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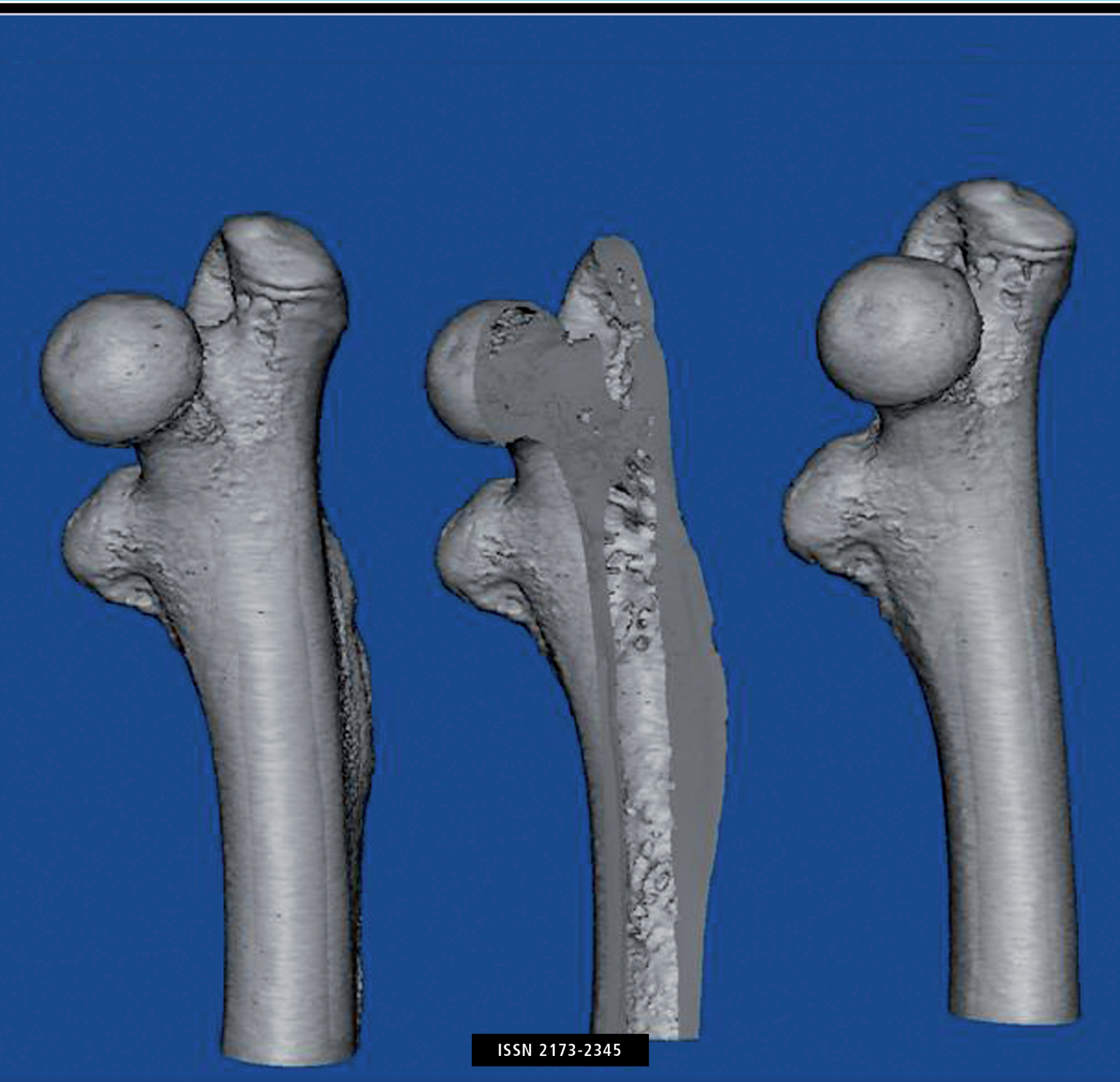

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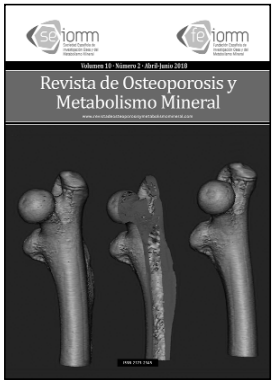

Our cover
3D reconstruction of the rat proximal femur not osteoporotic

Autor:
Image courtesy of Dr. José R. Cairo Rey, Trabeculae EBT S.L. San Cibrao das Viñas (Ourense)

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SUMMARY

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Editorial Committee

Hip fracture in Latin America. Is it approaching the European experience of recent years?

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The fracture of the proximal extremity of the femur, better known as hip fracture (HF), is a fragility or osteoporotic fracture that has some peculiarities that make it unique. On the one hand, due to its potential severity. In western countries, HF occurs mainly in the elderly, with an average age of 80 years, who present significant comorbidity[1] and require hospital admission and surgical intervention, since patients who are not operated on have a higher mortality rate[2]. All this conditions the existence of an important mortality, as has recently been confirmed in several studies carried out in Spain[1,3,4].

On the other hand, HF presents a different clinical behavior between men and women. Thus, although fragility fractures are generally more frequent in women, in the case of HF, in the most advanced age groups, the incidence becomes almost the same between both sexes and in some cases greater among men[5], mortality being greater among them6. This has also been observed in other European countries in our environment. In a study conducted in the Picardy region in France, mortality in the acute phase, immediately after the fracture, was reported to be 8.1% in women and 10.2% in men. At 2 years, male/female mortality showed a ratio of 1.94/1[7].

Another peculiarity of HF is that its presence as a clinical antecedent significantly increases mortality in patients who suffer a second heart attack. Thus, in the EPIDOS study, conducted in France, in the acute phase of hospital admission, mortality for women was reportely 112.4 per 1,000 women and year, whereas mortality had not previously suffered the same fracture. it was noticeably lower, 27.3 per 1,000 people and year[8].

Several studies carried out both in Spain and in other European countries have shown a tendency to stabilize the incidence of heart failure and even to decrease it[9-13]. But we must also be taken into account that, although incidence may decrease in absolute numbers, the number of fractures has increased, probably due to the aging of the population. For example, in Gran Canaria, comparing the incidence of HF cases in a period of 5 years separated by 20 years from each other, although the overall incidence showed a tendency to decrease, the number of fractures doubled in this period of time5. However, in other regions of Spain the exact opposite has been described: an increase in the incidence of HF[14].

Given HF's peculiarities in our environment, it is interesting to observe its behavior in other populations, its similarities and differences with Spain, as in the case of Latin America. In this issue, López Gavilánez et al.[15] publish data on HF epidemiology in Ecuador, after collecting cases of this fracture after a thorough search. Their data are similar to those published in Spain a few years ago. Ecuador, like all of Latin America, is seeing an increase in life expectancy and it is precisely in these countries that a change in the population pyramid is taking place, with an increase in the elderly population, where an increase in HF incidence is expected in the coming years, as happened in Europe around 30 years ago[11]. Perhaps the observation of our experience and evolution in recent years, both in the acquisition of preventive and therapeutic measures and epidemiological studies that have been carried out here, may serve Latin American countries to slow down, in a shorter period, the foreseeable and feared increase of HF incidence, as we have achieved on this side of the Atlantic over three decades.

Conflict of interests: The authors declare that there are no conflicts of interest.

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Osteoporotic hip fractures in older adults in Ecuador 2016

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Summary

Objectives: To ascertain the incidence of hip fractures in Ecuador in 2016, to determine whether there were variations according to geographic region, residence or season of the year.

Materials and methods: Epidemiological, descriptive and retrospective study. The Hospital Discharges Yearbook of Ecuador was used to determine the number of people aged 60 or more hospitalized for hip fracture from January 1 to December 31, 2016. To calculate the incidence per 100,000 inhabitants/year, the Ecuadorian population projection of the Economic Commission for Latin America and the Caribbean (CEPAL) was used as a denominator for the year 2016. The incidence standardized by age was calculated by the direct method using 2 reference populations: 1) the one of 60 or more years for America Latina made by the Latin American and Caribbean Demographic Center (CELADE) in 2016; 2) with the population of Ecuador in 2010.

Results: In total, 2,054 people were hospitalized with hip fracture diagnosis (1,470 women and 584 men) in 2016. The crude annual incidence was 123 cases per 100,000 inhabitants/year (74.6 per 100,000 men/year and 165.8 per 100,000 women/year). The age-adjusted incidence increased exponentially with age in both sexes. It was greater in women. The standardized incidence with with the population of Latin America was 165.4 and 80.1 per 100,000/year, in women and men respectively. In-hospital mortality was 5.1% and 3.8% in women and men, respectively.

Conclusions: The incidence of hip fractures is greater in women than in men, there being an exponential increase with age, more evident after 80 years. There were no differences by geographical region. In comparison with developed countries and other Latin America countries, incidence of hip fractures was lowest in Ecuador.

Key words: *hip fracture, epidemiology, incidence, osteoporosis, older adults, Ecuador.*

Introduction

As life expectancy increases throughout the world, the number of older adults is growing in each geographic region. In Ecuador, the proportion of older adults (≥60) among the overall population rose from 7.2% in 2000 to 10.2% in 2016 (from 912,695 to 1,669,800, respectively). By 2050, this figure is expected to increase to 21% (4,994,082)[1].

Osteoporosis worldwide is a serious health problem, especially in older populations. Its main consequences are fractures, with the hip being the most severe complication, associated with great morbidity and mortality. Its worldwide incidence will increase from 1.66 million in 1990 to 6.26 million in 2050, reports predict[2].

Although hip fracture is the least frequent (20%) of all osteoporotic fractures[3], it is considered an appropriate model for the epidemiological study of osteoporosis[4], since patients who undergo it practically always enter a hospital for their attention, what allows the epidemiological registry of them[5].

Most available epidemiologic data concerning hip fracture comes from research carried out in the USA or Europe, whereas Latin America is scarcely considered compared to countries in the northern hemisphere (USA, Europe)[6-12].

This study's main objective was to ascertain the hip fracture incidence rates –crude, age- and gender-specific, and standardized with a reference population– in adults aged 60 years and older in Ecuador in 2016. A secondary goal was to determine if there are incidence variations by geographic region, urban or rural residence, or time of year when hip fractures occur.

Material and methods

Ecuador is located on the northwest of South America, stretching from latitudes 1°N to 4°S. The country does not undergo a definite separation of 4 seasons, as in the northern and southern hemispheres. Rather, there is one dry and one rainy season. It has different climates: a tropical and subtropical climate in the Pacific and eastern coastal regions (23 to 36°C), and a cold, temperate climate (13 to 18°C) in the Andean region of the center of the country[13].

In 2016, Ecuador had 16,384,534 inhabitants, of which 1,669,800 (10.2% of the total population) are 60 years old and older[1].

We present a descriptive, retrospective study based on data from hospital discharges registered in 2016 by the National Institute of Statistics and Census of Ecuador (INEC)[14]. The Hospital Expenditures Yearbook 2016 was used to secure the information of people aged 60 and older hospitalized with the main diagnosis of hip fracture from January 1 to December 31, 2016.

The Hospital Expenditures Yearbook is part of the National Surveillance System conducted annually by INEC[14], which records the expenditures of all public and private hospitals in Ecuador. The data extracted from the hospital records contain information related to demographic and administrative data, hospital discharge status and primary diagnosis upon discharge[14].

The diagnosis of hip fracture was recorded according to the International Classification of Diseases, tenth revision, clinical modification (S72.0-S72.1 and S72.2)[15]. Crude and age-specific incidence rates and sex were calculated for age groups 60-64, 65-69, 70-74, 75-79, 80-84 and 85 and over. To calculate the incidence rates per 100,000 inhabitants/year, the projection of the Ecuadorian population by age and sex for 2016 prepared by the Economic Commission for Latin America and the Caribbean (ECLAC)[1] was used as the denominator. We calculated the incidence standardized by age by the direct method using two reference populations: 1) the one 60 or more years for Latin America prepared by the Latin American and Caribbean Demographic Center (CELADE) - Population Division of ECLAC, revision published in 2016[1]; and 2) with the population of Ecuador according to the 2010 population and housing census[16]. The cases were considered to come from urban or rural areas according to the territorial division classification of Ecuador[17].

Most epidemiological studies use data from Caucasian populations, generally from the USA and Europe, for standardizing incidence rates, or the World Health Organization (WHO) global ones. Latin America is a melting pot of nationalities, which share a diversity of climates, racial origin, ethnicities and socio-cultural characteristics. We used the ECLAC-published reference population for Latin America[1], because it better represents the similarities of our region's populations. In addition, for comparison purposes, we also standardize with Ecuador's own population 2010.

The hospital mortality rate was defined as the number of fatal events divided by the total number of patients hospitalized for hip fracture[18]. The statistical data analysis was carried out with the EPIDAT program, Version 4.2 (www.sergas.es/Saude-publica/EPIDAT). A value of $p<0.05$ was considered statistically significant.

Projects that involve surveys/research and bibliographic databases of public access and use (e.g., INEC)[14] was excluded as it requires approval by an ethics committee in research. Even so, this study was reviewed and approved by the Teaching Hospital of the Guayaquil National Police Center Number 2 Ethics Committee.

Results

In 2016 in Ecuador, the population of adults aged 60 or older was 1,669,800 inhabitants, 10.2% of the total population (n=16,384,534). During 2016, there were 183,191 hospitalizations among people 60 years of age or older, of which 2,054 (1.12) were attributed to hip fractures.

The mean age of the total cases is 80.7±10.8 (95% CI: 80.36; 81.2); and separated by sex, 81.5±9.6 years in men (95% CI: 80.7; 82.3) (n=584) and 82.7±8.6 years in women (95% CI: 82.2; 83.1) (n=1,470).

The annual crude incidence of hip fractures in older adults (≥60 years) was 123 cases per 100,000 inhabitants (95% CI: 117.7; 128.4); 74.6 per 100,000 men (95% CI: 68.7; 80.9) and 165.8 per 100,000 women (95% CI: 157.4; 174.4) (Table 1).

The incidence of hip fracture adjusted for age standardized with the Latin American population1 was 80.1 per 100,000 men (95% CI: 73.7; 86.9) and 157.3 per 100,000 women (95% CI: 149.2; 165.4) (Table 1), and the incidence adjusted for age standardized with the population of Ecuador in 2010 was 75 per 100,000 men (95% CI: 70-79.3) and 166 per 100,000 women (95% CI: 159-173) (Table 1).

The specific incidence by age increased significantly for men and women in the group of 80 years and older, a situation that was more noticeable in women (Figure 1).

The incidence was similar in men and women aged 60-64 years, but beyond this age range the incidence was consistently higher in women (Table 1). The incidence in both sexes increased in an exponential pattern with increasing age, from 14.6 per 100,000 men and 14.7 per 100,000 women in the age group of 60 to 64 years, to 551.9 per 100,000 men and 1,086 per 100,000 women in the age group 85 years or older (Table 1).

In men, the number of fractures increased from 38 in the age group of 60-64 years, to 248 in the group of 85 years and older, an increase of 6.5 times; and in women it increased from 41 in the age group 60-64 years to 693 in the 85-year-old and older, an increase of 16.9 times (Figure 2).

In the group of 80 or more years, the number of hip fractures was 48.4% and 17.6% in women (n=995) and men (n=361), respectively.

The female: male ratio of the general incidence was 2.22, and it was higher than 1 in all age groups, except in the group of 60 to 64 years in which it was similar (ratio=1). The highest ratio between women and men was found in the groups of 70-74 and 80-84 years (2.3 and 2.1, respectively) (Table 1).

The mean age of men with cervical and pertrochanteric fractures was 81.1±9.7 and 82.5±9.3 years respectively, and in women 82.6±8.5 and 83.3±8.6 years. Femur neck fractures were more numerous than the pertrochanteric fractures, 64.8% and 30.48% respectively, while the subtrochanteric fractures were scarcely 4.72%.

57% of hip fractures cases were treated in public sector hospitals, 35.7% in private sector hospitals, and 7.15% in charitable hospitals. The duration of the overall hospital stay was 8.6±8.3 days (95% CI: 8.28; 8.99); in public hospitals it was 9.9±8.6 (95% CI: 9.4; 10.3) (n=1,173) and for private hospitals, 7.02±7.9 days (95% CI: 6.5; 7.5) (n=881).

There were no significant differences in the number of fractures between the coastal and Andean regions, 49.03% (n=1.007) and 48.2% (n=990), respectively. The Amazon and insular regions represent only 2.58% (n=53) and 0.19% (n=4), respectively (Figure 3).

In the last quarter of 2016 there were more hip fractures than in the first 3 quarters of this: 27% vs. 24.7% (p<0.001).

