusted life years gained. On the other side, economical assessment reveal that the use of vitamin D/calcium-fortified foods or vitamin D/calcium supplements are cost-beneficial, at least for the population over aged 70 or with high fracture risk. In addition, these strategies seem to save direct costs, especially for elderly women with high fracture risk.

Conclusions: The use of vitamin D/calcium-fortified foods or vitamin D/calcium supplements reduces the amount of fragility-induced fractures, and so, it is a potentially favourable strategy, economically speaking.

Key words: vitamin D, costs and cost analysis, public health, dietary supplement, fortified foods.

Introduction

Osteoporotic fractures, especially those of the hip, are one of the main causes of disability in the elderly population, triggering a considerable decrease of life quality and lifespan. Besides, more than 30% of people die during the first year after suffering one of these fractures[1]. In 2010, the European Union recorded nearly 3.5 million fragility-induced fractures that led to 43,000 deaths. From an economical point of view, these fractures meant an expenditure of 37 billion euros, a sum that is expected to rise by 52% in 2025[2].

Vitamin D and calcium are essential compounds for bone metabolism and prevention of osteoporotic fractures. Two recent meta-analyses have reported that low levels of 25(OH)D are related to the increase of fragility-induced fractures due to bone mass loss and bone structure deterioration[3,4].

In Europe, the prevalence of vitamin D deficiency (defined as 25(OH)D <20ng/ml) is estimated to be 40%[5]. One of the natural sources of vitamin D is the exposure to the sun. However, due to factors such as latitude, physical inactivity or the use of sun creams, the synthesis of vitamin D obtained in this way tends to be insufficient. In fact, and contrary to what one might surmise, this deficiency is lower in the north of Europe than in the south (<20% vs. 30-60%, respectively)[6] despite receiving less sunlight. One of the reasons for this difference is the increased consumption of vitamin D-fortified foods or vitamin D/calcium supplements in Nordic countries[6].

With the aim of reversing this situation, it is paramount to ensure an adequate intake of vitamin D and calcium. The Spanish Society for Bone and Mineral Metabolism Research (SEIOMM), the Spanish Society of Rheumatology (SER) and the Spanish Society of Endocrinology and Nutrition (SEEN) consider key to ensure levels of 25(OH)D to be at least 30ng/ml[7-9]. To maintain an adequate level of 25(OH)D, an intake of 400-1,000 IU/day and 500-1,200 mg/day of calcium are required, preferably from foods. These amounts may vary according to age, patient type and guidelines consulted[7-9]. In the case of patients with osteoporosis and vitamin D deficit, SER recommends a daily intake of 800-2,000 IU of vitamin D supplements, depending on their baselines[9]. Unfortunately, calcium ingestion, and especially that of vitamin D, is not enough, as 38% and 93% of people do not consume them, respectively[10].

The aim of the vitamin D/calcium supplements is to decrease the fragility-induced fracture risk. Nevertheless, there is a debate about the benefits of supplementation on the general population. While some studies suggest that an ideal vitamin D/calcium intake reduces the fracture risk[11,12], other studies indicate that the decrease of this risk

