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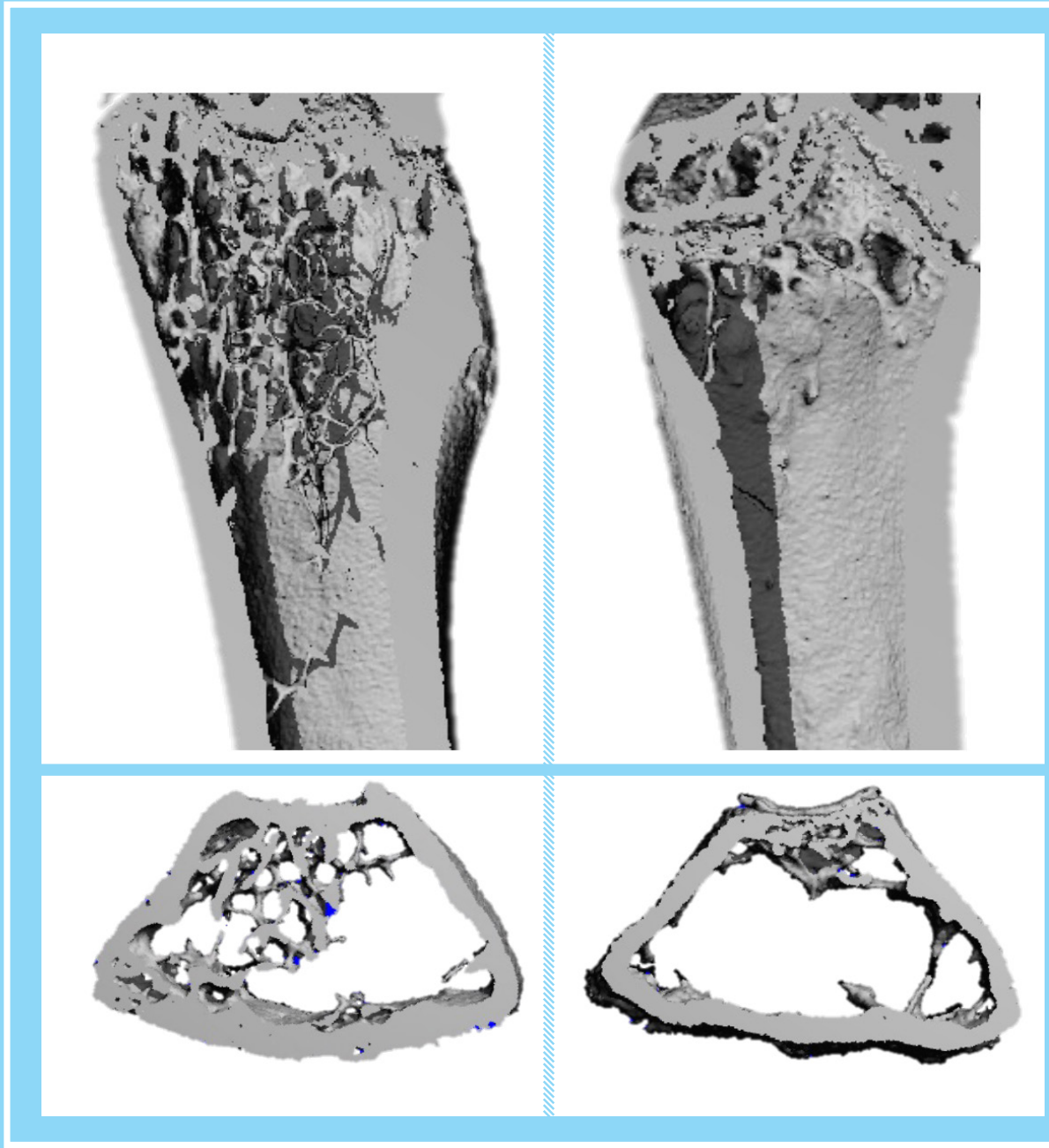

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Summary

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Originals

Special Article

Cover image:

Micro-CT images of longitudinal and transverse sections of femurs from control and osteoporotic CD1 mice.
Provided by Flor María Pérez-Campo. Universidad de Cantabria. Spain

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Original

Adherence to denosumab during the COVID-19 pandemic

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Abstract

Introduction: we aimed to analyze whether the SARS-CoV-2 pandemic has led to a decrease in denosumab (Dmab) adherence in the population, and assess the incidence of subsequent fractures in non-adherent patients.