Table 1. Incidence of hip fracture in older adults in Ecuador 2016

	Population		Fractures		Incidence[a]		Relation ship woman /man
Groups of age (years)	Women	Men	Women	Men	Women (IC 95%)[b]	Men (IC 95%)[b]	
60-64	278,930	261,139	41	38	14.7 (10.5-19.9)	14.6 (10.3-19.9)	1
65-69	199,991	183,813	92	43	46.0 (37.08-56.4)	23.4 (16.9-31.5)	1.9
70-74	156,318	138,828	135	53	86.4 (72.4-102.2)	38.2 (28.7-49.9)	2.26
75-79	113,050	95,486	207	89	183.1 (159-209.8)	93.2 (74.8-114.7)	1.96
80-84	74,727	58,775	302	113	404.1 (359.8-452.4)	192.3 (158.4-231.1)	2.10
≥85	63,808	44,935	693	248	1086.1 (1006.7-1170)	551.9 (485.3-625)	1.96
Total	886,825	782,975	1,470	584	165.8 (157.4-74.4)	74.6 (68.6-80.9)	2.22
IE1[c]					157.3 (149.2-165.4)	80.1 (73.8-86.9)	1.96
IE2[d]					166 (159-173)	75 (70-79.3)	2.21

[a]: incidence per 100,000 inhabitants/year; [b]: 95% CI = 95% confidence interval; [c]: standardized incidence for population ≥60 years of Latin America 2016[1]; [d]: standardized incidence for population ≥60 years of Ecuador 2010. 2010 Population and Housing Census.

In the urban area, more fractures occurred than in the rural area (91.7% vs. 8.3%, p<0.05). In-hospital mortality rates were 5.1% and 3.8% for men and women, respectively.

Discussion

In the 21st century, most hip fractures will occur in developing countries, and the largest increases will occur in the countries of Asia and Latin America[19]. Life expectancy is increasing in all Latin American countries, presaging the increased importance of osteoporosis as a public health issue. Ecuador is also undergoing an epidemiological and demographic transition, with a growing number of older adults and increased life expectancy. The country's life expectancy at birth in both sexes increased from 72 years in 1990 to 76 years in 2015, this increase being greater in women than in men (79 vs. 74 years, respectively). In the year 2000, the proportion of older adults in our population was 7.2% and increased to 10.2% in 2016. So, the number of fractures associated with aging and osteoporosis (fracture of hip and others) is expected to increase[1].

Hip fracture is the most serious complication of osteoporosis and is the one that is usually used worldwide as a model to carry out epidemiological studies[4]. The countries of Latin America have a common historical origin (invasion and colonization by Europeans), as well as similarities in racial origin (miscegenation), climate, economic, social and cultural development, which identifies us as a true subcontinent. These particularities are also reflected in the epidemiological trends of diseases of worldwide distribution, such as osteoporosis. For example, if one considers the specific ethnicity rates, the population of Hispanics in the US it would be in the low risk category in relation to the Caucasian population[5].

There is great geographic variation in the incidence of hip fractures across continents, as well as between different regions of the same country[20]. On average, hip fracture rates standardized by age are highest in North America and Europe, followed by Asia, the Middle East, Oceania, Latin America and Africa[2]. There is a north-south gradient in European studies, and more fractures are also observed in the northern US. than in the south[19,20]. These variations show that demographic, geographic, environmental and ethnic factors play an important role in the epidemiology of hip fracture[2]. In Latin America there are also differences between the different countries of the region, and even between regions of each country[6].

In a recent study of the trend in the incidence of hip fracture from 1999 to 2016 in our country[22], it is reported that a marked annual increase in hip fracture rates was observed predominantly among residents of the coastal region, surpassing those rates in the Andes region in 2016. However, in the current study no significant differences in the geographical distribution of the frequency of hip fractures between the coastal and Andean regions in 2016 are shown. It should be noted that the age group at which this study was conducted is different (60 vs. 65 years), which we estimate would explain this discrepancy.

Figure 1. Incidence of age-specific hip fractures in Ecuador

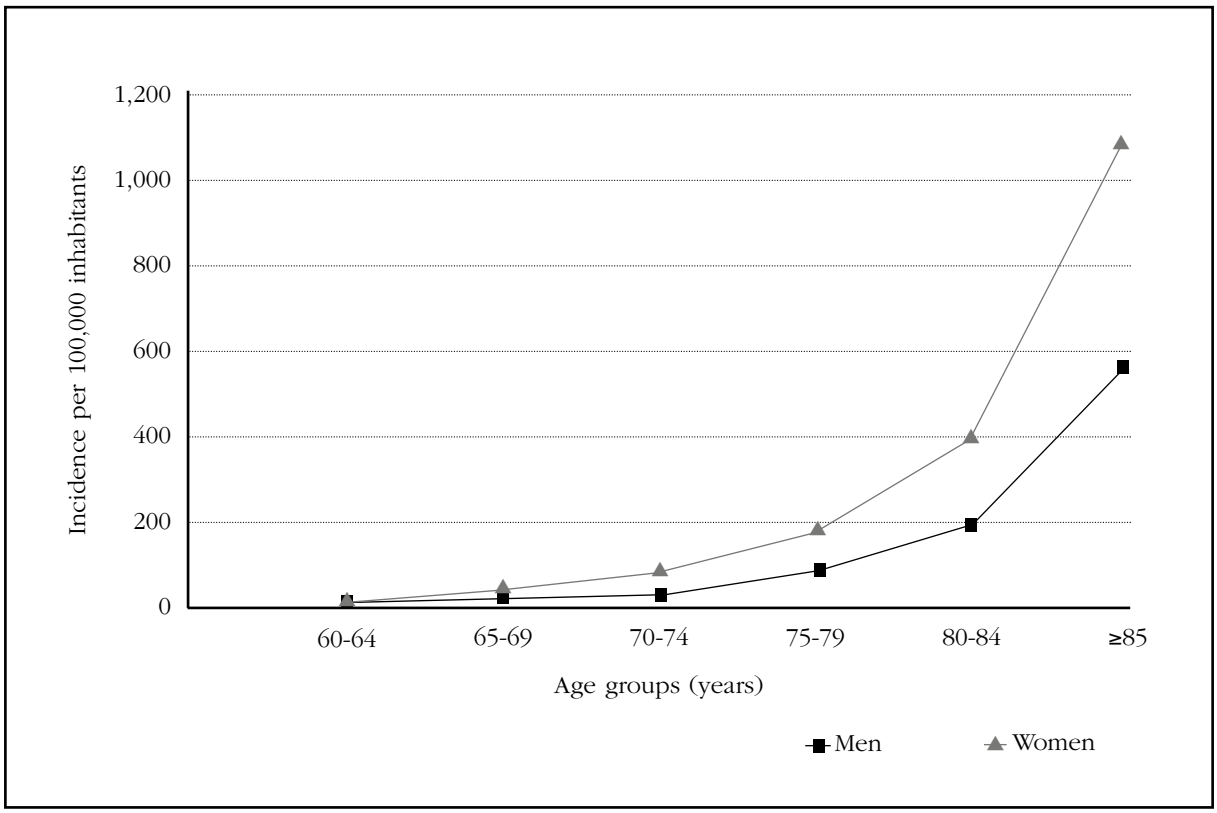


Figure 2. Number of hip fractures by age in Ecuador

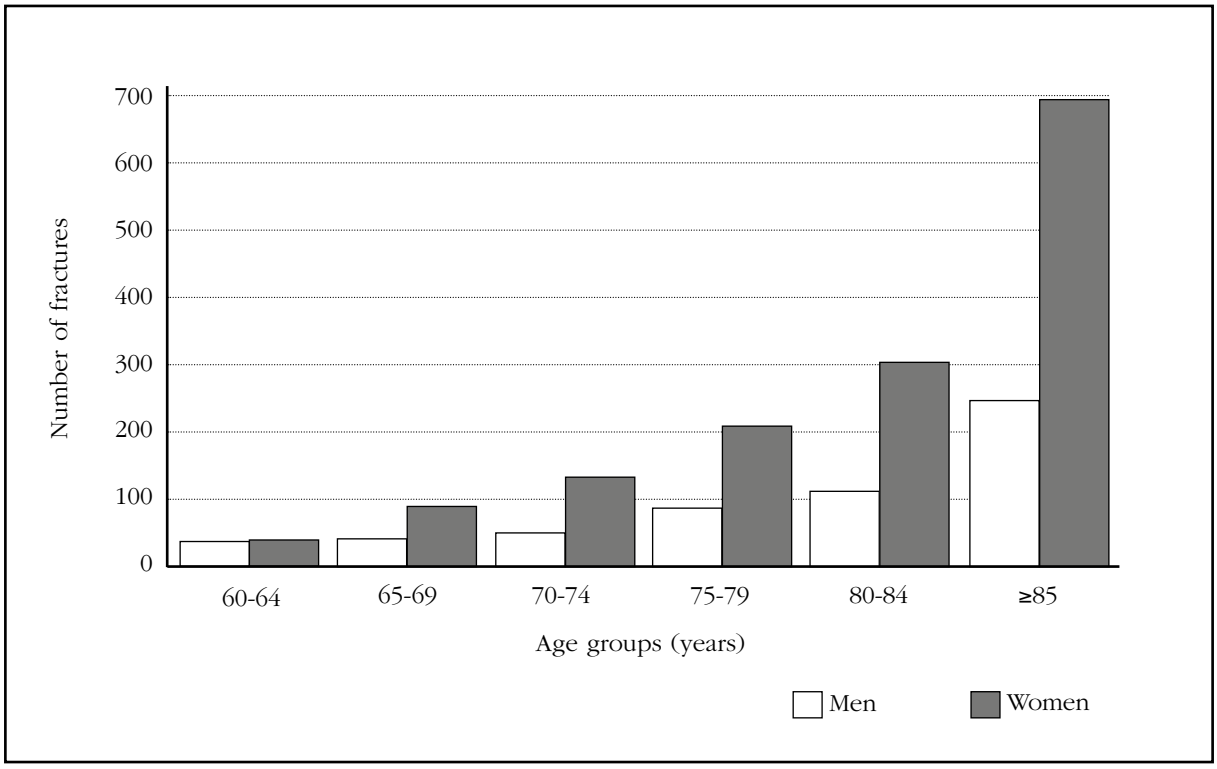
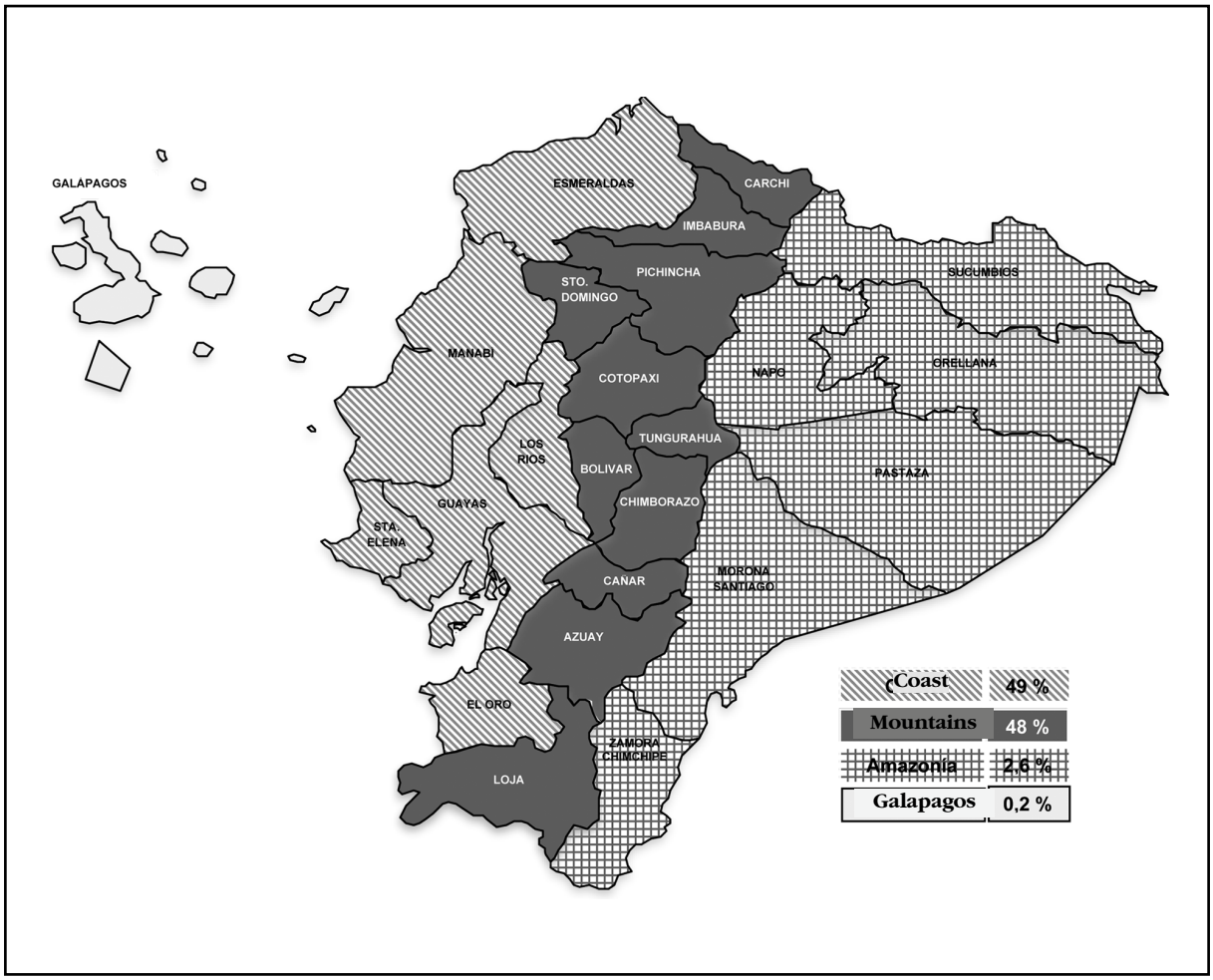


Figure 3. Hip fractures in the different geographic regions of Ecuador



From the available studies on the epidemiology of osteoporosis in Latin America, it can be inferred that there is a generally lower incidence of hip fractures than in developed countries[6]. Some of these population-based studies produced incidence figures between 263.6 and 304.5 fractures per 100,000 people aged 50 or older. While others based on hospital data reported values between 40 and 362 fractures per 100,000 people aged 50 or older[6-8,26]. The country with the highest incidence is Peru, followed by Brazil and Argentina. Those with the lowest incidences are Venezuela and Ecuador[6,11]. In our study, the incidence rate in adults aged 60 and over increases with respect to a previous study[11], although it is still among the lowest in the region and far below that in the northern hemisphere[5]. According to the classification of Kanis et al., Ecuador would be among the low risk countries[5]. In a previous study carried out in 2005 in our country[11], a substantially lower annual incidence was reported (49.5 per 100,000) than that described in the present study. Given the different design (adult population ≥50 years), such work is not directly comparable to ours. Furthermore, there is a difference of 11 years compared to the present.

Although most studies in Latin America have shown hip fracture rates lower than those found in the population of the US, Canada and Europe, these results may be due to the selected population studied, differences in the populations' life expectancy[1], differences in the definition of the cases and other methodological factors[6].

A seasonal variation in hip fracture incidence has been described, being higher in winter[27]. Changes in weather conditions could explain these seasonal differences[27]. In effect, a fall –usually from the individual's own height– is the underlying mechanism of hip fracture in most cases. This would be favored by bad weather conditions (rainy season and/or cold, wet climate). Another mechanism involved is the metabolic alteration of vitamin D and the absorption of calcium that occurs during the winter months, although it would be less likely in the short term. Ecuador does not have a winter season similar to that of the Nordic countries, as our rainy season starts in January and ends in April. We have no explanation for the prevalence of fractures in the last 3 months of the year in the present study. In Ecuador, a vitamin D deficit has been reported in the population both in residents of the coast and in the Andean region, which could be a contributing factor to the incidence found in this study[28,29]. However, this evidence would not necessarily be related to the seasonality in which most fractures occurred. One limitation is, perhaps, the short period of study, which makes it difficult to investigate temporal changes in both the trend, as well as the seasonality of hip fractures. In subsequent studies, the influence of weather conditions on this seasonal distribution should be analyzed.

Although it is true our country's Andean region dress habits and cold climate conditions[13] mean less exposure to sunlight, no regional differences are found in the frequency with which hip fractures occur (coast vs. Andes).

Globally, hip fracture rates are higher in women than in men, with an average ratio of approximately 2:1[3,21], which concurs with our data where we find on average a 2:1 ratio.

A lower incidence of hip fracture in the rural population has been described[30]. Since hip fractures require immediate medical attention and, in most cases, a surgical procedure, it would be very rare for a rural inhabitant who suffers a hip fracture to not receive hospital care. This would be in accordance with our results, in which the proportion of hip fractures in rural areas is lower than in urban areas (8.3% vs. 91.7%, respectively). Similar results are observed in other countries[31]. A more physically active lifestyle reportedly protects them against osteoporosis and fractures[31]. However, it should be noted that the generally great distance from urban centers, the limited availability of transport or lack of specialized health personnel may prevent these rural patients from receiving care.

There are discrepancies in the literature on the proportion of cervical fracture/pertrochanteric fracture. For some authors, this relationship varies widely, while for others it is approximately equal. Together they comprise more than 90% of the fractures of the proximal femur[2]. In our study, the number of femoral neck fractures is greater than that of the pertrochanteric fractures in all age groups, 64.8% and 30.5% respectively, while the subtrochanteric fractures are only 4.72%. This probably depends on the type of population studied, since in some previous studies the average age of subjects with trochanteric fractures is greater than that of cervical fracture cases[7,11]. However, our results do not support this claim, as we did not find differences in the average age between cases with fracture of the femoral neck and that of other sites. We have no explanation for the prevalence of femoral neck fractures reported in the present study, but these fractures may be more sensitive to the effects of nutrition, socioeconomic and environmental factors.