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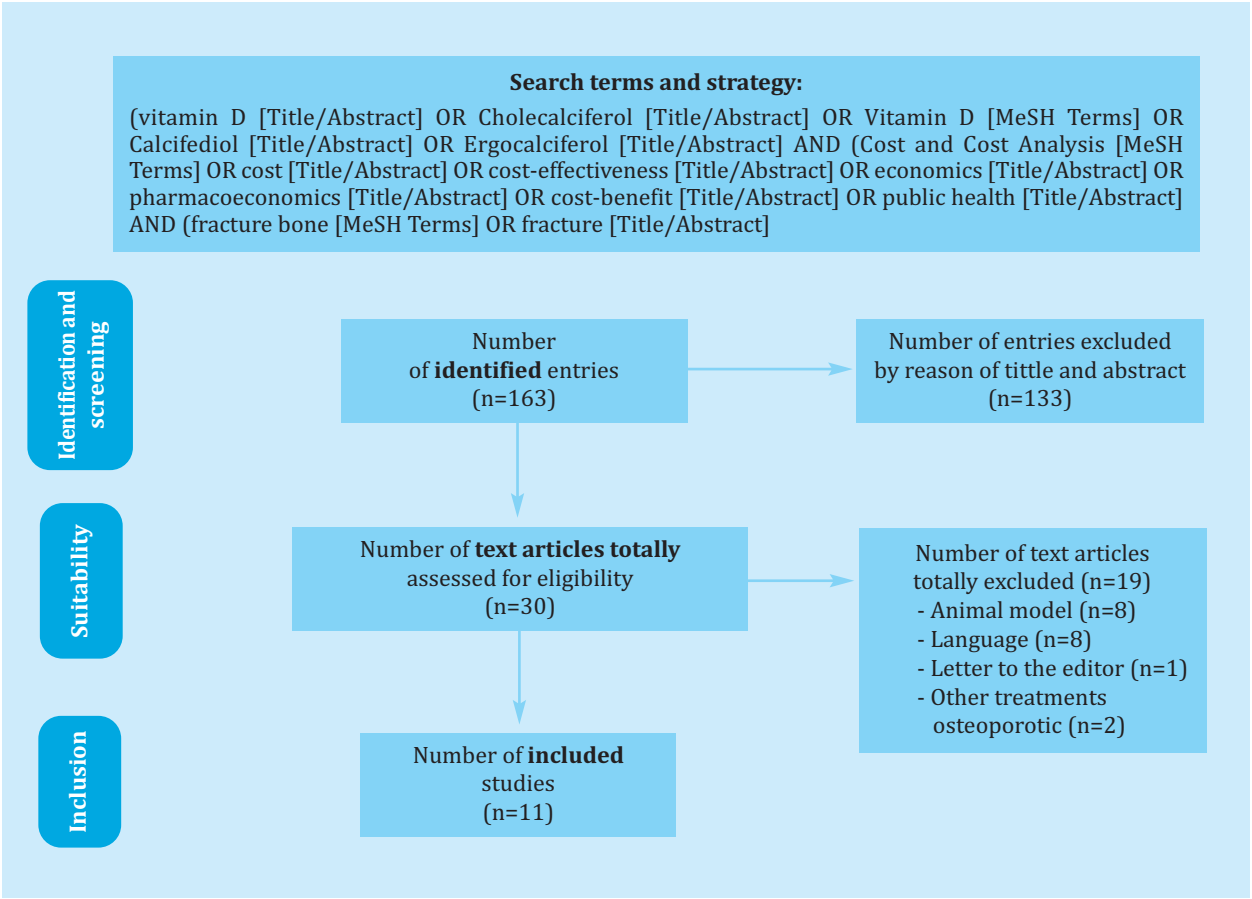
Figure 1. Search strategy and PRISMA diagram



is not of statistical significance[13,14]. Despite this, it is important to state that a considerable proportion of people with optimal vitamin D values are included in most of the studies aimed to analyse the effects of supplementation. That is it cannot be assumed that supplements lack of effect on people with hypovitaminosis. In fact, the lower the concentration of 25(OH)D, the higher the response[15]. Likewise, combined analyses imply that the decrease of fractures thanks to vitamin D supplements would only be possible in people with vitamin D deficit[13].

Vitamin D supplements are not expensive, however, given the high number of people who need them, the monitoring of these people's 25(OH)D serum levels could pose a considerable cost. In order to assist those responsible for the shaping of health policies on preventive nutrition programs, it is important to gauge the health and economic impact that consuming vitamin D-fortified foods or vitamin D/calcium supplement has.

This review aims to identify and outline the available evidence on vitamin D/Ca supplements in order to prevent fragility-induced fractures in terms of health and cost-effectiveness benefits.

Materials and methods

We carried out a review of the PubMed international database in order to identify those studies regarding the economical or health impact of vitamin D/calcium- fortified foods or vitamin D/calcium supplements intake. In order to do that, we have used a combination of MeSH terms and less specific terms related to vitamin D, costs and fragility-induced fractures.

We selected all the articles published in English or Spanish over the past 10 years (up to December, 2020). We excluded those articles assessing vitamin D/calcium supplements in combination with other drugs, as well as those about the effects of vitamin D/calcium supplements, clinical trials, letters to the editor and speeches at conferences.