Methods: we assessed all patients who should have required the administration of a dose of Dmab in Cantabria (Spain), during the lockdown Sociodemographic variables, risk factors for osteoporosis, data on Dmab administration, and the reason for drug discontinuation were collected. Furthermore, the development of a subsequent clinical fracture during the following year was also analyzed.

Results: a total of 2948 patients should have received a new dose of Dmab during the lockdown months, but 546 (18.5 %) discontinued the drug. The main reason for withdrawal was the patient's own doing (65 %). The incidence of clinical fractures in the overall group was low (n = 45; 1.46 %) with only 4 vertebral and 3 hip fractures being reported. When the group that did not receive more doses of Dmab or an alternate antiosteoporotic agent was analyzed (n = 147), it was revealed that 2 patients (1.36 %) sustained a vertebral fracture and another one (0.68 %) a hip fracture during the year following the last dose of the drug.

Conclusions: there was a non-negligible percentage of patients who did not receive the dose of Dmab on time during the lockdown period. However, the incidence of clinical vertebral and non-vertebral fractures was low, even in the non-compliant subjects who did not receive a different antiosteoporotic agent. None of the patients sustained multiple vertebral fractures at the 1-year follow-up.

Keywords:
Denosumab.
Adherence.
Osteoporosis.
Fractures.

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INTRODUCTION

Osteoporosis is the most frequent metabolic bone disease and fragility fractures represent a major health problem (1,2). Among the available therapeutic schemes, denosumab (Dmab), a monoclonal antibody that acts as an inhibitor of the RANK ligand (RANKL), is usually administered via subcutaneous injection every 6 months, frequently in primary care centers. Dmab Discontinuation without subsequent antiosteoporotic therapy leads to significant changes in bone remodeling, the so-called "rebound phenomenon", and is associated with an increased risk of vertebral fractures (3,4).

The SARS-CoV-2 pandemic, which hit Spain with extraordinary virulence, has had a huge impact on the management of chronic diseases, including osteoporosis. The strict lockdown imposed by the Spanish government within the first months of the pandemic, changed the classic health care model, leading to an increase in telemedicine, delays in performing densitometric studies, and interruptions in drug supply and administration of parenteral drugs (5). Moreover, the potential risk of a flu-like reaction that could be mistaken for a COVID-19 infection after IV zoledronic acid administration (6) or fear of visiting the primary care center in association with the administration of Dmab represented an important dilemma for both clinicians and patients with osteoporosis (7).

Taking into account the above-mentioned considerations, we aimed to analyze whether the lockdown period has led to a decrease in adherence to Dmab and to study the features of non-adherent subjects. Besides, we also studied the potential development of subsequent clinical fractures in non-adherent patients vs those fully compliant with this monoclonal antibody.

PATIENTS AND METHODS

All patients from our region (Cantabria, Sppain) who should have required the administration of a dose of Dmab during the COVID-19 lockdown period in Spain, from March through June 2020 were included in the study. Nine patients were excluded because of incomplete data on the clinical chart. To detect non-compliant subjects, drug withdrawal in the pharmacy was analyzed and later checked in the patient's health history.

The study was conducted in Cantabria, a region in northern Spain, with a population of 581,641 inhabitants, an area of 5321 km^2, and a population density of 109 inhabitants per km^2. As Dmab is administered biannually, the data of those who received the last dose from September to December 2019 were collected. The study was approved by Cantabria Ethics Committee (No. 2022.004).

STUDY VARIABLES

Age, sex, the financial contribution of the patient to pharmacy costs, and the place of residence were collected as sociodemographic variables. An urban area was considered if the size of the population size exceeded 10,000 inhabitants.