The differences found in the days of hospital stay are probably due to the structure of the general medical care system and to the practices of hip fracture care in different countries. In our cohort, they were somewhat shorter (8.6±8.5 days) than those reported in other studies (11-16 days)[32].

Mortality associated with hip fractures is substantial, with rates reported from 16% to 23% within 1 year after the event[3,23]. The risk of mortality appears to be higher in men than in women. In men, the general mortality rates in 1-year range between 18% and 31%[3]. This disparity in the rates was confirmed by a systematic review of the excess mortality a year after the hip fracture[24]. In Latin America, between 17% and 37% of patients with a hip fracture die in the year following the fracture[6]. In this study, the in-hospital mortality rate due to hip fracture was 5.1% in men and 3.8% in women, which is in the range reported in other studies[11,23,25].

Given the nature of the design, our study presents some limitations that should be acknowledged. First, the number of cases could be underestimated if the patients had been treated outside a hospital. However, this eventuality is unlikely, given that it is estimated that practically 100% of hip fractures are treated in hospitals[5]. In Ecuador, the number of patients treated outside a hospital after a hip fracture is indeterminate.

Secondly, some cases may have been treated outside their habitual residence because of the fracture in another place. This constitutes an unpredictable and inevitable bias, but for reasons of probability it should not be significant. Other cases may have been transferred to another region, which is likely due to the conditions in which the communication routes with the neighboring regions are located.

Third, the design of this study does not allow to identify the mechanism that triggers the fracture, nor the presence of a previous fracture. The comorbidity of the hip fracture was not studied in this work, so we cannot infer the impact of these on mortality.

Despite these limitations, the main strength of the present study is that information was obtained from official sources that are updated every year and that collect data from all public and private hospitals throughout the country. Thus, such data are very reliable.

In our study then, we observed that the number and incidence of hip fractures increased with age in both sexes. This increase was greater among women, consistent with patterns described in most populations where there is a female predominance, an exponential increase with age and no differences according to geographic region.

Due to the aging of the population in Ecuador, it is expected that the number of hip fractures will increase considerably among people aged 80 or over. Accurate, updated epidemiological data are essential to design strategies for preventing and treating osteoporosis and its most feared consequence, hip fracture, in our country.

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Using free access bioinformatic tools to identify potential vascular calcification biomarkers in patients with diabetes mellitus type 2

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Summary

Objectives: Identify potential biomarkers involved in vascular calcification processes to improve DM2 diagnosis and treatment in its subclinical stages.

Methods: This experimental study included 5 patients suffering diabetes mellitus type 2 (DM2) with peripheral arterial disease and critical ischemia. Protein extraction and identification of the proteome were carried out using liquid chromatography and mass spectrometry (LC-MS/MS) of calcified femoral artery sections. The identified proteins were analyzed through gene ontology and compared with other specific proteins of related vascular pathologies through the DisGeNET database. Cytoscape software analyzed the network of biological functions of the proteins selected for classification based on the disease in which they are involved.

Results: 530 proteins were identified in the analyzed samples with functions mainly of calcium binding and catalytic. 37 of them were common in other related vascular pathologies. The exploration of the biological networks of the 37 proteins identified, led to the identification of 2 potential specific markers of vascular calcification in atherosclerotic processes, such as 10-kDa thermal shock mitochondrial protein, and the flavoprotein subunit of succinate dehydrogenase.

Conclusions: There is significant expression of proteins involved in processes of bone mineralization in calcified vascular tissue, suggesting the existence of common molecular mechanisms between bone regulation and vascular. The use of bioinformatics tools suggests the involvement of the mitochondrial 10 kDa heat shock protein and the subunit of the succinate dehydrogenase as potential biomarkers of vascular calcification in patients with DM2, although additional studies are needed to confirm this hypothesis.

Key words: *diabetes mellitus type 2, cardiovascular disease, vascular calcification, biomarkers, proteomics, bioinformatics.*

Introduction

Diabetes mellitus type 2 (DM2) is a significant public health problem throughout the world which affects a large sector of the population. An increased incidence of DM2 cases is predicted worldwide, involving more than 300 million people in 2025[1]. DM2 is associated with various complications, with cardiovascular disorders being one of the main causes of mortality in this disease[2]. Among the complications, chronic ischemia of lower limbs due to atherosclerotic processes is among the most frequent vascular problems in DM2 patients, with a prevalence of calcified plaques in the lower limbs that may reach 44.6% in this population[3]. Several studies reveal that DM2 is an independent risk factor in cardiovascular disease (CVD) development in both men and women[4,5]. The advance of these vascular complications is determined by the presence of common risk factors in DM2 such as obesity, chronic hyperglycemia, insulin resistance, dyslipidemia and states of inflammation and oxidation, among others. However, only some of the patients with DM2 will develop these vascular complications while others, despite presenting the same risk factors, will not suffer cardiovascular events during the disease course. This suggests that the pathway that links dysglycemia and CVD is not well established, requiring in-depth study of the factors involved in the development of CVD associated with DM2. Evidence has shown a process of regulation of vascular calcification similar to bone mineralization, suggesting a connection between bone metabolism disorders and the vascular system.

In this context, the identification of new biomarkers involved in the development of vascular complications in patients with DM2 may be of great importance to improve the prognosis and quality of life of this population, as well as to design new preventive and therapeutic strategies that prevent the development of cardiovascular events, thus reducing the morbidity and mortality of diabetic patients.

Proteomic analysis is a hypothesis-free approach that integrates genetic and epigenetic influences by examining protein expression profiles, and is not limited by prior knowledge. The majority of biomarker search studies have been carried out at the serum level, due to their easy collection by non-invasive techniques. However, there are proteins that are not released into the bloodstream but are expressed specifically in the affected tissues, and therefore cannot be detected by serum proteomic studies. Therefore, the aim of this study is to identify potential biomarkers of CVD in calcified vascular tissue of patients with DM2 through the use of proteomic techniques. One of the main limitations in studies of this type is the obtaining of tissue samples from healthy subjects, or patients without associated complications, which makes it very difficult to carry out a comparative study of the protein profile between cases and controls for the identification of proteins involved in the study. The introduction of bioinformatics tools in the scientific field in recent years has helped to overcome these limitations. Bioinformatics is crucial to combine information from multiple sources and to generate new knowledge from existing data. It also has the potential to simulate the structure, function and dynamics of molecular systems, among other utilities. Therefore, it may help researchers formulate hypotheses to guide experimental work.

In our study, different bioinformatic tools are applied that allow us to obtain a large amount of information published for free online. In this way, a classifier has been used according to certain characteristics of the proteins identified, such as its molecular function, biological process or its behavior as a cellular component. This allows us to study the proteins as a whole, and their proportion according to these characteristics in the subjects under study.

On the other hand, the use of protein repositories identified as biomarkers of different conditions potentially related to the calcification of the femoral artery allows us to establish protein links between different disorders, as well as to identify specific proteins of this disease.

Therefore, applying these tools allows us to deepen the knowledge of the pathways involved in vascular disorders, offering, at the same time, an experimental orientation aimed at identifying potential biomarkers involved in the atherosclerotic processes of the femoral artery.

Material and methods

Population study

Our experimental work included five subjects. These patients are diagnosed with DM2 according to the criteria of the American Diabetes Association (2011). The criterion for inclusion of peripheral arterial disease was the presence of critical ischemia with indication of a lower limb amputation because they were not candidates for revascularization or because it has failed. Critical ischemia criteria according to recommendation nº 16 of the consensus document on peripheral arterial disease, TASC II [1], are: persistent ischemic pain and relapsing at rest requiring analgesia with opiates for at least 2 weeks; ulcers or gangrene standing or on toes; systolic blood pressure in the ankle less than 50 mmHg or systolic pressure in the toe less than 30 mmHg; chronicity excluding acute ischemia.

The patients came from Granada's Campus Hospital of Health Studies and were evaluated in the Service of Angiology and Vascular Surgery. All patients were Caucasian, with normal serum levels of calcium and phosphorus and no kidney, liver, gastrointestinal or thyroid diseases. All received medication for DM2, including metformin, sulfonylureas, insulin or a combination of these drugs.

Samples of vascular tissue from the femoral artery with presence of calcification were obtained. The samples were immediately cryopreserved after extraction. All the samples used for the

study were managed by the Biobank of the Andalusian Public Health System (SSPA Biobanco) of the San Cecilio University Hospital, in accordance with the Biobank procedures approved by the Ethics Committee for Biomedical Research of Andalusia.

All subjects included in the study signed the acceptance and understanding of informed consent. The study was carried out with the approval of the Ethics Committee of San Cecilio University Hospital and in accordance with the relevant ethical guidelines for human and animal research included in the Helsinki Declaration.

Proteomic study

Protein extraction

Samples corresponding to 45 sections of femoral artery tissue were homogenized at 4°C after the addition of 500 µL of soluble protein extraction buffer (20 mM Tris-HCl pH 7.6, 10 mM NaCl, 0.5 mM deoxycholate sodium, 1 mM EDTA, 4% SDS, 30% glycerol, 5 mM PMSF, 200 mM DTT, benzonase -1µL/10 mL of buffer-). The samples were centrifuged for 15 minutes at 6,000 r.p.m. and 4°C and the supernatant representing the cytosolic fraction of the cells where the soluble proteins are found was collected. These samples were stored at -80ºC until further processing for protein analysis.

Sample processing

The frozen samples were sent for analysis to the Proteomics Unit of the University of Córdoba's Central Research Support Services (SCAI). Once thawed, the samples were concentrated by ultrafiltration using an Amicon Ultra-0.5 Centrifugal Filter spin column of 3 kDa. Protein extracts were cleaned by SDS-PAGE 1D 10% polyacrylamide electrophoresis. For this, the samples were loaded on the concentrator gel, to which a voltage of 100 V was applied until the electrophoresis front reached the separating gel. The assay was stopped when the protein extract had entered 1 cm into the separating gel. The gel was stained with Coomassie blue, and then the cutting and extraction of the gel bands that were kept in water until digestion was carried out.

For protein digestion, the gel portions were preserved in 50 mM ammonium bicarbonate (BA)/50% acetonitrile for 15 minutes, with subsequent incubation for 5 minutes in 100% acetonitrile. The protein was maintained under reducing conditions by the addition of 20 mM DTT in 25 mM BA, and was incubated for 20 minutes at 55°C. The mixture was cooled to room temperature, with the subsequent alkylation of the free thiol groups by the addition of 40 mM iodoacetamide in 25 mM BA for 20 minutes in the dark. Subsequently, the gel portions were washed twice in 25 mM BA. Proteolytic digestion was carried out by the addition of trypsin (Promega, Madison, Wisconsin, USA) to 12.5 ng/µL of enzyme in 25 mM BA, and incubated at 37°C overnight. The digestion of proteins was stopped by adding trifluoroacetic acid to a final concentration of 1%. The digested samples were finally dried by SpeedVac.

Analysis by Mass Spectrometry/Mass coupled with liquid nano-Chromatography (nLC-MS/MS)

The Nano-LC was carried out on a UPLC Dionex Ultimate 3000 nano (Thermo Scientific) with an Acclaim PepMap100, C18, 3 µm, 100 Å, 75 µm i.d. x 50 cm, nanoViper (Thermo Scientific). The peptide mixture was preloaded in a 300 µm x 5 mm Acclaim Pepmap precolumn (Thermo Scientific) in 2% acetonitrile/0.05% trifluoroacetic for 5 min at a flow of 5 µL/min. Peptide separation was carried out at 40°C for all runs. The elution buffers were: buffer A (0.1% formic acid in water) and buffer B (20% acetonitrile and 0.1% formic acid). Samples were eluted at a flow of 300 nL/min with the following gradient: 4-35% B for 120 minutes; 35-55% of B for 6 minutes; 55-90% of B for 3 minutes, followed by 8 minutes of washing with 90% B and re-equilibration from 15 minutes to 4% of B. The total time of the chromatography was 150 minutes.

The eluted peptide cations were converted to gas phase ions by nanoelectrospray ionization and analyzed in a mass spectrometer, Thermo Orbitrap Fusion (Q-OT-qIT, Thermo Scientific) operated in positive mode. The recognition scan of the peptide precursors from 400 to 1500 m/z was carried out at 120,000 FWHM resolution with the objective of counting 4 x 10^5 ions. Tandem mass spectrometry was carried out by isolation at 1.2 Da with the quadrupole, CID fragmentation with normalized collision energy of 35 and fast scanning MS analysis in the ion trap. The AGC ion count target was adjusted to 2 x 10^3 and the maximum injection time was 300 ms. Only those precursors with loading status of 2-5 were sampled for MS/MS. The dynamic exclusion duration was set at 15 s with a tolerance of 10 ppm around the selected precursor and its isotopes and activating the selection of monoisotopic precursors.

Identification of proteins

The raw data was processed using the Proteome Discoverer software package (version 2.1.0.81, Thermo Scientific). MS/MS spectra were searched with the SEQUEST™[6] engine (Thermo Fisher Scientific) against a Uniprot_Homosapiens_ dateofanalisys database (www.uniprot.org). A theoretical tryptic digestion of the peptides was carried out, cysteine carbamidomethylation was introduced as a fixed post-translational modification and methionine oxidation as a variable post-translational modification. The tolerance of the mass of the precursors was 10 ppm and the ions of the product were recorded with a tolerance of 0.1 Da. The spectral matches of the peptides were validated using a filter based on q values at 1% FDR (False Discovery Rate), obtaining a list of proteins identified in each of the processed samples (Proteome Discoverer).

Data analysis using bioinformatics tools

Analysis by gene ontology (GO)

The gene ontology resource (gene ontology) is an important initiative of bioinformatics that aims to standardize the representation of protein attributes between species and databases[7]. The online system

provides a vocabulary of terms to describe characteristics of the gene products and annotation data of gene products of the members of the GO consortium, as well as tools to enter and process this data. Each protein is characterized in terms of three ontologies: molecular function, cellular component and the biological process involved[8]. Using the GO database (http://www.geneontology.org) and the OntoExpress analysis, the genes involved were classified in order to obtain an overview of the potential functions of the expressed genes corresponding to the proteins identified. in the study.

Analysis by comparison of pathologies

The proteins identified in this study were confronted with the proteins already described in other vascular pathologies with a possible relationship with the vascular calcification of the femoral artery. This information was obtained thanks to the DisGeNET database (http://www.disgenet.org) that contains one of the largest collections of genes and variants associated with human diseases, publicly available[9]. The pathologies from which the information was obtained were: Carotid Stenosis, UMLS CUI: C0007282; Supravalvular aortic Stenosis, UMLS CUI: C0003499; Aortic Valve Stenosis, UMLS CUI: C0003507; Esophageal Stenosis, UMLS CUI: C0014866; Laryngostenosis, UMLS CUI: C0023075; Mitral Valve Stenosis, UMLS CUI: C0026269; Pulmonary Valve Stenosis, UMLS CUI: C0034194; Pyloric Stenosis, UMLS CUI: C0034194; Aortic Calcification, UMLS CUI: C1096249; Calcification of Mitral Valve, UMLS CUI: C0919718; Aortic Valve Calcification, UMLS CUI: C0428791; Renal Artery Stenosis, UMLS CUI: C0035067 and Vascular Calcification, UMLS CUI: C0342649. The visualization of these proteins was carried out using the Cytoscape tool[10].

Analysis by protein-protein interaction and functional networks

To investigate the direct (physical) and/or indirect (functional) relationships between identified genes, we used the search tool for the retrieval of interacting genes (STRING) in databases to analyze the functional network (https://string-db.org) . The STRING database provided a score for each gene-gene interaction, calculated as the joint probability of the different evidence channels (protein interaction, fusion, coexpression, etc.), correcting the random probability of observing an interaction. A high database score meant that there was high experimental or predicted evidence for functional gene-gene interaction. Therefore, a functional associated network was constructed on the basis of the expression profile of the proteins identified in the present study.

Results

Gene ontology

We identified 530 proteins from the processed samples ($p<0.05$, q value=0). All the identified proteins were classified using the gene ontology annotations and grouped into the three functional groups: biological processes, cellular components and molecular functions (Figure 1).

The analysis of the gene ontology showed that the two most common molecular functions in the selection carried out were functions related to catalytic activity (GO: 0003824) and with binding (GO: 0005488). Regarding the analysis of the cellular component, the most frequent was the cellular portion (GO: 0044464), followed by organelle (GO: 0043226) and extracellular region (GO: 0005576); as biological processes highlighted those related to cellular processes (GO: 0009987), metabolic processes (GO: 0008152) and organization or biogenesis of cellular components (GO: 0071840).

Table 1 shows the main classification according to GO of the proteins that have been identified classified in protein classes. It should be noted a high proportion of calcium binding proteins.

Protein comparison

Of the 530 proteins identified in the proteomic study, 37 proteins coincided with proteins already described in vascular pathologies, some of which were recurrent in different vascular pathologies (Table 1). These proteins were selected as candidates to perform a study of their biological network in order to know their relationship with vascular calcification in the femoral artery (Figure 2).