Results

Characteristics of the studies

163 studies were found in total, 11 of which were finally included (Figure 1)[16-26].Their characteristics are shown in table 1.

The studies assess the use of vitamin D supplements [n=1], vitamin D/calcium supplements [n=5] and vitamin D/calcium-fortified foods [n=5] for fracture prevention from two angles:

1) Health benefits [n=10] (deaths prevented, fractures prevented, life years gained, quality adjusted life years gained -QALYs- and net cost).
2) Economical assessment [n=11] (€//QALY gained, €/life years gained -LYG- and cost-effectiveness incremental ratio -ICER-).

The studies were carried out in France (n=3), The Netherlands (n=3), Germany (n=1), United Kingdom (n=1), Norway (n=1), USA (n=1) and USA/Europe (n=1). We did not find any studies conducted in Spain.

All studies followed a very heterogeneous methodology: economic model, population characteristics, supplementation type, doses, strategy effectiveness and cost-effectiveness threshold (Table 1).

Table 1. Characteristics of the selected articles

Author, year	Country	Method	Age	Sex	Pathology	Study	Intervention	Dosis	HFRr
Childs, 2016[16]	USA	Hindcasting and economic	All	W/M	PF	E	VitD + Ca supplements	VitD: 1.600 IU/day Ca: 1.200 mg/day	5%*
Hiligsmann, 2015[17]	The Nether-lands	Markov microsimulation	≥60	W/M	O	PH/E	VitD + Ca supplements	VitD: 800 IU/day Ca: 1.000 mg/day	18%
Poole, 2014[23]	UK	Economic	≥65	W/M	G	PH/E	VitD supplements	VitD: 800 IU/day	30%
Weaver, 2019[25]	EU USA	Analysis cost benefit	≥50	W/M	O	PH/E	VitD + Ca supplements	VitD: 600 IU/day Ca: 1.000 mg/day	14%
Zarca, 2014[26]	France	Markov microsimulation	≥65	M	G	PH/E	VitD + Ca supplements	VitD: 100.000 IU/ 15-90 days	10%
Hagen, 2016[19]	Norway	Markov state-transitoin	65	M	G	PH/E	VitD + Ca supplements	VitD: 200 IU/day Ca: 1.000 mg/day	16%
Ethgen, 2015[17]	The Nether-lands	Population-based (Markov microsimulation)	≥50	W/M	G/O/RF	PH/E	VitD + Ca fortification	VitD: 800 IU/day Ca: 1.000 mg/day	18%
Ethgen, 2016[18]	The Nether-lands	Markov microsimulation	≥65	M	G/O/RF	E	VitD + Ca fortification	VitD: 400 IU/day Ca: 800 mg/day	18
Sandmann, 2015[24]	Germany	Spreadsheet	≥65	M	G	PH/E	VitD + Ca fortification	VitD: 800 IU/day Ca: 200 mg/day	19%
Hiligsmann, 2017/2018[21,22]	France	Markov microsimulation	≥60	W/M	G	PH/E	VitD + Ca fortification	VitD: 800 IU/day Ca: 1.000 mg/day	16%

HFRr: hip fracture risk reduction; W: woman; M: man; PF: previous fracture; O: osteoporosis; G: general population; RF: high risk of fracture; PH: public health; E: economical assessment; VitD: vitamin D; Ca: calcium; IU: international units *: unwelded fracture.

Health benefits

Different authors have estimated the number of fractures prevented annually attributed to vitamin D/calcium food fortification or vitamin D/calcium supplements: 323,566 (USA), 544,687 (EU), 64,932 (France), 45,800 (United Kingdom), 36,705 (Germany), 30,376 (The Netherlands), 16,130 (Norway) (Table 2). Considering the mortality linked to hip fracture (adjusted by age and sex) Poole et al. esteemed that vitamin D supplements should prevent 1,700 deaths per year in Great Britain[23]. Simultaneously, Ethgen et al. and Hiligsmann et al. suggest, based on the excess of mortality, that 6,605 years (The Netherlands) and 29,169 years (France) could have been gained1[7,21].