Risk factors for osteoporosis including smoking, alcohol consumption, diseases or drugs affecting bone metabolism, previous drug use, history of fractures, and use of calcium and/or vitamin D supplements were also collected, as well as serum 25-hydroxyvitamin D levels (ng/mL) and bone densitometry parameters. Dmab onset and last dose date, the initial prescribing physician, and the reason for drug withdrawal were also gathered from the clinical charts.

The number and characteristics of the patients who did not receive the corresponding dose of Dmab or who received it with a delay of more than 1 month, as well as the variation in Dmab pick-up in pharmacies vs the previous year were also assessed. Finally, we analyzed the occurrence of subsequent clinical fractures at the 1-year follow-up. Data were obtained from the patients' health history. A total of 9 patients from the non-compliant group were excluded from the analysis because they did not present sufficient valid data on their clinical charts.

STATISTICAL ANALYSIS

Results were expressed as numbers and percentages, mean ± standard deviation (SD), or median and interquartile range (IQR), as appropriate. To compare quantitative and qualitative variables Student t-test and the chi-square test or Fisher's exact test were used, when appropriate. A two-tailed p-value < 0.05 was considered significant in all calculations.

RESULTS

During the study period, a total of 2948 patients with osteoporosis should have received their correspondent dose of Dmab. Of these, 546 patients did not receive the subcutaneous injection (18.5 % of the entire sample).

The sociodemographic and clinical variables of the compliant and non-compliant groups are summarized in table I. The mean age was 76 years, female sex was predominant (*n* = 2732; 92.7 %), as well as the urban residence (*n* = 2165; 73.4 %) and a financial contribution < 10 % to the cost of the drug (*n* = 2639; 89.5 %). Significant differences were found regarding the contribution to the cost of Dmab in pharmacy (*p* = 0.009).

Figure 1 shows the distribution reason-wise of the non-compliant group. As can be seen, most patients discontinued their therapy on their own doing. Characteristics of Dmab prescription and reason for withdrawal in the group of patients who did not receive the drug at the scheduled time are shown in table II. Regarding pick-up Dmab data in pharmacies, -12.4%, -7.2%, -4.1%, and +10.8% were reported in March, April, May, and June 2020 vs the same months of the previous year. These figures represent a 12.9 % decrease in the entire study period. Table III shows the risk factors for osteoporosis and fragility fractures of the non-compliant group.

When stratifying these data by sex (Table IV), statistically significant differences were found for age, alcohol intake, and previous vertebral fractures (higher in men), and previous antiosteoporotic treatment and history of non-vertebral fractures (higher in women).

Table V summarizes the incidence of a subsequent fracture at the 1-year follow-up in the studied patients. When analyzing the group that did not receive more doses of Dmab (excluding deaths; *n* = 41, or 36 patients shifted to an alternate antiosteoporotic agent; *n* = 147 [27.4 %]), 2 patients (1.36 %) sustained a vertebral fracture, and 1 (0.68 %) a hip fracture during the year following Dmab discontinuation. All fractures occurred in women. The first patient with a vertebral fracture after Dmab discontinuation was an 85-year-old woman who had sustained multiple previous vertebral fractures, and the second case was a 77-year-old woman who had sustained a previous vertebral fracture. The reason for Dmab withdrawal was mainly the patient's own doing (76.2 %; *n* = 112), followed by the physician's decision (17 %; *n* = 25) and the odontologist´s advice (6.8 %; *n* = 10).

Considering the overall group of patients on Dmab (*n* = 2402), 21 patients (0.87 %) sustained a vertebral fracture, 10 (0.41 %) a hip fracture, and 4, other non-vertebral fractures (0.16) at the follow-up. One patient (2.7 %) of the non-compliant group with alternative antiosteoporotic therapy sustained a vertebral fracture within the next year, and another one (2.7 %) a hip fracture. Of note, the non-compliant group without alternative antiosteoporotic therapy was 15 older vs the group with Dmab administration delay, 11 years older vs the non-compliant group that did receive alternative therapy, and 6 years older vs the overall group.