Functional networks

The 37 proteins of interest resulted in an extensive functional network where a nucleus representing the calcification of the femoral artery is observed, where numerous proteins that are also part of other related diseases converge (Figure 3).

To this first functional network, 5 more proteins were added related to the initial network (Figure 4). The addition of this second level allowed us to find two of the five proteins included in the network as possible specific biomarkers of femoral artery calcification, as they were not found in the rest of the related diseases studied.

These two proteins were also identified in our proteomic analysis (flavoprotein subunit of succinate dehydrogenase (SDHA, P31040), mitochondrial 10 kDa heat shock protein (HSP1, P61604), expressed in samples from patients with femoral artery calcification Therefore, they were selected as the first two candidates to begin their study in greater depth.

Discussion

DM2 currently represents a serious health problem as it affects a large proportion of the population. Patients with DM2 have a higher risk of developing various complications, mainly cardiovascular events that represent the main cause of mortality of this disease. The probability of developing vascular complications in this population depends on a series of traditional factors and others that are not completely known. The identification of new factors involved in these disorders can facilitate the early diagnosis of high-risk subjects before irreversible damage occurs. The aim of the present study was to identify proteins in vascular tissue

that could be related to processes of vascular calcification and, therefore, to increased cardiovascular risk in subjects with DM2.

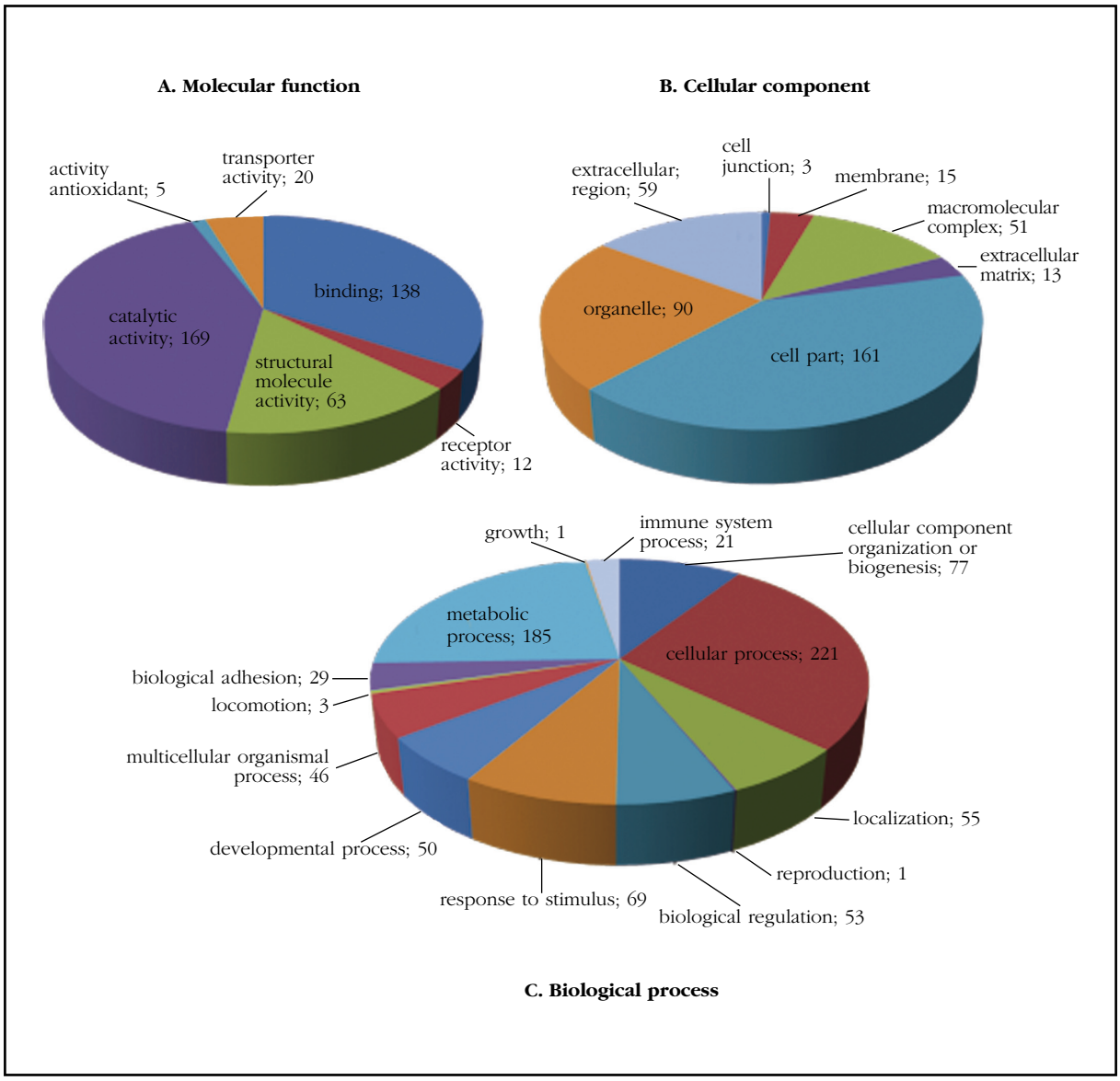
Studies into biomarker identification at the proteomic level present a high complexity due to the large amount of proteins and pathways involved in the different diseases, the interaction between proteins, protein differences depending on the study tissue, high technological costs, among others. All this, in addition to the large amount of data to be processed that are generated in these studies, makes it difficult, in many cases, to identify real biomarkers of certain diseases. To all this we must add the difficulty of obtaining tissue samples from control subjects in order to carry out a comparative study between the different study groups.

The use of bioinformatics tools allows, to a large extent, to solve many of the problems that arise in biomarker identification studies. The use of these tools facilitates the study, comparison and correlation of data found experimentally; the prediction of domains, structures and phylogenetic relationships of the sequences examined; the development of algorithms and statistics necessary for the understanding of biological information, interconnecting information from different approaches. The information provided by these tools allows, in addition, an orientation of the scientific work offering keys that allow a saving of time and economic and human resources.

Today, bioinformatics tools, available for free on the net and increasingly efficient for in silico studies, are booming.

This study establishes a simple and intuitive method to locate biomarkers related to vascular calcification processes. After the identification of 530 proteins corresponding to the characteristic femoral artery protein profile with presence of calcification of patients with DM2 using nLC-MS/MS,

Figure 1. Protein classification based on gene ontology according to molecular functions (A), cellular component (B) and biological process (C)



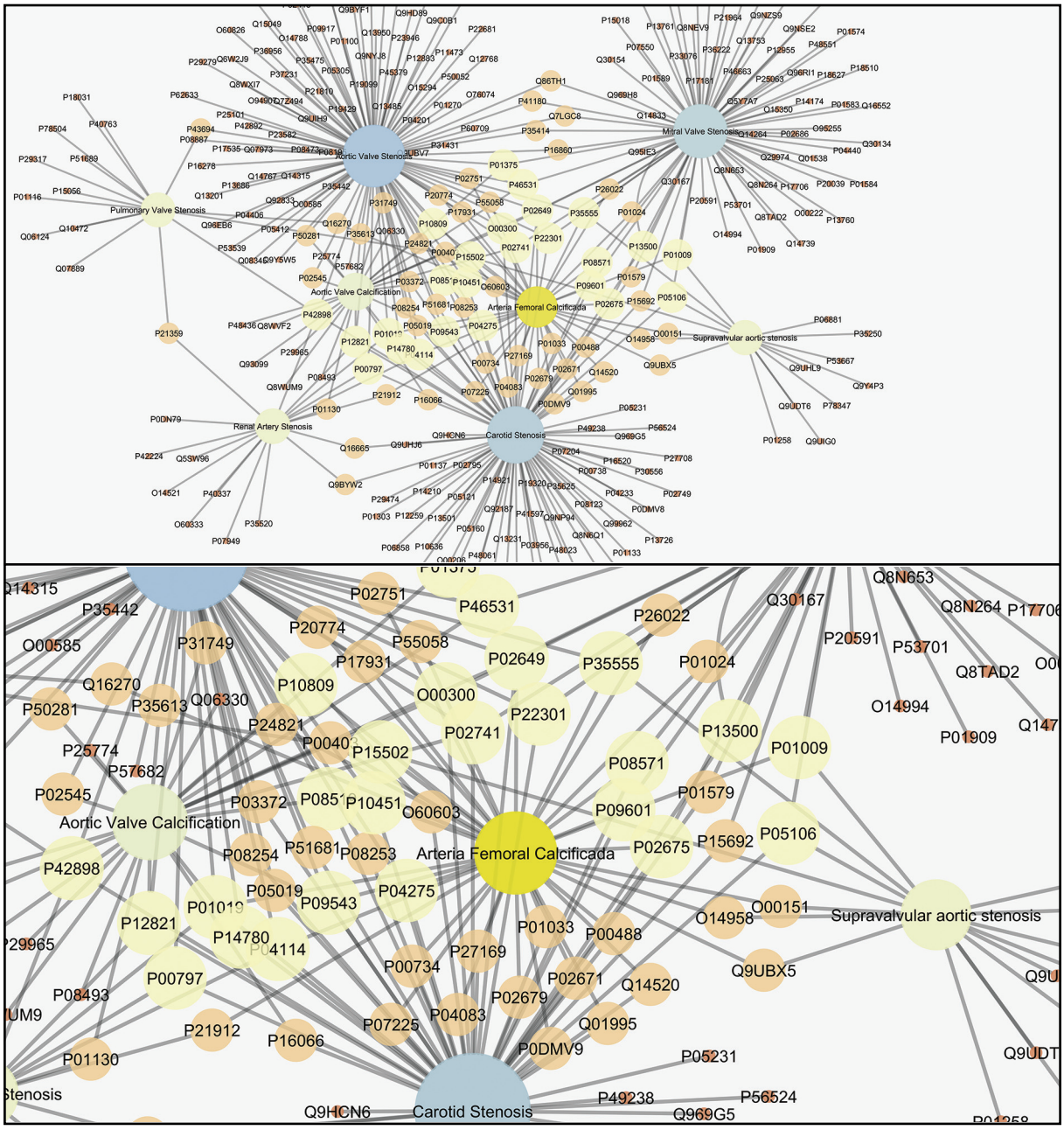
various bioinformatic strategies have been used to limit and filter the search for biomarkers, as well as discard those that do not were related to vascular pathologies. In the first place, the gene ontology methodology was used, which focuses on the study of the functions of the group of genes corresponding to the identified proteins, in order to select those functions most related to processes involved in vascular calcification. The majority molecular function of the genes coding for the identified proteins was the catalytic function and the binding function. Recent studies have shown that, in a calcifying environment, vascular smooth muscle cells are able to undergo a phenotypic transition to osteocyte-like cells capable of expressing typical bone markers, contributing in this way to arterial mineralization[12]. It is not surprising that, under these conditions, there is an increase in the proteins that bind calcium, thus contributing to the calcium deposition in the arterial wall. These processes lead to the development of atherosclerotic processes so frequent in patients with DM2. Recent studies show that macrophages surrounding calcium deposits in human atherosclerotic plaques are phenotypically defective and incapable of reabsorbing calcification[13]. In this line, the identification of proteins with catalytic activity in the vascular samples analyzed, with the presence of calcifications, could reflect a defense mechanism of the organism to try to disintegrate the components of the atheroma plaque generated in these situations. According to this hypothesis, there are studies that show an increase in markers of bone resorption in patients with vascular disorders[14,15].

With the aim of directing and delimiting the biomarker identification study, a comparison was made of the 530 proteins identified in the proteome of the study subjects with those described as proteins involved in various vascular diseases, in

Table 1. Classification of proteins by Classes according to GO

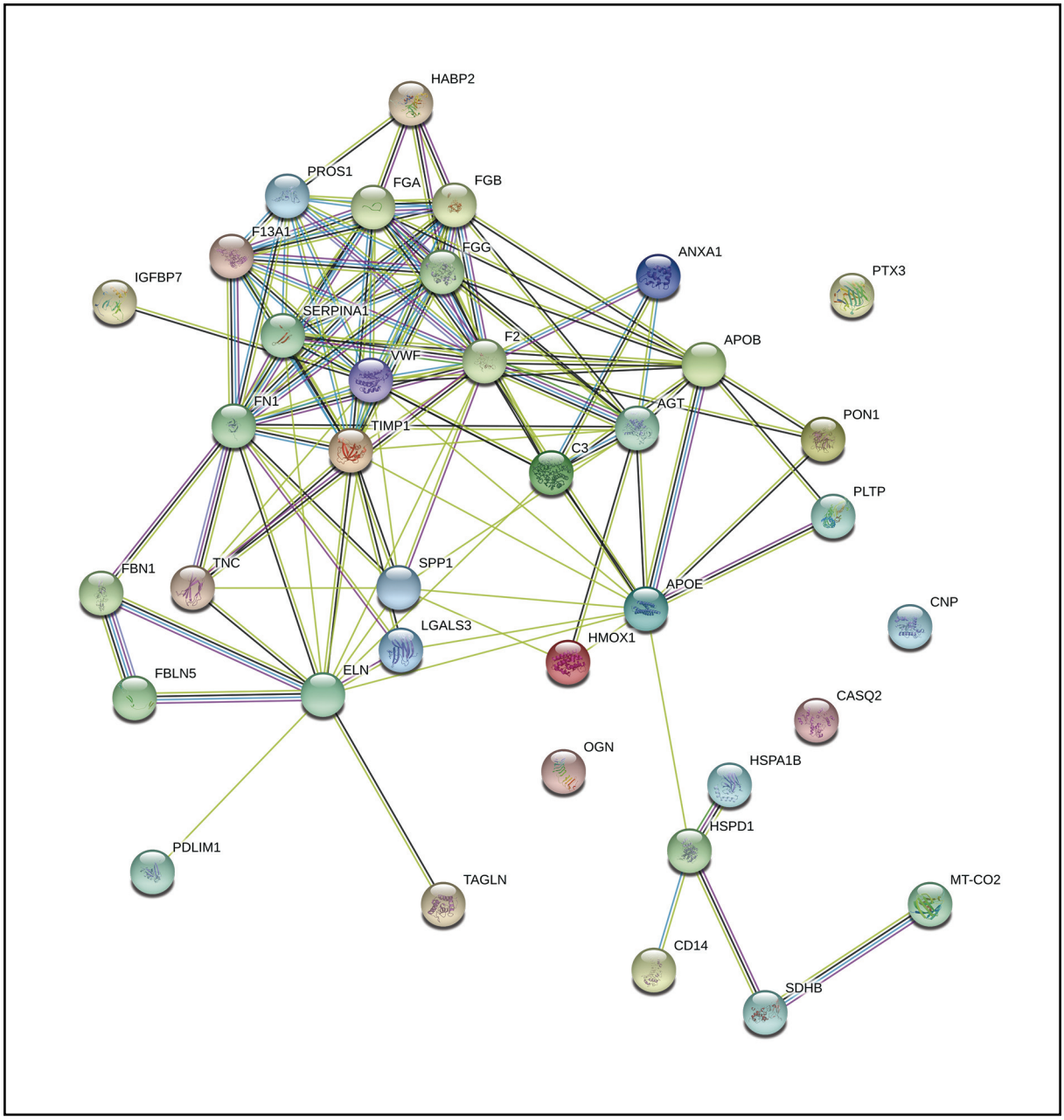
Protein Class	ID Class	Quantity	%
Transmembrane receptor regulatory/adaptor protein	PC00226	1	0,2%
Storage protein	PC00210	2	0,4%
Cell junction protein	PC00070	3	0,6%
Ligase	PC00142	8	1,6%
Chaperone	PC00072	9	1,8%
Isomerase	PC00135	9	1,8%
Cell adhesion molecule	PC00069	11	2,2%
Lyase	PC00144	12	2,4%
Membrane traffic protein	PC00150	12	2,4%
Transcription factor	PC00218	12	2,4%
Structural protein	PC00211	12	2,4%
Defense/immunity protein	PC00090	13	2,7%
Receptor	PC00197	13	2,7%
Extracellular matrix protein	PC00102	15	3,1%
transporter	PC00227	16	3,3%
Calcium-binding protein	PC00060	17	3,5%
Transfer/carrier protein	PC00219	17	3,5%
Transferase	PC00220	22	4,5%
Nucleic acid binding	PC00171	31	6,3%
Signaling molecule	PC00207	31	6,3%
Oxidoreductase	PC00176	34	6,9%
Enzyme modulator	PC00095	37	7,6%
Hydrolase	PC00121	48	9,8%
Cytoskeletal protein	PC00085	61	12,4%

Figure 2. Visualization of the protein linkages between various vascular related diseases, by means of Cytoscape



the repository of molecular markers (DisGeNet). By using this tool, the number of candidate proteins was reduced to 37, those commonly expressed in various vascular disorders associated with vascular calcification processes. The disposition of these protein nexuses in a biological network shows a potential capacity as a biomarker of some of the proteins, which participate in various vascular conditions. The design of specific interaction networks between each of the 37 selected proteins showed a well-formed interaction network, in which a close relationship is observed between most of these proteins. Among these proteins, we find some that could be indirectly related to calcification processes, such as osteopontin and osteoglycine, both of which are involved in biomineralization and bone remodeling processes. Likewise, we also identify muscle proteins typical of vascular smooth muscle cells such as transgelin or tropoelastin, which could indicate the loss of the vascular phenotype. The presence of other proteins of interest identified, but which did not pass the level of statistical significance, as is the case of osteoprotegerin or the GLA protein of the matrix, could be due to the low number of study subjects, which generates a variability that is high enough to be statistically discarded. However, by increasing the interaction network by 5 proteins, adding the first five proteins closely lin-