One of the most used indicators for public health is the QALYs, which take into account the quantity and quality of life gained. A QALY is a year lived in perfect health. Three of the studies predict a gain of 0.008 to 0.022 QALYs per patient thanks to vitamin D/calcium-fortified foods or vitamin D/calcium supplements[19,20,26]. This fluctuation seems to depend on factors such as age and sex. Ethgen et al. stated that the older the subject, the superior the QALYs gained, regardless of the group of women observed (osteoporosis-free, low bone density or high risk of fracture)[17]. Similar results were reported by Hiligsmann et al., who also noticed that the QALYs gained due to the intervention were higher in women than in men (23,067 vs. 9,502, respectively)[20].

Hagen et al. considered three scenarios for cardiovascular risk due to vitamin D/calcium supplements:

1) Risk-free
2) Medium risk
3) High risk.

The results showed that, while there was a gain of 0.022 QALYs per patient in the first scenario, in the second and third scenarios there would be a net health loss (-0.052 y -0.078, respectively)[19].

Economic impact

From an economic perspective, this supplementation/fortification addressed to the general population seems to be cost-effective from the 70[17,21]-80[18] year age range. In the case of people with osteoporosis, this intervention could be cost-effective from 60[20]-70[18] years of age, and in people with a high risk of fracture, from 50-60 years[17]. Regarding sex, the assessed strategies were even more cost-effective in women, except for the case of those men with high risk of fracture (Table 3).

Zarca et al. analyzed the ICER of four different strategies:

1) Do not treat (comparative value)
2) General treatment with no monitored effectiveness
3) Treating and monitoring
4) Screening and treating the population with vitamin D defiency.

As a result, "treating and monitoring" and "screening and treating" turned out to be cost-effective strategies (5,219 and 9,104 €/QALYs, respectively) and of dominance over "treating but not monitoring". In addition, the acceptability curves showed the screening to be the highest chances to be cost-effective (around 6,000 €/QALYs)[26]. Acceptability curves present the probability that a stra-

Table 2. Main public health results

Autor	Country	Intervention-population	Prevented fractures/year	Prevented fractures/year/100,000	Prevented deaths/year	LYG	Increased gain in QALYs
Childs[16]	USA	S-PF	NE	NE	NE	NE	NE
Hiligsmann[20]	The Netherlands	S-O	NE	NE	NE	NE	0.008-0.021
Poole[23]	UK	S-G	45,800 (hip)	71.2*	1,700	NE	NE
Weaver[25]	EU USA	S-O	544,687 (EU) (hip) 323,566 (USA) (hip)	122.1* (EU) 98.6* (USA)	NE	NE	NE
Zarca[26]	France	S-G	NE	NE	NE	NE	0.015-0.020
Hagen[19]	Norway	S-G	16,130* (all)	306.8*	NE	NE	No CVE: 0.022 Medium CVEr: -0.077 High CVEr: -0.078
Ethgen[17]	The Netherlands	F–G/O/RF	30,376 (hip and vertebral)	178.9*	NE	6,605	NE
Ethgen[18]	The Netherlands	F–G/O/RF	NE	NE	NE	NE	2 servings daily: 0.006-0.026
Sandmann[24]	Germany	F-G	36,705 (all)	45.2*	NE	NE	NE
Hlligsmann[21,22]	France	F-G	64,932 (all)	97.2*	NE	29,169	NE

LYG: life years gained; QALYs: quality-adjusted life years; S: supplementation; F: fortification; PF: previous fracture; O: osteoporosis; G: general population; RF: high risk of fracture; NE: not evaluated or specified; CVE: cardiovascular event; CVEr: cardiovascular event risk*: calculated from the study's original data and population's data at the time of the study.

tegy is optimal for a given cost-effectiveness threshold. To this effect, the percentages of simulations in which the assessed alternative has an incremental cost-effectiveness lower than the threshold for different values of it are calculated.