Table I. Sociodemographic variables of the compliant and non-compliant groups

		Compliant group (*n* = 2402)	Non-compliant group (*n* = 546)	*p*
Age (years)		75.8 ± 9.6	76.5 ± 10.6	0.15
Sex	Female	*n* = 2233 (93 %)	*n* = 499 (91.4 %)	0.20
	Male	*n* = 169 (7 %)	*n* = 47 (8.6 %)	
Residency	Urban	*n* = 1847 (76.9 %)	*n* = 318 (76.8 %)	0.94
	Rural	*n* = 555 (23.1 %)	*n* = 127 (23.2 %)	
Financial contribution to the cost of drug	< 10 %	*n* = 2167 (90.2 %)	*n* = 472 (86.4 %)	0.009
	> 40 %	*n* = 235 (9.8 %)	*n* = 74 (13.6 %)	

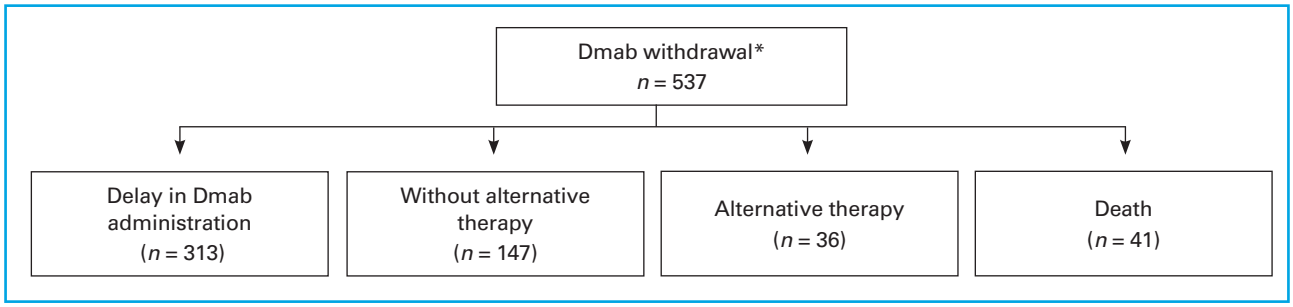

Figure 1. Flowchart of patients who discontinued Dmab. *A total of patients were excluded because they did not have sufficient valid data on their clinical charts.

Table II. Initial and final prescriber and cause of Dmab withdrawal in the non-compliant group

			n	%
Physician who starts treatment	Rheumatology		195	36.3
	Internal Medicine		142	26.4
	Primary Care		123	22.9
	Endocrinology		28	5.2
	Traumatology		19	3.5
	Gynecology		18	3.4
	Other		12	2.3
Physician who withdraws	**Primary Care**		16	23.9
	A different specialist		41	61.2
		Rheumatologist	19	46.3
		Internist	14	34.1
		Palliative care physician	3	7.3
		Other	5	12.3
	Odontologist		10	14.9
Cause of withdrawal	Patient´s own doing		353	65.7
	Postponed by the nurse		76	14.2
	Primary care physician		16	3
	A different physician		41	7.6
	Odontologist		10	1.9
	Death		41	7.6

**A total o 9 patients were excluded because they did not have sufficient valid data on their clinical charts.*

Table III. Risk factors for fracture in the non-compliant group

	n	%
Previous osteoporosis treatment	266	49.5
Alcohol intake	39	7.3
Current smoking	42	7.8
Secondary osteoporosis	136	25.3
Corticosteroid use	69	12.8
No. of previous treatment		
1	164	62.4
2	53	20.2
3	24	9.1
4	5	1.9
> 4	20	6.4
Oral pharmacological calcium intake	173	32.2
Oral vitamin D intake	422	78.6
Previous vertebral fracture	172	32.1
1	81	47.9
2	47	27.8
3	21	12.4
4	12	7.1
> 4	8	4.7

(Continues on next page)

Table III (cont.). Risk factors for fracture in the non-compliant group

	n	%
Previous non-vertebral fracture	146	27.2
Hip	43	29.9
Distal forearm	36	24.7
Rib	10	24.7
Humerus	5	3.4
Other	52	13.7
Serum 25OH D level (*n* = 317); mean ± SD (ng/mL)	27.9 ± 14.2	

Table IV. Sex-related sociodemographic and clinical variables of the non-compliant group