Figure 3. Functional network of the 37 proteins identified by nLC-MS/MS in calcified femoral artery samples from patients with DM2, previously described in vascular diseases

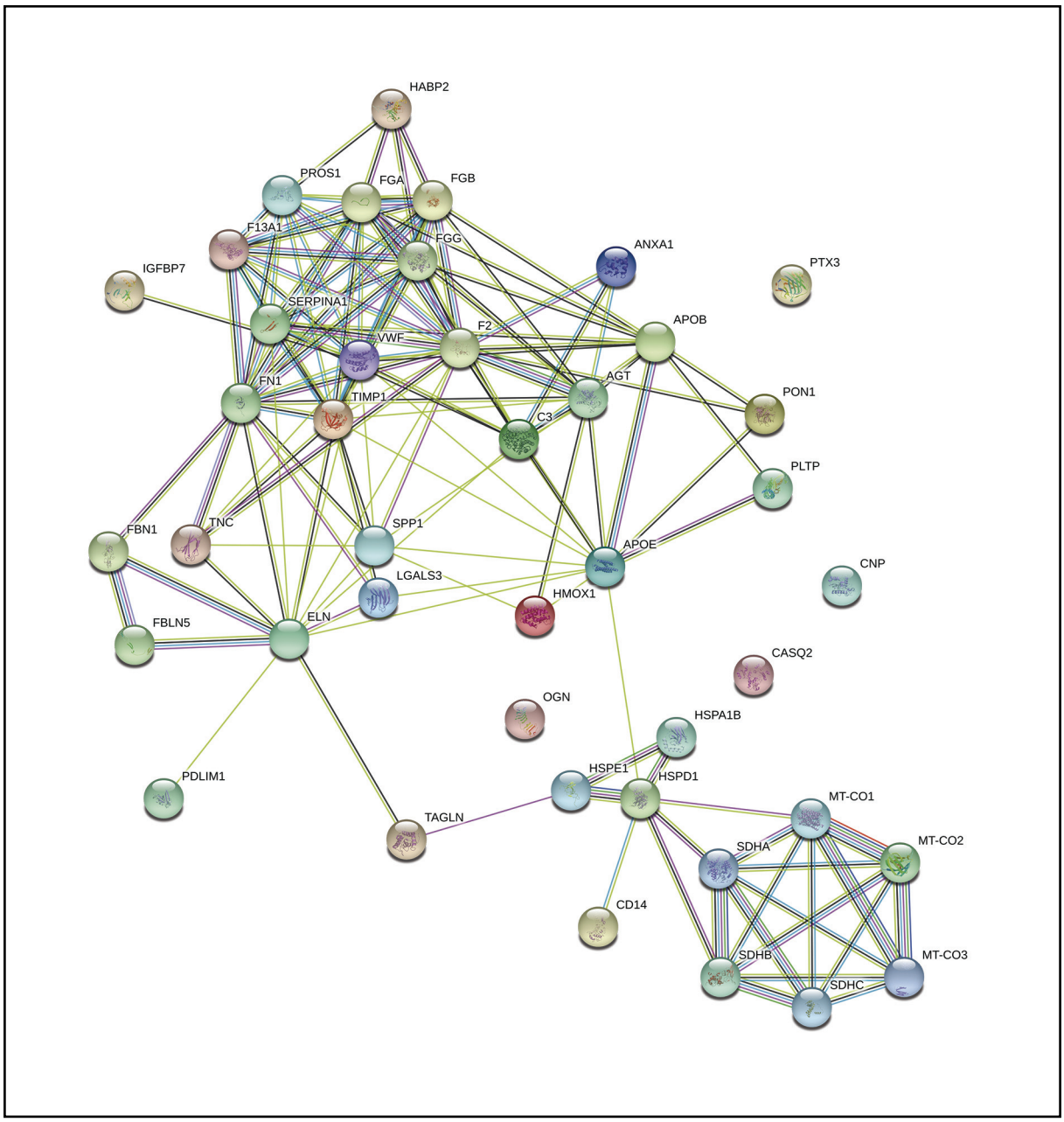


ked to those arranged in the first network, we observed that 2 of them are part of the proteome characteristic of the vascular tissue samples analyzed. One of them, the flavoprotein subunit of succinate dehydrogenase (P31040), is involved in several processes, such as the synthesis of fumarate to succinate in the tricarboxylic acid cycle of carbohydrate metabolism, or in oxide-reduction processes, among others. The deficiency of succinate dehydrogenase is related to a lower flow of electrons and a decrease in antioxidant activity in the respiratory chain, increasing oxidative stress and hypoxia, with important consequences in the process of vascular calcification.

The presence of this protein in calcified vascular tissue could be related to a defensive mechanism to preserve the production of aerobic energy in the state of hypoxia generated in the process of vascular calcification during atherosclerosis[16]. The other, a 10 kDa, mitochondrial heat shock protein (HSPE1, P61604), is a co-chaperone involved in the importation of mitochondrial proteins and macromolecular assembly. In addition, it is also involved in osteoblastic differentiation and in the activation of endopeptidase activity during the apoptotic process.

The fact that these two proteins do not appear in the rest of the related pathologies that were compared in the study, suggests that they could be two good candidates to suggest them as specific biomarkers of atherosclerosis in the lower limbs in DM2.

Figure 4. Functional network of the 37 proteins identified by nLC-MS/MS in calcified femoral artery samples from patients with DM2, previously described in vascular disorders, after the addition of 5 new related proteins



Our study presents certain limitations. First, the limited number of control subjects makes comparison with a reference study group impossible. Secondly, the small number of study subjects leads to a high variability of the results, limiting the identification of potential proteins of interest.

Although future studies are needed to confirm the role of the proteins identified as possible biomarkers of vascular calcification in diabetic patients, the use of bioinformatics tools has allowed us to generate hypotheses to work on thanks to the simplification of the large amount of data obtained. The identification of proteins with a high degree of expression in calcified vascular tissue in patients with high cardiovascular risk could offer information to achieve an early and targeted treatment.

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Conflict of interests: The authors declare that there are no conflicts of interest, as well as having read and acted in accordance with the precepts of the Helsinki declaration on clinical studies.

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Study of the genetic basis of Trabecular Bone Score reduction related to aromatase inhibitors

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Summary

Objectives: Aromatase inhibitors (AI) are effective adjuvant endocrine therapies for breast cancer patients, although they have been associated with an increased risk of osteoporotic fracture. Trabecular Bone Score (TBS) loss has been previously demonstrated, although it may vary among AI-treated patients. This study aims to identify the genetic basis associated with TBS change by studying steroidogenic pathway genes. Material and methods: The B-ABLE cohort studies prospectively postmenopausal women with breast cancer under treatment with AI. TBS is calculated from the raw data acquired in dual-energy x-ray absorptiometry (DXA) scan at the outset of the study and at the end of AI-treatment. The relative TBS change was calculated as the percentage variation of the TBS value at the end of treatment from baseline. To study the possible genetic association, nucleotide polymorphisms (SNPs) were genotyped in genes *CYP11A1, CYP17A1, HDE3B2, HDE17B3, CYP19A1, CYP2C19, CYP2C9, ESR1, GC, CYP27B1, VDR* and *CYP24A1*. The possible relationship between genes and TBS changes was studied by multiple linear regression, considering models of dominant, recessive and additive genetic inheritance.

Results: The study included 212 women that had not been treated with bisphosphonates and had available TBS data. Half of the patients had been treated previously with tamoxifen. The percentage of intra-individual TBS change was -0.04% [95% CI: -0.05 to -0.03; p<0.001] at the end of AI treatment. The SNP rs6013897 in the gene *CYP24A1* showed a significant association with TBS reduction [p=0.03565; coefficient β (95% CI) = -1.55 (-2.98 to -0.11)].

Conclusions: The *CYP24A1* gene could be involved in the phenotypic variability found in bone microarchitecture deterioration during AI treatment.

Key words: *aromatase inhibitors, breast cancer, TBS, genetic association study.*

Introduction

Aromatase inhibitors (AI) are widely regarded as effective adjuvant endocrine therapies for breast cancer patients with positive hormone receptors. These patients generally present a good prognosis, with an overall survival rate higher than 80%[1,2]. However, these therapies have been associated with side effects that may affect quality of life, such as bone loss and increased osteoporotic fracture[3]. Clinical guidelines for treating AI-related bone loss (AIBL) recommend strict control of bone mineral density (BMD) and other risk factors to assess the need for treatment with antiresorptive therapies[4].

The Trabecular Bone Score (TBS) has been proposed as a tool for skeletal evaluation, complementary to conventional BMD, which has proven to be clinically useful for predicting fracture risk[5]. The TBS is a non-invasive measure of bone microarchitecture extracted from bone densitometry by means of dual radiological absorptiometry (DXA) of the lumbar spine. Interestingly, most individuals with a fragility fracture have BMD in the osteopenic or even normal range, which indicates the importance of bone quality in fracture risk[6]. High TBS values indicate a better microarchitecture, while lower values reflect poor bone quality with greater susceptibility to fracture[7]. Regarding the treatment of breast cancer with AI, it has been shown that TBS could be affected with a significant microarchitecture reduction, comparable to BMD loss[8].

The B-ABLE cohort is a prospective clinical cohort of postmenopausal women with early-stage breast cancer receiving AI[9] in whom an association between *CYP11A1* gene polymorphisms and bone loss after 2 years of treatment with AI[10] has been reported. This indicates that the AIBL variability observed among patients could be partly genetically determined.

As with AIBL, a great variability in TBS changes has also been observed among patients treated with AI[8], which suggests that there may also be a genetic basis in the response to AI. No genetic study has yet been conducted on TBS loss in AI-treated patients, so it is not yet known if this parameter is also genetically determined. Our study aims to determine the genetic basis of this variability in the B-ABLE cohort by genotyping genetic variants in genes of the steroidogenic pathway.

Material and methods

Participants

From December 2005 to February 2013, postmenopausal Caucasian women diagnosed with early breast cancer, positive for hormone receptors and candidates for AI treatment (cohort B-ABLE) were consecutively recruited. The postmenopausal state was defined as patients aged >55 years with amenorrhea for more than 12 months, or those aged ≤55 years with luteinizing hormone levels >30 mIU/ml or values of follicle stimulating hormone >40 mIU/ml. We excluded women with a history of bone disease, rheumatoid arthritis, metabolic or endocrine diseases, previous diagnosis of osteomalacia or Paget, concurrent or previous treatment with bisphosphonates, oral glucocorticoids, or any other drug with bone activity except tamoxifen.

Ethical approval

The study protocol was approved by the Ethics Committee of the Mar health facilities (2013/5283/I), and written informed consent was obtained from all participants after having read the information sheet of the study and responded to all the questions.

Study design and interventions

B-ABLE is a prospective, non-selected clinical cohort study carried out in the Breast Cancer Unit and the Hospital del Mar (Barcelona, Spain) bone metabolism unit. The participants were treated with AI (letrozole, exemestane or anastrozole) in accordance with American Society of Clinical Oncology recommendations[11]: 5 years of AI beginning within 6 weeks after surgery or 1 month after the last cycle of chemotherapy or alternatively, switching to an AI after taking tamoxifen for 2 to 3 years, to complete 5 years of hormone therapy.

All participants were supplemented with calcium tablets and 25(OH) vitamin D3 (1,000 mg and 800 IU per day, respectively), and those with vitamin D deficiency (<30 ng/ml) received an additional dose of 16,000 IU. calcifediol (HIDROFEROL® FAES FARMA) every 2 weeks.

Variables and measures

Trabecular Bone Score

TBS measurements in the spine were carried out using the program installed in the densitometer (TBS iNsight® v2.1, Med-Imaps, Pessac, France). The TBS is calculated on the basis of the raw data acquired by DXA, evaluating the same regions used for the study of the BMD of the lumbar spine and without additional administration of ionizing radiation to the patient. The TBS data were obtained at the beginning and at the end of the AI treatment.

Other measurements

Information was recorded on clinical variables at the time of inclusion, including the age of recruitment, the age of menarche and menopause, the number of children, breastfeeding, previous chemotherapy and radiotherapy, adjuvant treatments, weight, height and plasma levels of 25(OH) vitamin D.

Selection of candidate genes

For the study of genetic association, genes were selected that code for the key factors in the synthesis and response of estrogen and vitamin D (Figure 1). Eight candidate genes were selected in the case of estrogen: *CYP11A1, CYP17A1, HDE3B2, HDE17B3, CYP19A1, CYP2C19, CYP2C9* and *ESR1* and four genes of vitamin D: *GC, CYP27B1, VDR* and *CYP24A1*.

Selection of polymorphisms of change of a nucleotide (SNPs)

The SNPs were selected on the basis of the following criteria: 1) frequency of the minority allele (MAF) >0.05; 2) tag-SNPs according to the

HapMap project in the population of CEU (Residents of Utah with European ancestry of the North and the West, coming from the CEPH collection); 3) putative functional polymorphisms; and 4) previous association with other musculoskeletal phenotypes: plasma concentrations of 25(OH) vitamin D[12], BMD[13-16] and AI-related arthralgia[17].

Genomic DNA extraction and genotyping of polymorphisms

The extraction of DNA from peripheral blood was carried out at the LGC genomic facilities. The genotyping of the polymorphisms was carried out using the KASPar v4.0 genotyping systems in the LGC genomic facilities. To guarantee the quality of the genotyping, a random sample (5% of the total number of samples) was also genotyped on a separate control plate. There was a 100% agreement between these results.

Statistical analysis

Relative TBS change was calculated as the percentage change of the TBS value at the end of treatment compared to the baseline TBS value. Changes of intra-individual TBS were evaluated using the Student t test for paired samples.

To assess the association between the SNPs studied and the change in TBS, linear multivariable regressions were used (log-additive, dominant and recessive models). The models were adjusted for age, body mass index (BMI) and previous treatment with tamoxifen. The collinearity, the interaction and the linear trend of the covariates were checked. The possible confounding factor for baseline and final concentrations of 25(OH) vitamin D was also evaluated. To minimize error due to multiple tests, the False Discovery Rate (FDR)[18] correction was used, accepting all predictions with $p<0.05$ as significant.

All the analyzes were two-tailed. Statistical analyzes were performed using R for Windows version 3.3.3 (packages: foreign, car, compareGroups, SNPassoc, and multi-test).

Results

Baseline characteristics of the patients and evaluation of the TBS

The study included 212 women in the B-ABLE cohort with TBS values and who had not been treated with bisphosphonates. Half of the patients had received previous treatment with tamoxifen. The initial clinical characteristics of the study participants are shown in table 1.

The percentage of cumulative intra-individual change of TBS was -0.04% [95% CI: -0.05 to -0.03; $p<0.001$] at the end of the AI treatment.

Genetic association with the change of TBS

Table 2 shows the minor allele frequency (MAF) and the p-value of the Hardy-Weinberg equilibrium (HWE) for each SNP genotyped in the B-ABLE cohort. The SNP rs6013897 showed a nominally significant association with TBS reduction attributed to AI (Table 3). The AA genotype of this SNP is associated with greater loss of TBS (mean: -5.32, SD: ±6.15) with respect to the TT genotype (mean: -2.20, SD: ±6.27). These results do not vary after adjusting for both baseline and final vitamin D levels.

Discussion

The results of this study in which genetic variants were genotyped in genes involved in the estrogen and vitamin D hormone response pathways show that a downstream SNP of the *CYP24A1* gene is significantly associated with the change of TBS at the end of treatment with AI. This same SNP has recently been associated with total hip BMD where the A allele was associated with lower levels of BMD[19].

The *CYP24A1* gene encodes the mitochondrial protein that initiates the degradation of 1,25-dihydroxyvitamin D3, the physiologically active form of vitamin D3. It catalyzes the NADPH-dependent 24-hydroxylation of calcidiol (25-hydroxyvitamin D3) and calcitriol (1-alpha, 25-dihydroxyvitamin D3). The enzyme can perform up to 6 rounds of hydroxylation of calcitriol leading to calcitroic acid. It also shows 23-hydroxylation activity which produces 1-alpha, 25-dihydroxyvitamin D3-26.23-lactone as the final product. In the regulation of vitamin D3 level, this enzyme plays a role in calcium homeostasis and in the endocrine system of vitamin D. This enzyme is expressed in several tissues mainly in the adrenal cortex and bladder according to the GTEX portal.

The SNP rs6013897 is associated with the serum levels of 25(OH) vitamin D[12], so a possible mechanism for the effect of rs6013897 on TBS could be through the changes of 25(OH) vitamin D serum; therefore, 25(OH) vitamin D levels were subsequently included as covariates in the linear regression. After adjusting for vitamin D the association of the SNP did not vary, which shows that the effect of the SNP on the change of TBS is independent of serum levels of vitamin D. This may imply that the action of *CYP24A1* may be more locally of the bone tissue.