The net cost of the use of supplements or foods fortified with vitamin D/calcium in order to prevent fragility-induced fractures has been analysed in 7 of the 11 articles included in this review, and we can say there is a great diversity of results among them[16,19-21,23-25]. Hiligsmann et al. appraised the net cost of the intake of fortified foods by the general population over 65 years of age in France to be 1,556 million euros. By contrast, Poole et al. witnessed a net cost of -22 million pounds, due mainly to the savings on over 80-year-olds. In patients with osteoporosis or high risk of fracture, three studies predict savings on the population over 50-65 years of age[16,24,25], while Hiligsmann et al.'s model opts for those over 80[20]. Conversely, Hagen et al. estimated a 322 €/patient net cost, but as high as 1,033 €/patients when there is high risk of cardiovascular events led by calcium supplements[19].

Lastly, Hiligsmann et al. evaluated the economic impact of vitamin D and calcium fortification in France during the next 40 years and they witnessed a progressive benefit intensification, in particular, in 2060, when the cost per QALY gained will fall from 58,244 € in 2015 to 42,616 €[22].

Discussion

This literature review has identified 11 studies assessing the health benefits and the economic impact of vitamin D/calcium-fortified foods or vitamin D/calcium supplements.

The available evidence suggests that the intake of adequate amounts of vitamin D or calcium via fortified foods or vitamin D/calcium supplements may have substantial benefits for the public health (fractures and deaths prevention, LYG and QALYs). From an economical perspective, all models indicate these interventions to be cost-effective, at least regarding the elderly population or those with a higher risk of fracture, even leading to an economical saving.

One aspect to be considered is the economical evaluation is the role of complementary strategies, such as the screening and monitoring of levels of 25(OH)D. This way, only patients with a deficiency in vitamin D could be treated or the treatment effectiveness could be monitored in order to adapt the dosage. Zarca et al.[26] address this perspective when stating that the hypovitaminosis screening, followed by treatment, would be the most cost-effective strategy.

At the moment, there is some controversy about the effect of calcium supplements on cardiovascular risk[27]. Interestingly, Hagen et al. assessed three risk scenarios, concluding that the benefits (on the health and economical) will only appear if vitamin D and calcium supplementation do not lead to a cardiovascular risk rise[19]. Anyhow, it is important to note that the supplementation/fortification negative effects for cardiovascular risk have not yet been proven, and if it exists, it could be related to higher than recommended vitamin D or calcium levels[28,29]. On another note, the vitamin D's potential extraosseous benefits (ex. Pre-eclampsia, diabetes, cancer etc.) at the moment for debate, could lead to improving the registered data or even make up for possible side effects.