		Male (*n* = 48)	Female (*n* = 489)	*p*
Age (years), mean ± SD		79.0 ± 11.2	75.5 ± 10.4	0.03
Residence (%)	Urban	52.1 (*n* = 25)	54.4 (*n* = 266)	0.88
Financial contribution to the cost of the drug (%)	Reduced (< 10 %)	93.8 (*n* = 45)	87.3 (*n* = 427)	0.28
Current alcohol intake (%)		18.8 (*n* = 9)	6.1 (*n* = 30)	0.004
Current smoking (%)		10.4 (*n* = 5)	7.6 (*n* = 37)	0.67
Secondary osteoporosis (%)*		18.8 (*n* = 9)	25.9 (*n* = 127)	0.36
Corticosteroid use (%)		12.5 (*n* = 6)	12.9 (*n* = 63)	0.88
Previous osteoporosis treatment (%)		22.9 (*n* = 11)	52.1 (*n* = 255)	0.0002
No. of previous antiosteoporotic agents (%)	1	12.5 (*n* = 6)	32.3 (*n* = 158)	0.007
	2	4.2 (*n* = 2)	10.4 (*n* = 51)	0.26
	3	2.1 (*n* = 1)	4.7 (*n* = 23)	0.64
	4	4.2 (*n* = 2)	0.6 (*n* = 3)	0.09
	> 4	0 (*n* = 0)	3.5 (*n* = 17)	0.38
Pharmacological calcium intake (%)		35.4 (*n* = 17)	31.9 (*n* = 156)	0.74
Pharmacological vitamin D intake (%)		72.9 (*n* = 35)	79.1 (*n* = 387)	0.42
Serum 25OH D level (ng/mL), (mean ± SD)		28.9±14.1	27.9±14.2	0.10
Presence of previous vertebral fracture (%)		50.0 (*n* = 24)	30.3 (*n* = 148)	0.008
No. of previous vertebral fractures (%)	1	14.6 (*n* = 7)	15.1 (*n* = 74)	0.91
	2	27.1 (*n* = 13)	6.9 (*n* = 34)	0.0001
	3	4.2 (*n* = 2)	3.9 (*n* = 19)	0.76
	4	4.2 (*n* = 2)	2.0 (*n* = 10)	0.66
	5	0 (*n* = 0)	1.6 (*n* = 8)	0.49
Previous non-vertebral fracture (%)		12.5 (*n* = 6)	28.6 (*n* = 140)	0.02
Site of previous non-vertebral fracture (%)	Hip	2.1 (*n* = 1)	8.6 (*n* = 42)	0.19
	Distal forearm	0 (*n* = 0)	7.4 (*n* = 36)	0.10
	Rib	0 (*n* = 0)	2.0 (*n* = 10)	0.66
	Humerus	0 (*n* = 0)	1.0 (*n* = 5)	0.93
	Ankle	2.1 (*n* = 1)	0.8 (*n* = 4)	0.93
	Knee	0 (*n* = 0)	0.4 (*n* = 2)	0.43
	Foot	0 (*n* = 0)	0.2 (*n* = 1)	0.15

**Secondary osteoporosis: hypogonadism, endocrine disorder (hyperparathyroidism, hyperthyroidism), GI, rheumatologic (rheumatoid arthritis), or organ transplantation.*

Table V. Occurrence of a subsequent fractures at the 1-year follow-up

	Overall (*n* = 2939)*	Compliant group (*n* = 2402)	Delay in Dmab administration (*n* = 313)	Non-compliant group with alternative therapy (*n* = 36)	Non-compliant group without alternative therapy (*n* = 147)
Age (years; mean ± SD)	78.9 ± 8.2	79.3 ± 7.6**	70.5 ± 19.1**	74.0 ± 11.9**	85.0 ± 2.8
Total fractures (*n*)	43	35	2	3	3
Clinical vertebral fracture (*n*)	25	21	1	1	2
Overall clinical vertebral fractures (%)	0.85	0.87	0.31	2.7	1.36
Hip fractures (*n*)	13	10	1	1	1
Overall hip fractures (%)	0.44	0.41	0.31	2.7	0.68
Non-vertebral fractures (*n*)	5	4	0	1	0
Overall non-vertebral fractures (%)	0.17	0.16	0	2.7	0

**A total of 9 patients were excluded because they did not present sufficient valid data on the clinical chart. **p < 0.0001 vs the non-compliant group without alternative therapy.*

Given the high risk of age-related fracture related in the non-compliant group without alternative treatment and the incidence of fracture in this group vs the other ones, it seems reasonable to suggest that there is no increased risk of fractures.