This is the first study to find an association between *CYP24A1* and TBS reduction due to AI treatment, which highlights the important role played by vitamin D in the prevention of bone deterioration. Interestingly, in the case of AIBL, a significant association was found with the *CYP11A1*[10] gene, whereas in the case of the loss of TBS, the associated gene is *CYP24A1*. These data suggest that both the loss of bone mass and the loss of TBS related to AI are genetically determined, although the genes involved in the two bone phenotypes are different. In any case, these two genes belong to the same metabolic pathway, which highlights the importance of vitamin D in the modulation of the adverse effects of IA in bone tissue. This gives more weight to the fact of supplementing patients with vitamin D, as is taking place in the B-ABLE cohort.

Our study included a limited number of patients in this specific analysis of our cohort, which did not allow the detection of subtle allelic effects. However, as far as we know, this is the lar-

Figure 1. Diagram of the metabolites and enzymes of the steroidogenic pathway. The genes selected for the association analysis are marked in bold in a gray box

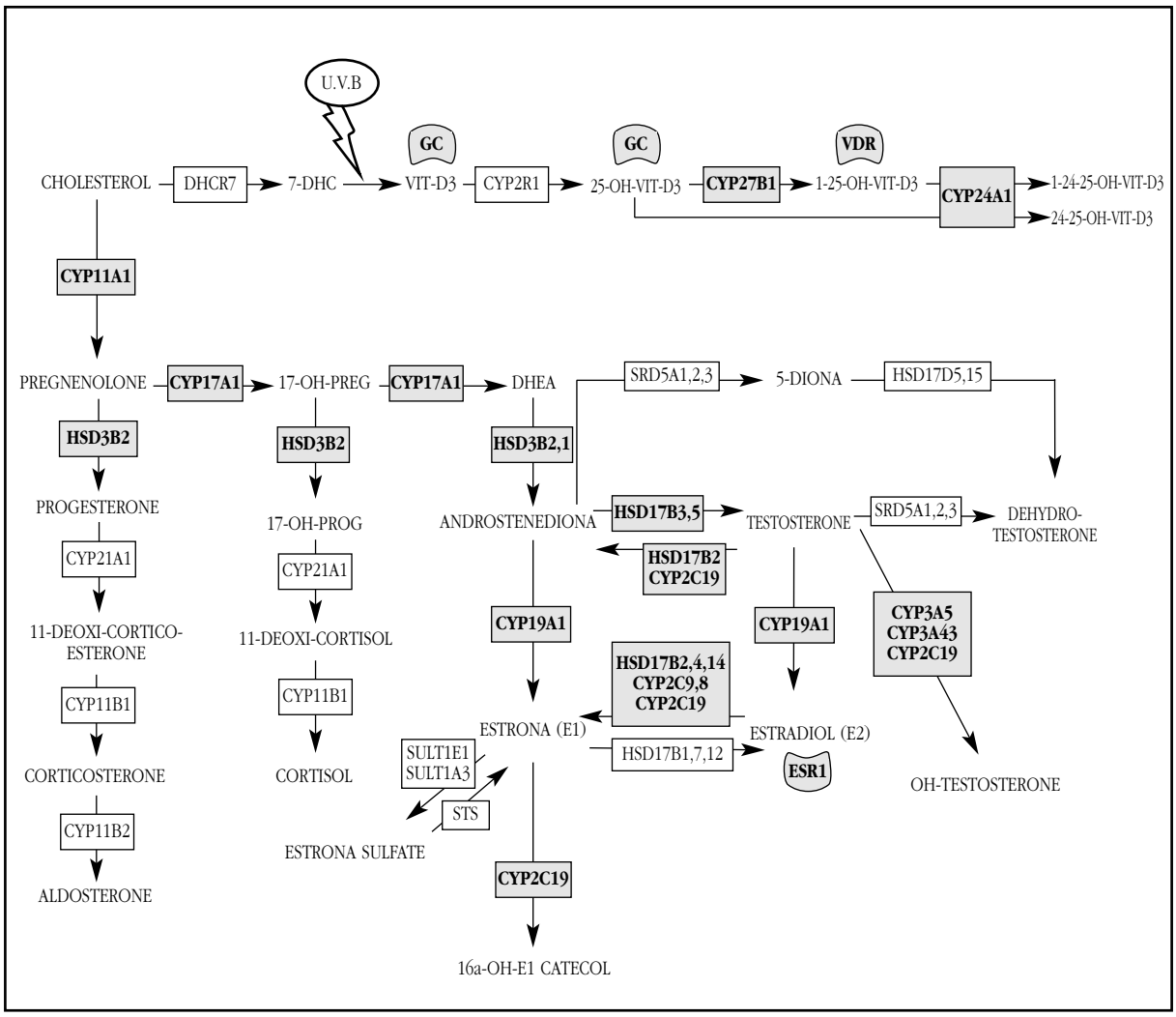

Table 1. Characteristics of patients from the B-ABLE cohort

Variables	N=212
Age (mean ± SD)	59.7±8.39
BMI (mean ± SD)	29.2±5.19
Previous treatment with TAM (n (%))	118 (55.7%)
Absolute value of baseline TBS (mean ± SD)	1.22±0.13
Absolute value of TBS at the end of treatment (mean ± SD)	1.18±0.12
TBS relative change percentage from start to finish of treatment (mean ± SD)	-2.95±5.93

BMI: body mass index; DE: standard deviation; TAM: tamoxifen; TBS: Trabecular Bone Score.

gest cohort available to date for genetic studies of TBS in women treated with AI. In addition, being a population study with data obtained from the usual clinical practice reinforces the observed results. Finally, the fact of working with candidate genes that have previously been associated with bone phenotypes or hormonal levels facilitates the finding of reliable associations and reduces the number of SNPs in the study.

In conclusion, an association was found between the SNP rs6013897 near the *CYP24A1* gene, previously associated with serum 25(OH) vitamin D levels, and the bone microarchitecture deterioration related to AI treatment, measured through TBS.

Table 2. BMI: body mass index; DE: standard deviation; TAM: tamoxifen; TBS: Trabecular Bone Score

Locus	ID SNP	Alleles (R/A)	MAF (*)		HWE Value p (*)	Efficiency of genotyping (%)
CYP19A1	rs1062033	C/G	0.45	(G)	0.67	96.70
	rs4775936	C/T	0.48	(T)	1.00	95.28
CYP17A1	rs4919687	G/A	0.28	(A)	0.06	96.23
	rs6163	C/A	0.44	(A)	0.16	97.17
CYP11A1	rs4077581	T/C	0.38	(C)	0.07	96.23
	rs900798	G/T	0.41	(T)	0.06	96.23
	rs11632698	G/A	0.49	(A)	0.32	94.81
HDE3B2	rs2854964	A/T	0.37	(T)	0.65	96.70
	rs3765948	T/C	0.15	(C)	0.43	97.64
HDE17B3	rs408876	G/A	0.15	(A)	0.18	96.70
	rs2066474	A/G	0.19	(G)	0.37	97.17
	rs2183009	A/G	0.36	(G)	0.36	97.17
CYP2C19	rs12248560	C/T	0.22	(T)	1.00	97.17
	rs3758581	G/A	0.06	(A)	0.00	94.81
	rs4244285	G/A	0.13	(A)	0.76	96.23
CYP2C9	rs28371674	C/T	0.16	(T)	0.60	96.23
ESR1	rs2504063	G/A	0.44	(A)	0.48	95.28
CYP27B1	rs10877012	G/T	0.24	(T)	0.83	78.30
	rs4646536	A/G	0.26	(G)	0.69	79.25
GC	rs3755967	C/T	0.33	(T)	1.00	94.81
CYP24A1	rs6013897	T/A	0.21	(A)	0.68	95.28
	rs11907350	G/A	0.04	(A)	0.05	97.17
	rs4809957	A/G	0.25	(G)	1.00	96.23
VDR	rs2544037	A/G	0.43	(G)	0.32	96.7
	rs11568820	C/T	0.25	(T)	0.57	94.81
	rs1544410	G/A	0.33	(A)	0.34	96.23
	rs17879735	C/A	0.44	(A)	1.00	95.75

R: reference allele; A: alternative allele; MAF: frequency of the minority allele; HWE: balance of Hardy-Weinberg, *(in the B-ABLE cohort).

Table 3. SNP associated with the change of TBS in an adjusted linear regression analysis

Locus	SNP ID	Genotypes	n	β coefficient (95% CI)[a]	P value
CYP24A1	rs6013897	T/T	121	ref.	0.03565
		T/A	63	-1.55 (-2.98 a -0.11)	
		A/A	8		

[a]: adjusted for age, body mass index and previous treatment with tamoxifen; n: number of patients; ref: reference value.

Conflict of interests: The authors declare they have no conflicts of interest in this work.

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Monthly versus biweekley calcifediol in the treatment of osteoporotic patients. Study in real life

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Summary
Objectives: To assess serum concentrations of 25-hydroxyvitamin D, 25(OH)D, in osteoporotic patients treated for one year with calcifediol.
Methods: We have studied 156 patients with osteoporosis (23 males and 133 females), aged 71,9±9,6 years who had received treatment with calcifediol for at least one year. Ninety-two of them received 0.266 mg of calcifediol every fifteen days and the remaining 64 the same dose once a month. Serum levels of 25(OH)D, intact PTH (iPTH), procollagen type 1 amino-terminal propeptide (PINP) and C-terminal cross-linked telopeptide of type I collagen (CTX) were determined before and one year after starting treatment.
Results: A significant increase in the concentration of 25(OH)D was observed with both treatment regimens ($p<0.001$). The percentage of patients who reached levels of 25(OH)D higher than 20 and 30 ng/ml was similar with both guidelines, while the percentage of patients exceeding 60 ng/ml was higher with the biweekly dose ($p<0.01$). The concentration of iPTH decreased significantly after the administration of calcifediol, although on this occasion there were no differences between the two forms of treatment. Both bone remodeling markers, PINP and CTX, decreased similarly in patients treated with antiresorptives ($p<0.0001$), without these changes being related to the calcifediol regimen.
Conclusions: The monthly administration of 0.266 mg of calcifediol is adequate to achieve effective levels of vitamin D, and it is also safe enough to avoid reaching potentially harmful levels of it, so it would be preferable to the biweekly schedule in the usual clinical practice.

Key words: *calcifediol, PTH, osteoporosis, vitamin D, bone remodeling markers..*

Introduction

Opinions vary as to what constitutes normal levels of vitamin D in the general population[1-5]. According to some authors, severe vitamin D deficiency is when serum levels of 25-hydroxyvitamin D (25(OH)D or calcifediol) are below 10 ng/mL, moderate deficiency when they are between 10 and 20 ng/mL, insufficiency between 20 and 30 ng/mL and adequate values if they are above 30 ng/mL[1-3]. Others, such as the US Institute of Medicine, propose values for the healthy general population above 20 ng/mL[4]. Nor is there an established excessive, potentially harmful level, although there is a tendency to set this at around 50-60 ng/mL[1,5,7,8].

As for patients with osteoporosis, most clinical practice guidelines indicate that treatment with antiresorptive or anabolic drugs must be accompanied by an adequate supply of vitamin D, in addition to an appropriate amount of calcium[9-11]. The latter, whenever possible, should be administered through diet, while vitamin D is recommended to be administered as supplements. An aspect also debated is the serum 25(OH)D levels that patients with this disease should reach, although most authors and scientific societies, including Spanish ones, recommend serum concentrations above 30 ng/mL[2,3,5,6,9-12]. To achieve these aims, a daily dose of 800-1,000 IU of vitamin D (in Europe the vitamin D used is vitamin D3 –cholecalciferol–) is advised, although its weekly, biweekly or monthly equivalent can also be administered[10]. The biological potency of vitamin D is established as international units (IU), so that 1 µg of cholecalciferol equals 40 IU[13]. In some countries, as in the case of Spain, 25-hydroxyvitamin D3, a metabolite of vitamin D, also called calcifediol or calcidiol, is also commercially available. It is prepared in dispensing units containing 0.266 mg. The exact equivalence of this dose with vitamin D in terms of metabolic activity (how many IU of vitamin D activity is 1 µg of calcifediol) is not known with total precision, but the most widespread therapeutic regimen has been to administer once to the month or every fifteen days, compared to the 800-1,000 IU of cholecalciferol daily, as mentioned above.

Calcifediol is more hydrophilic than cholecalciferol, with a shorter half-life, and causes a more rapid, sustained increase in serum levels of 25(OH)D[14,15]. A recent study carried out in our country in 40 women with postmenopausal osteoporosis[16] reported 25(OH)D levels around 80 ng/mL (basal: 15.2 ng/mL) after the administration of a weekly dose of 0.266 mg of calcifediol for six months, and around 65 ng/mL (basal: 15.8 ng/mL) with the same dose in a fortnightly schedule. In another study carried out by our group[17] in osteoporotic women receiving treatment with alendronate, the administration of 0.266 mg of calcifediol weekly for three months also managed to increase the levels of 25(OH)D in a similar way (82±31 ng/mL basal 21 ng/mL).

These data suggest that the 25(OH)D levels reached with the weekly or biweekly therapeutic regimen could generate concentrations higher than the desirable levels of vitamin D[1,5-8]. It is therefore possible to think that administering a more spaced dose of calcifediol over time is sufficient to maintain adequate levels of 25(OH)D in routine clinical practice. Thus, we have proposed: a) to evaluate the serum concentrations of 25(OH)D in osteoporotic patients treated for a year with biweekly or monthly doses of 0.266 mg of calcifediol; and b) determine if there are changes in intact parathyroid hormone levels (iPTH) and remodeling markers, amino-terminal propeptide of type I procollagen (PINP) and carboxyterminal telopeptide of type I collagen (CTX), after administration of both doses of calcifediol.

Material and methods

Patients

We retrospectively reviewed the data of the last 200 osteoporotic patients who had been treated until the time of study in the Bone and Mineral Metabolism Unit of our Center and who had received treatment with 0.266 mg of calcifediol (Hydroferol®) every two weeks or monthly for at least one year (mean ± SD: 15±3 months). Exclusion criteria were the suffering of certain processes (malabsorption, renal failure [Glomerular filtration <45 mL/min/1.73 m^2], uncontrolled hyperthyroidism, primary hyperparathyroidism, chronic liver diseases, chronic inflammatory diseases) or the follow-up of treatments that they could interfere with bone metabolism (glucocorticoids, anticonvulsants, antihormonal treatments), as well as previous treatment with vitamin D supplements. Those who recognized inadequate therapeutic compliance were also excluded. The diagnosis of osteoporosis was based on the presence of fragility fractures (vertebral or hip) or the existence of a bone densitometry with T≤2.5 values in the lumbar spine, femoral neck or total hip (DXA, Hologic QDR 4500). The majority of patients also received treatment with antiresorptives (63% with oral bisphosphonates, 9% with zoledronate and 28% with denosumab). Clinical and analytical data were collected through the electronic medical record. In all cases, the month in which the two analytical determinations were made (baseline and after treatment) was collected. The body mass index (BMI) was obtained by dividing the weight in kg. between the height in meters squared. Weight and height were measured while the patient was in underwear and without shoes.

The study was approved by our clinical research ethics committee (CEIC) (2017.023).

The criterion for deciding the therapeutic regimen, biweekly or monthly, was based on the baseline of 25(OH)D, preferring the first in cases in which this figure was lower. However, a precise cutting value was not established, leaving the decision to the opinion of the doctor responsible for the patient. The retrospective analysis showed that 90% of the patients who received the biweekly regimen had baseline values lower than 20 ng/mL, while this percentage was only 53% in the case of the monthly regimen.

Analytical determinations

Serum levels of 25(OH)D, iPTH, PINP and CTX were determined before (baseline) and at least one year (12-25 months) after starting treatment with calcifediol (mean ± SD: 15±3 months).

All determinations were made on an empty stomach early in the morning. The routine determinations (glucose, creatinine, calcium, albumin, phosphate, alkaline phosphatase) were carried out by automated methods in a TechniconDax autoanalyzer (Technicon Instruments, Colorado, USA). The serum concentrations of 25(OH)D, iPTH and markers of remodeling (PINP and CTX) were carried out by an automated chemiluminescence system. In the case of 25(OH)D, the chemiluminescence assay of DiaSorin LIAISON (DiaSorin, Stillwater, Minnesota, USA) was used, and in the remaining tests the IDS-ISYS assay (Immunodiagnostic Systems Hokding PLC, London, RU). The limit of detection of 25(OH)D was 4 ng/mL and the intra- and inter-assay variation coefficients (CV) of 4.4% and 8.3%, respectively. Regarding iPTH, the limit of detection was 6 pg/mL, with a normal range of 10 to 45 pg/mL. The intra-assay and inter-assay CVs were 3.7% and 5.4%, respectively. The detection limit of the PINP was 5 ng/mL (reference range between 18 and 102 ng/mL) and its intra-assay and inter-assay CV of 4.6% and 9.2%, respectively. Finally, CTX intra-assay and inter-assay CVs were 5.9% and 10% respectively, and their reference range was 0.152-0.761 ng/mL.