Table 3. Main economical results

Autor	Country	Intervention-population	Cost-effectiveness threshold	ICER	Net cost
Childs[16]	USA	S-PF	NA	NE	**-65 866 $/hospital/year** **-27,9 $/patient/years***
Hiligsmann[20]	The Nether-lands	S-O	45,000 €/QALY	60 years: **40,578** (W)/**23,477** (M) €/QALY 65 years: **16,266** (W)/**19,695**(M) €/QALY 70 years: **7,912** (W)/**10,250** (M) €/QALY 80 years: **-12,815** (W)/**-6,723** (M) €/QALY	60 years: 316 (W) /274 (M) €/patient 65 years: 211 (W)/230 (M) €/patient 70 years: 127 (W)/138 (M) €/patient 80 years: **-270** (W)/**-99** (M) €/patient
Poole[23]	UK	S-G	NA	NE	65-69 years: 10,115 £ million/year 70-74 years: 56,1 £ million/year 75-79 years: 12,6 £ million/year 80-84 years: **-39,0 £ million/year** ≥85 year: **-153,6 £ million/year** ≥65 year:: **-22,4 £ million/year**
Weaver[25]	EU USA	S-O	NA	NE	50-59 years: **-1.011 € million/year** (EU); **-700 $ million/year** (USA) 60-69 years: **-1.323 € millon/year** (EU); **-729 $ million/year** (USA) 70-79 years: **-1.911 € million/year** (EU); **-904 $ million/year** (USA) ≥80 years: **-1.462 € million/year** (EU); **-978 $ million/year** (USA) Total: **-5.710 € million/year** (EU); **-3.312 $ million/year** (USA)
Zarca[26]	France	S-PF	WHO threshold	"Treating and monitoring": **5,219 €**/QALY "Screening and treating": **9,104 €**/QALY	NE
Hagen[19]	Norway	S-O	60,000 €/QALY	No CVE: **14,453 €**/QALY Medium CVEr: Controlled High CVEr: Controlled	65 years: a) 322 €/patient (no CVE) b) 322 €/patient (CVEm) c) 1,033 €/patient (CVEa)
Ethgen[17]	The Nether-lands	F-G/O/RF	120,000 €/LYG	2 servings daily (G/O/RF), women : 50 years: 296,532/300,277/168,701 €/LYG 60 years: 184,479/174,359/**96,744 €**/LYG 70 years: **91,430**/**74,707**/**35,687 €**/LYG 80 years: **30,759**/**19,910**/**3,369 €**/LYG 2 servings daily (G/O/RF), men: 50 years: 255,753/203,563/**118,823 €**/LYG 60 years: 151,392/121,582/**70,057 €**/LYG 70 years: **85,627**/**61,349**/**31,423 €**/LYG 80 years: **37,048**/**24,231**/**8,916 €**/LYG	NE
Ethgen[18]	The Nether-lands	F-G/O/RF	45,000 €/QALY	2 servings daily (G/O/RF): 65 years: 123,122/56,498/48,018 €/QALY 70 years: 62,975/**32,467**/**32,685 €**/QALY 80 years: **15,576**/**6,868**/**3,390 €**/QALY	NE
Sandmann[24]	Germany	F-O	NA	NE	≥65 years: **-314.8 € million/year**
Hiligsmann[21]	France	F-G	30,000 €/QALY	2 servings daily: 60-69 years: 155,006 (W)/218,176 (M) €/QALY 70-79 years: **24,997** (W)/92,676 (M) €/QALY ≥80 years: **1,907** (W)/**27,683** (M) €/QALY ≥60 years: 38,256 (W)/106,113 (M) €/QALY	≥60 years: 1.556 € million
Hiligsmann[22]	France	F-G	30,000 €/QALY	2 servings daily, ≥60 years (2015/2040/2060): 58,244/45,732/42,616 €/QALY	NE

In bold: cost-effectiveness or net saving; ICER: cost-effectiveness incremental ratio; S: supplementation; F: fortification; PF: previous fracture; O: osteoporosis; G: general population; RF: high risk of fracture; NE: not evaluated or specified; NA: not applicable; W: woman; M: man; QALYs: quality-adjusted life years; LYG: life years gained; CVE: cardiovascular event; CVEr: cardiovascular event risk; WHO: World Health Organization.

In long-term health policies, forward-looking projections could be very interesting, even more so considering that population's aging leads to an increase in the incidence of osteoporosis, and this to fragility-induced fractures[2]. In this light, Hiligsmann et al. noticed a clear tendency towards the increase of health benefits (with the avoidance of up to 78% of the fractures) and the rising of cost- effectiveness[22].

Our literature review, however, has a series of potential limitations. On the one hand, it is limited to a single database (PubMed/Medline) and we were not able to assess the quality of the evidence. Besides this, and due to the different methodology of the practices, we were not able to directly compare them.

At the same time, many of the identified studies present their own limitations:

1)The studies assume the effectiveness of vitamin D and calcium supplements shown in the literature this effectiveness is thus extrapolated to the fortification of foods, sometimes following different doses.

2) Hardly any possible side effects have been taken into consideration (only one study does so), nor different potential benefits of the supplementation/fortification.

3) Most of the studies showed 100% adherence.

4) Those articles assessing fortified foods calculate the costs for the Health System and do not take into consideration people's established practices. In other words, the percentage of people who are already consuming the recommended fortified portions and that of those who are not. Regarding the latter, only the difference between the cost of fortified and non-fortified products should be calculated.

In conclusion, the available evidence suggests that the intake of vitamin D/calcium-fortified foods or vitamin D/calcium supplements reduce the fracture risk (with resulting health benefits) and it is a cost-effective strategy which can even allow cutting costs regarding certain sub-populations. Nevertheless, more studies should be needed, especially observational studies concerning the Spanish context, in order to assess these strategies' real impact over the Spanish public health.

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

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