Statistical study

The quantitative variables were expressed as mean ± SD or median (interquartile range) and were compared using Student's t test or Mann-Whitney U test, according to the distribution of the data. The qualitative variables were expressed as number and percentage, and for comparison the χ^2 test or Fisher's test was used, as appropriate. The differences between the basal and annual values of calcitrope hormones and markers of remodeling were analyzed using the Wilcoxon test. The association between the percentage of change (baseline-annual) of serum levels of 25(OH)D, iPTH, PINP and CTX, and age, sex, BMI, weight and month of the year of the determination of the laboratory was analyzed using the Pearson/Spearman correlation coefficient. Data analysis was carried out using the SPSS v20 statistical package (Chicago, IL). A $p<0.05$ was considered significant in all calculations.

Results

After applying the exclusion criteria, we finally studied 156 patients (23 men and 133 women) between the ages of 49 and 93 years (mean ± SD: 71.9±9.6). All had received treatment with calcifediol for at least one year, with an average of 15±3 months. Ninety-two of them had received 0.266 mg of calcifediol every fortnight and the remaining 64 received the same dose once a month (Table 1). The patients who received the biweekly regimen were older (4.6 years older, $p<0.05$). There were, however, no differences in weight or BMI.

As can be seen in table 2, and in accordance with the criterion that had led to the choice of one or another therapeutic regimen, the baseline levels of 25(OH)D were lower in patients who received fortnightly calcifediol than in those who received the monthly dose ($p<0.01$). In absolute terms, the difference was approximately 7 ng/mL (16.7 ng/mL vs. 23.3 ng/mL). With both treatment regimens, a significant increase in the concentration of 25(OH)D was observed ($p<0.001$), although these values were higher with the biweekly regimen than with the monthly regimen (in absolute terms, 39.5 ng/mL and 15.5 ng/mL, which meant in relative terms increases of 323% and 85% [$p<0.0001$]). The final concentration was clearly higher in patients receiving the biweekly regimen (56.2±18.5 ng/mL vs. 38.8±12.5 ng/mL, $p<0.01$).

The percentage of patients who reached 25(OH)D levels above 20 and 30 ng/mL was slightly higher with the biweekly treatment (100% and 92%, respectively) than with the monthly regimen (97% and 80%, respectively). However, the percentage of patients exceeding 60 ng/mL was higher in patients who received the biweekly regimen of calcifediol, 38%, compared to 6% in patients with the monthly regimen ($p<0.01$).

The concentration of iPTH decreased significantly after the administration of calcifediol with both treatment regimens. It is interesting to note that, despite the existence of differences in the values of 25(OH)D at the end of the study, the PTH values at that time were similar with the two treatment regimens (Table 2, Figure 1). The changes in the levels of iPTH in absolute terms (-19% vs. -20%) were also similar. Therefore, a clear discrepancy is observed in the behavior of 25(OH)D and PTH.

All patients had normal calcium values at baseline and no case of hypercalcemia was detected with both treatment regimens.

The results of the remodeling markers (PINP and CTX) are also shown in table 2. As expected, both markers, PINP and CTX decreased significantly in patients treated with antiresorptives ($p<0.0001$), without these changes will be related to the dosage regimen of calcifediol (biweekly or monthly).

Finally, the changes in the levels of 25(OH)D, iPTH, PINP and CTX had no relationship with age, sex, weight, BMI or the month of the year in which the samples were obtained.

Discussion

In most of the clinical practice guidelines of osteoporosis, recommended serum levels of 25(OH)D are above 20 ng/mL, and even exceed 30 ng/mL[9-11]. In our study, the vast majority of patients who received monthly or biweekly doses of calcifediol for at least one year exceeded these figures. Specifically, the percentage of patients who reached levels 25(OH)D higher than 20 and 30 ng/mL was 100% and 92% with the biweekly regimen and 97% and 80% with the monthly, respectively.

As expected, the average concentrations of 25(OH)D reached by patients treated with calcife-

diol for at least one year were lower on the monthly than on the biweekly schedule. However, it can be concluded that both guidelines are effective for attaining metabolically sufficient 25(OH)D concentrations. A criterion conventionally used to assess the efficacy of serum levels of 25(OH)D involves its relationship with serum PTH, a negative relationship, since it inhibits the production of serum PTH. Since PTH is thought to be harmful to bone, its inhibition by vitamin D should be beneficial. Although there is no general agreement regarding the exact characteristics of this relationship, the most accepted idea is that as the levels of 25(OH)D rise, PTH decreases until it reaches a value above which the hormone is not suppressed more[2,3,18-22]. According to what we have been commenting, above this value of 25(OH)D, the beneficial effect does not continue to increase, as the PTH decreases. Although it is not known exactly at which concentration of 25(OH)D this value corresponds, it seems that there is agreement that it should be between 20 and 30 ng/mL, or close to these figures[20-22]. Our group studied more than 1,800 people (1,154 postmenopausal women and 657 men 50 years and older) and found that the threshold of 25(OH)D needed to prevent the increase of PTH (and the loss of bone mass) would be around 30 ng/mL[22]. The results of the present work point to an equivalent idea when showing that the concentration of iPTH decreases significantly after administering calcifediol without evident differences in the behavior of the hormone between the two treatment regimens. In fact, the reduction obtained with the biweekly schedule was 19% and 20% with the monthly pattern. So, from a certain value, vitamin D no longer exerts beneficial effects.

Studies concerning vitamin D doses and desirable 25(OH)D serum levels have usually focused on aspects related to their efficacy. The possible toxicity of vitamin D has traditionally been related to the development of hypercalcemia, which only occurs with high doses of vitamin D (several thousand-day units) and levels of 25(OH)D in ranges greater than 100 ng/mL[3]. It was thus considered a safe substance. However, over the last few years, data have been appearing suggesting that vitamin D could develop other pernicious effects, independent of hypercalcemia, with much lower levels. So, the idea that, in general terms, the relationship between the beneficial or harmful effects of vitamin D and its levels have a U-shaped relationship (that is, detrimental effects develop both with low levels and with high levels of the vitamin), with the particularity that in the latter case they would begin to settle down with levels of 25(OH)D very inferior to the 100 ng/mL previously commented, having indicated figures of around 50-60 ng/mL. Michaelson et al.[23], for example, followed a cohort of 1,194 males observing a U-ratio between vitamin D concentration and total mortality. The morphology of the curve was also met in cancer mortality specifically. Durup et al.[8] also described in 247,574 people a reverse association in the form of J between serum levels of 25(OH)D and mortality. Smith et al.[24], in a study conducted with various doses of vitamin D supplements, observed that the effect of the same in

Table 1. Baseline characteristics of the patients included in the study (biweekly dose and monthly dose)

	Biweekly	Monthly	Total
Nº patients	92	64	156
Age (years) #	73.8 (9.3)	69.2 (9.6)	71.9 (9.6)*
Sex (W/M)	75/17	58/6	133/23
Weight (kg) #	64.9 (13.5)	63.5 (9.9)	64.3 (12.0)
Size (m) #	1.55 (0.06)	1.56 (0.08)	1.55 (0.07)
BMI (Kg/m²) #	26.9 (5.3)	26.2 (4.5)	26.6 (4.9)

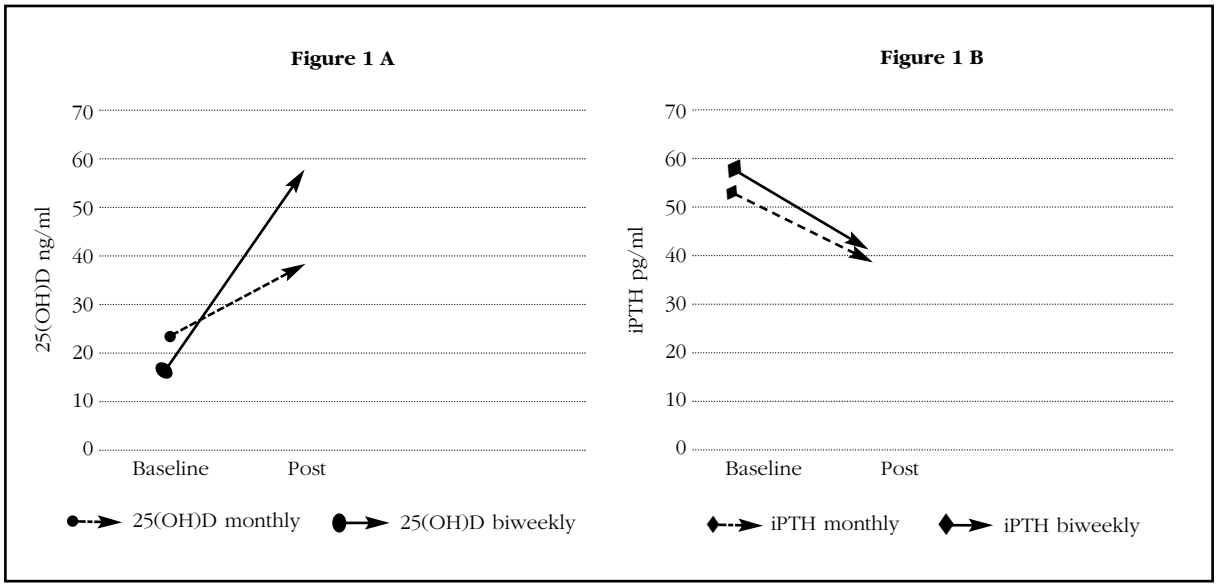
#: mean (SD); *: p<0,05.

Table 2. Concentrations of 25(OH)D, iPTH, PINP and CTX before (baseline) and after at least one year of treatment (after treatment) with 0.266 mg of fortnightly (biweekly) or monthly (monthly dose) calcifediol. Expressed as mean (SD)

	Biweekly dose		Monthly dose	
	Baseline	**After treatment**	**Baseline**	**After treatment**
25(OH)D (ng/mL)	16.7 (9.3)	56.2 (18.5)**	23.3 (8.3)	38.8 (12.5)**ǂ
iPTH (pg/mL)	57.9 (26.0)	40.7 (17.5)**	52.9 (25.0)	38.6 (15.9)**
PINP (ng/mL)	47.1 (27.4)	26.9 (19.8)**	36.5 (17.0)	26.7 (18.1)**
CTX (ng/mL)	0.573 (0.347)	0.288 (0.333)*	0.484 (0.337)	0.243 (0.219)*

Differences between baseline values and after treatment: (*) p<0.01; (**) p<0.001.
Differences between the biweekly and monthly doses: (ǂ) p<0.01.

Figure 1. A) Average concentrations of 25(OH)D, before (baseline) and after at least one year of treatment (post) with 0.266 mg of monthly or fortnightly calcifediol. B) Mean concentrations of iPTH, before (baseline) and after at least one year of treatment (post) with 0.266 mg of calcifediol monthly or biweekly



the reductions also shows a U curve, both the relationship in terms of dose and levels of 25(OH)D analyzed. The various research studies carried out in this line do not coincide exactly with the values that could be preferable, but in general terms, reviews and consensus documents indicate that values greater than 50-60 ng/mL should be avoided[5,7].

The results of our study show that while the figures reached with the monthly guideline are located in a safety zone (38.8±12.5 ng/mL), those obtained with the biweekly schedule do so in potentially harmful values (56.2±18.5 ng/mL), since the percentage of patients who exceeded 60 ng/mL among those who received the biweekly regimen was 38%, compared to 6% of those who received the monthly ($p<0.01$). Consequently, our work suggests that the therapeutic regimen with 0.266 mg of calcifediol monthly is the most appropriate. This does not mean, at all, that such a guideline should be considered rigidly, since in the achievement of one or other levels of 25(OH)D, factors of an individual nature are involved (for example, genetic) as circumstantial, which may advise modifying the recommended dose in a specific patient. Hence, the periodic measurement of serum 25(OH)D is recommended to check whether the regimen is adequate.

Regarding the behavior of remodeling markers, as expected, both markers, PINP and CTX, decreased significantly in patients treated with antiresorptives, both in those who received bisphosphonates and in those who received denosumab, without these changes being related to the dosage schedule of calcifediol (biweekly or monthly). This should not be interpreted in the sense that the provision of vitamin D does not influence the response to antiresorptive drugs. In a study carried out by our group in osteoporotic women receiving treatment with alendronate[17], some of which were supplemented with 0.266 mg of calcifediol weekly for three months and others not, we found that the response was higher in the supplemented patients, especially when they started from a situation of basal hypovitaminosis (25(OH)D <20 ng/mL).

Among the limitations of our study, it should be noted that this is a retrospective observational study, so we cannot rule out the existence of certain biases (selection, allocation, etc.), as well as confounding factors. For example, in our study, the assignment of patients to one or another treatment regimen was not carried out randomly, but rather in accordance with the criteria of the physician in charge of the patient's care. It is not surprising, therefore, that patients who received the biweekly regimen were older and had baseline values lower than 25(OH)D than those who received the monthly regimen. However, one of the strengths of our study is that it has been carried out under conditions of normal clinical practice.

In conclusion, our results indicate that in patients with osteoporosis being treated for it, the monthly administration of 0.266 mg of calcifediol is, firstly, adequate to achieve effective levels of vitamin D, and secondly, sufficiently safe for avoid reaching potentially harmful levels of it, so it would be preferable to the biweekly schedule in routine clinical practice.

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

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Gorham Syndrome

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We present images of a 50-year-old man who is monitored in our Internal Medicine Ward for lumbago and coxalgia over two years, subsequently diagnosed as Gorham-Stout disease. In the MRI of the hip an altered bone pattern is detected in a diffuse form with no osteolytic or sclerotic lesions. Bilateral necrosis of the femoral head was observed in the hip (Figures 1 and 2).

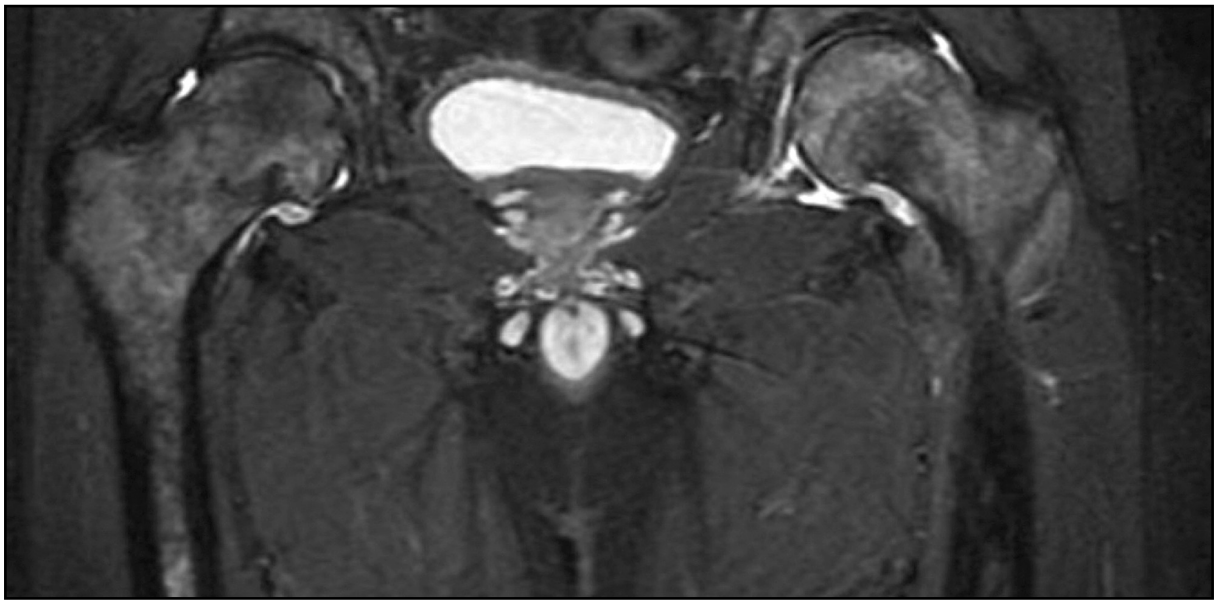
In the blood analysis, only 185 IU/L alkaline phosphatase stood out. The result of the biopsy of the femoral head reported vasodilation with an irregular contour with a malformation; staining with marker D2-40 reported vascular and non-lymphatic proliferation (Figure 3).

Gorham-Stout disease or Gorham syndrome, also known as vanishing bone disease, is a clinical condition characterized by progressive destruction of bone tissue (osteolysis) and growth of lymphatic and vascular tissue in the affected areas.

Although any bone structure can be affected, the most frequent involvement is at the level of ribs, vertebrae and pelvis. The cause is unknown, although it is suspected that alterations in vascular growth factors, changes in pH and oxygen deficit may be involved in the pathophysiology of the disease, since the biochemical changes that occur in the tissue due to these causes favor altered growth and remodeling of affected structures.

The clinical course usually begins with fractures and severe pain, there being a variety of

Figure 1



symptoms according to the diseased bone (loss of teeth in the case of the jaw or neurological signs in the case of the vertebrae).

Treatment involves bisphosphonates and interferon α-2B. Sirolimus is also considered as a therapeutic alternative, although the series are testimonial. In addition, the adoption of support measures that may reduce or stop chylothorax or stabilize the affected skeletal regions is indicated.

Radiotherapy can be used in combination with these therapies but, in general, it is reserved for refractory or rapidly progressive disease.

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

Figure 2

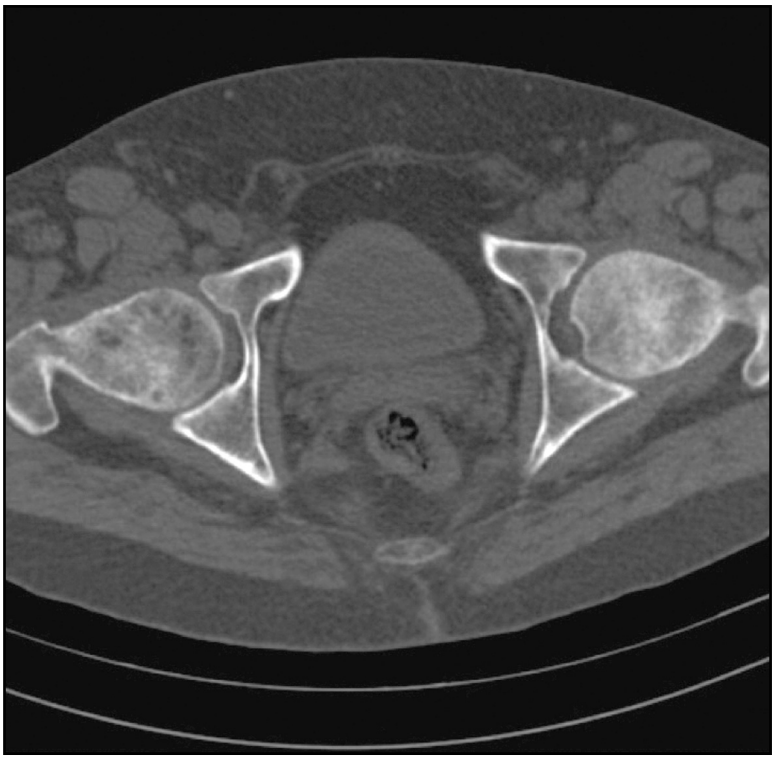
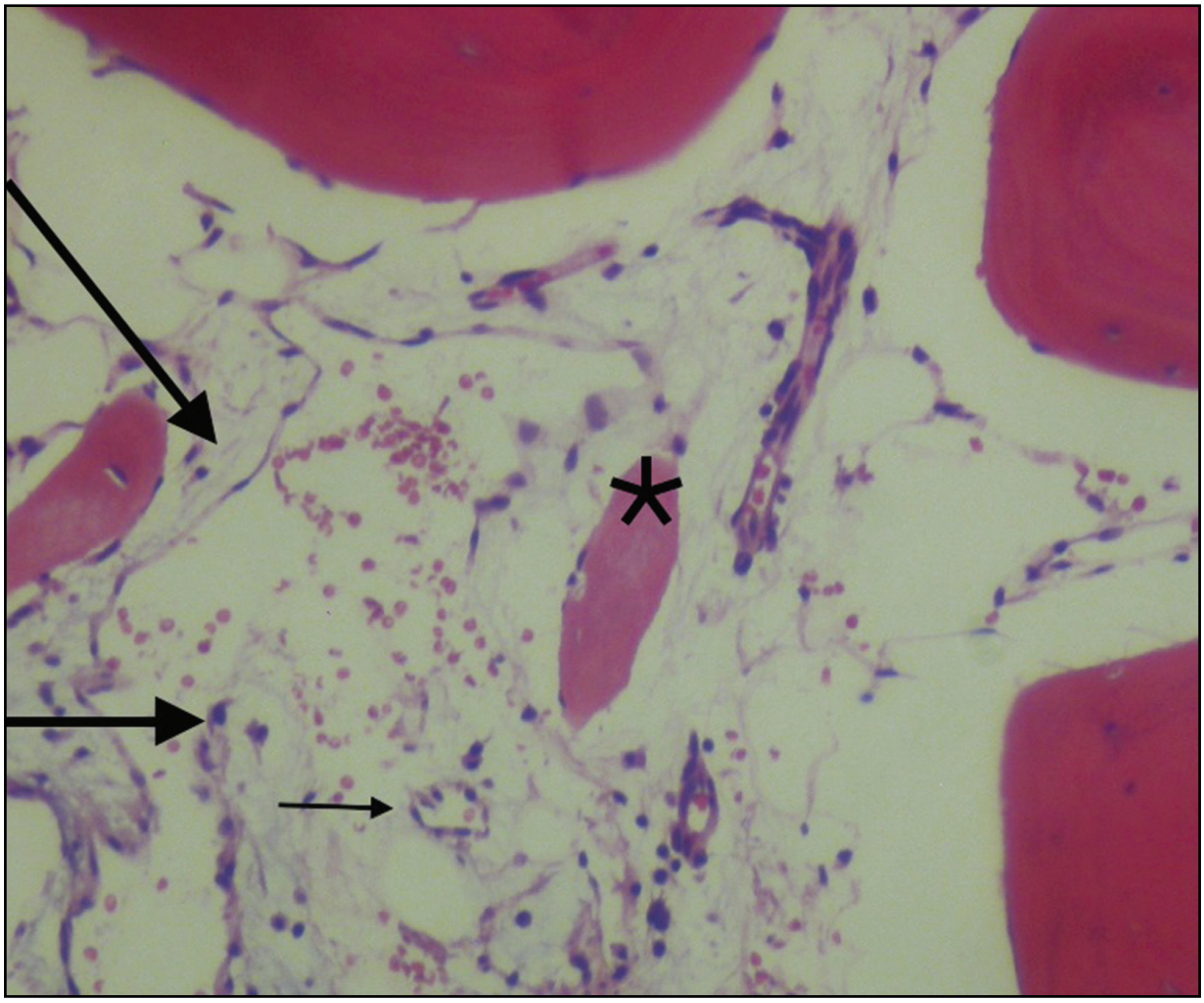


Figure 3



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The usefulness of preoperative traction in hip fracture

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Summary

Hip fracture is a serious clinical event which requires surgery in the vast majority of cases. In the period of time between admission and intervention, which may vary depending on many factors, skin traction in the affected member is traditionally applied to immobilize the limb and thus relieve pain. Furthermore, some surgeons maintain that this practice better reduces fracture risk, avoiding muscular contraction, which may facilitate the surgical process. Its use is recommended in managing protocols for this patient group in some hospitals, although some question this practice as lacking evidence to support it. On the other hand, it is not harmless, with a number of reported complications which raise questions about its routine use. This paper reviews the main studies carried out, with the objective of making a recommendation for the clinical management of this issue based on available evidence.

Key words: *hip fracture, skin traction, pain.*

Introduction

Hip fracture is among the most feared consequences related to bone fragility, a serious process that implies high morbidity and considerable mortality that use resources. Most of these patients undergo surgery in an effort to recover the functionality they previously had. Quality standards recommend carrying out surgery within the first 48 hours, since early intervention is associated with fewer complications, better functional results, shorter hospital stay, fewer readmissions, and even, some studies suggest, to a decrease in mortality1. However, in many cases this surgery is delayed for an undesirably long period. The average delay of surgery in Spain is usually about 3 days[1,2], due largely to clinical and organizational causes. In this preoperative interval, patients in some centers are routinely given skin traction with a variable weight depending on their constitution, aimed at reducing pain by immobilizing the limb (Figure 1). Some surgeons also believe there are other benefits. In theory, traction may reduce muscle contracture, which is potentially beneficial for the surgery itself, especially if the intervention consists of an osteosynthesis of the fracture, whose reduction and management would be hypothetically less difficult. But there is really no evidence in either one way or the other. The bibliography is compelling. Additionally, not only a series of adverse effects are described that make this practice apart from not being really beneficial, it can even be harmful, but also, the basic management of these patients in simple matters such as hygiene or the prevention of ulcers. Pressure can be more complicated if patients are "subject" to this immobilization system. Thus, we believe it is worthwhile to undertake this review and offer recommendations based on what is described in the literature.

Material and methods

For this review, a bibliographic search was carried out in January 2018, in both English and Spanish. The following databases were consulted: Pubmed, Scopus and EMBASE. The search strategy included the following terms in English: hip fracture, skin traction, pain and preoperative traction. It was extended with the terms in Spanish: *fractura de cadera*, *tracción cutánea*, *dolor*, *tracción preoperatoria*. In conclusion, the last review carried out by the Cochrane Library in 2011 is consulted. Of all the articles found, only those whose design consists of randomized prospective studies are selected.

Results

We selected 14 studies that are summarized in Table 1, ordered by date of publication and with their main characteristics[3-16].

Discussion

In one of the first published studies[3], 80 patients were studied with the three classic types of fractures, cervical, intertrochanteric and sub-trochanteric, for which they underwent skeletal traction, skin traction or simply placing a pillow under the affected limb. Regarding required analgesia, it was greater in the skeletal traction group, being similar in the other two groups. The reduction of the fracture was carried out in less time in patients without previous traction, and the bleeding was greater in patients with skeletal traction. The authors concluded that there are no advantages in the placement of any type of traction. As for skeletal traction, it has already been shown in other studies6 that its placement in patients with hip fracture does not show differences in terms of pain relief. It is a painful procedure for most patients who are being treated. In a similar study by other authors, 67 patients were evaluated not only for analgesic need, but also pain intensity with pain measurement scales[5], they found no differences between the groups with and without traction. In another study involving a larger number of patients[4], 137 with extracapsular fractures versus 115 with intracapsular fractures were prospectively randomized, so that 101 patients were placed on traction and 151 were not. Pain perception and the need for analgesia were similar in all groups and the authors recommended abandoning the routine of placing traction prior to surgery. This was not only because of the absence of benefits, but as they considered it a waste of time and money.

In this study, they also considered the subjective surgeons' "sensation" regarding the difficulty of reducing the fracture, without finding significant differences between the groups. In addition, the correction of the femoral head position in the displaced cervical fractures with the placement of the traction was emphasized. In theory, the correction of the external rotation of the limb could prevent the occlusion of the posterior capsular vessels. On the other hand, intracapsular pressure could increase, which could compromise the vascularization of the femoral head. These arguments are based on previous studies of intracapsular pressure measurements in non-displaced femoral cervical fractures[17], in which it is determined that the semi-flexion and external rotation position of the limb is the one with the lowest pressure and, probably, the greatest patient comfort. The position in extension and medial rotation (which is induced with traction) is the one that presents higher figures of intracapsular pressure. The authors already recommended not placing traction in this type of fracture, since it better corrects the patient's physiological posture.

In another study with a similar design in 120 patients[7], similar conclusions were reached. There is no beneficial effect with traction, in the improvement of pain or in the quality of fracture reduction.

In addition, they also did not find differences in their consolidation process. They influenced the possibility of complications with traction; in five of

Figure 1. Image of a classic soft tissue traction in a patient with a hip fracture waiting for a surgical intervention

the patients, secondary skin lesions appeared. Cutaneous lesions are usually the most frequent, since the skin of the elderly is more fragile and easier to lacerate. Sometimes they can be serious[18,19]. Neurological complications of a certain entity have also been described[20].

In the first work proposed to compare the use of a skin traction with the placement of a pillow under the affected limb, well designed, randomized and prospective, carried out in 50 patients in each group[8]. The patients with the pillow were more comfortable and needed less medication than those who had traction. In another prospective randomized study, carried out in 201 patients with traction and 172 without traction, in which pain was measured at various times of admission[9], an increase in pain was observed at the time of admission, with an increased need for pain medication in patients without traction versus those who had traction, but without significant differences, since there is also more pain and more need for analgesia in patients with traction upon admission. The presented graphs are significant. This causes us to question whether it is logical that in the initial moments there is more pain. The patient suffers the fall, is taken to hospital (by ambulance in the best of cases) and transferred several times (emergency stretcher, x-rays) until finally placed in the traumatology bed awaiting their intervention. But over time that pain is reduced, whether you have traction or not. This may explain many surgeons' "impression" that their patients improve with traction. They improve because the pain lessens with time, regardless of whether they have traction or not.

This issue is also seen in a randomized study conducted in the field of nursing only in patients with pertrochanteric fractures, and with the aim of assessing their degree of pain depending on whether or not they have traction14. A similar graph was observed, with pain decrease in the analog visual scale with the passage of hours, but in a parallel way and without differences in both groups. One issue that was raised in this study was the perception of difficulty in mobilizing the patient, which initially was higher in patients without traction, but that decreased over time, although the authors acknowledge that it is a question that is difficult to measure. In that sense, there are works that contemplate the opinion of nursing staff in reference to the care and cleanliness of patients, more difficult when the patient has a skin traction, presenting the same discomfort when mobilized, whether or not they have traction[10].

There are studies into the effect of traction with several days delay. In a Japanese study in which the average delay was 7.5 days before surgery, no significant differences were found in patients who had traction compared to those who did not[15]. In addition, despite spending several days, they also found no difference in the quality of fracture reduction after surgery. Again, the initial pain scheme is established upon admission, but it decreases over time, regardless of whether the patient has traction or not.

In a curious study that evaluated the possible placebo effect in the placement of skin traction[13], patients with hip fracture were separated into three groups: some patients were placed with a certain weight, others were placed without weight, and in a third, a pillow was placed under the extremity. The three groups presented a reduction in pain, but strikingly, this reduction was greater in the patients to whom the traction was placed without the corresponding weight.

We have only found an article that recommends the use of traction[16]. He analyzed only 40 patients with intertrochanteric fractures and only in the first 24 hours. Of the five pain measurements carried out on patients with and without traction, only in one of them, at the end of the day, presented a significant difference in favor of patients with traction. The authors acknowledged that there was no greater demand for analgesia in either group. Despite the discreet results of the work, they recommended the use of traction to provide "greater comfort and relaxation."

Finally, the Cochrane review, both in its 2006 edition and the last edition of the year 2011[21,22], establishes that the use of traction apparently does not provide any benefit and that the evidence in this aspect is increasing, inviting clinicians that persist in their use to stop doing it or to do it under the favorable results of a well-designed randomized trial.

Table 1. Summary of the studies selected after the bibliographic search

Study	Design	Number of patients	Type of fracture	Comparison	Measurement results	Conclusions
Finsen V (1992)[3]	Randomized prospective	80	Cervical, pertrochanteric, sub-trochanteric	Skeletal traction vs skin traction vs pillow	Need analgesics, hemorrhage, intervention duration	Traction does not bring advantages
Anderson GH (1993)[4]	Randomized prospective	252	Intracapsular extracapsular	Skin traction vs no traction	Need analgesics, reduction of fracture, complications	Traction does not bring advantages
Needoff M (1993)[5]	Randomized prospective	67	Cervical, pertrochanteric	Skin traction vs pillow	Intensity of pain, need analgesics	Traction does not bring advantages
Resch S (1998)[6]	Randomized prospective	78	Cervical, pertrochanteric	Skeletal traction vs skin traction	Pain control	No differences between both methods
Jerre R (2000)[7]	Randomized prospective	120	Cervical, pertrochanteric	Skin traction vs no traction	Intensity of pain, need analgesics, consolidation fractures, complications	Traction does not bring advantages
Rosen JE (2001)[8]	Randomized prospective	100	Cervical, pertrochanteric	Skin traction vs pillow	Intensity of pain, need analgesics	Traction does not bring advantages
Yip DK (2002)[9]	Randomized prospective	311	Proximal femur fractures	Skin traction vs pillow	Intensity of pain, need analgesics	Traction does not bring advantages
Pozzo A (2002)[10]	Randomized prospective	100	Cervical, pertrochanteric	Skin traction vs no traction	Intensity of pain, need analgesics, complications, ease surgical procedure	Traction does not bring advantages
Maldonado EH (2007)[11]	Randomized prospective	96	Proximal femur fractures	Skin traction vs no traction	Intensity of pain, complications	Traction does not bring advantages
Resch S (2009)[12]	Randomized prospective	123	Proximal femur fractures	Skin traction vs pillow	Intensity of pain, need analgesics	Traction does not bring advantages
Saygi B (2010)[13]	Randomized prospective	108	Cervical, pertrochanteric	Skin traction with weight vs skin traction without weight vs no traction	Intensity of pain	Traction does not bring advantages
Estrada-Masllorens JM (2011)[14]	Randomized prospective	40	Pertrochanteric fractures	Skin traction vs no traction	Intensity of pain	Traction does not bring advantages
Endo J (2013)[15]	Randomized prospective	81	Cervical, pertrochanteric	Skin traction vs no traction	Intensity of pain, need analgesics, reduction of fracture	Traction does not bring advantages
Manafi Rasi A (2015)[16]	Randomized prospective	40	Pertrochanteric fractures	Skin traction vs no traction	Intensity of pain, need analgesics	The traction yes it brings advantage

Conclusions - Recommendations

Based on the current evidence and in accordance with the best practices for the management of hip fracture in the elderly[23,24], we believe that the application of skin traction in patients with hip fracture waiting to be operated on should be eliminated from routine practice in those centers that still perform it[25]. There is no evidence to support that their supposed benefits, the improvement of pain and a better reduction of the fracture that facilitates the surgery, are real. A pillow under the affected limb seems the most effective measure to provide greater comfort to these patients in the preoperative period.

Conflict of interests: The authors declare that there are no conflicts of interest.

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