DISCUSSION

Our study found that there was a non-negligible percentage of patients (18.5 %) who did not receive the correspondent dose of Dmab during the lockdown due to the COVID-19 pandemic in Cantabria, Spain.

Kocijan et al. (8) found a decrease in the prescription of this monoclonal antibody in Austria from March (22 %) and April (23 %) 2020 vs the previous 6 months. The same trend was noted in this period regarding IV zoledronate (36 % and 49 % decrease vs the previous year).

Fuggle et al. (5) noted that 43% of 209 health professionals from different parts of the world reported difficulties in treating osteoporosis during the COVID-19 pandemic. The main issues were problems obtaining the drug, delays in the administration of parenteral agents, and the reluctance of patients to go to their health center. In the specific case of parenteral drugs, 46% were administered appropriately, 3% had to switch these treatments to an alternative area, 21% delayed treatment until there was a lower risk of COVID-19, 13% switched to an oral drug, 8% were administered at home and 9% had some other issues such as self-administration of the dose by the patient at home. Primary care physicians prescribed Dmab in 15% of the cases, which involves shorter delays in drug administration than the one we found in the present study (59.6%) (5). Additionally, these authors also found a lower percentage of switching to a different antiosteoporotic treatment (4.2 %), although they saw a greater percentage of change to an oral bisphosphonate (2.2 %) (5).

Peeters et al. (11) surveyed a total of 77 health care professionals in The Netherlands and found that 49% of patients on Dmab were properly treated by their family physician, and 33.4% were followed in the hospital outpatient clinics or at home via self-injection. Some 6.3% of patients reported a delay in Dmab administration, 8.3% were taught via video conference to self-administer the drug, and 1% discontinued treatment without starting another antiosteoporotic agent. These data indicate a lower percentage of withdrawal or Dmab administration delay than the one we found (5.0% and 10.6%, respectively).

Dmab discontinuation causes rebound high bone turnover and rapid bone loss within the first year, increasing the risk of major osteoporotic fractures, especially multiple vertebral fractures, particularly among subjects with previous vertebral fractures (3). Besides, the delayed administration of subsequent Dmab doses by more than 16 weeks has been associated with an increased risk for vertebral fractures vs on-time dosing. Nevertheless, evidence for an increased risk of fractures at different anatomical sites with long delays is insufficient (9).

Based on all this, Dmab should not be discontinued without switching to an alternative agent, usually bisphosphonates (4). This approach was very important during the SARS-CoV-2 pandemic, and the Joint Guidance on Osteoporosis Management in the Era of COVID-19 from the ASBMR, AACE, Endocrine Society, ECTS & NOF recommended that "for patients in whom

continued treatment with denosumab is not feasible within 7 months of prior denosumab injection, strongly consider transition to oral bisphosphonate if possible" (www.asbmr.org).

Although we found no differences in the rate of clinical vertebral or nonvertebral fractures after Dmab discontinuation, we observed a crude higher percentage of vertebral fractures in the group of patients who discontinued Dmab and switched to other alternative therapy (2.7%) vs the compliant group (0.87%). The differences reported were not statistically significant, mainly because only 1 patient sustained a vertebral fracture in the latter group. There was also a slight, albeit non-significant increase in the group of withdrawal patients without alternative therapy (1.36%) while in those who delayed the dose the incidence of fractures was very similar to that of patients who received the scheduled dose of Dmab. It may be that the low frequency of fractures following the non-administration of Dmab at the scheduled time may be due, in most cases, to switching to a different drug. Indeed, in many cases, a delayed administration was reported (exceeding 2 months from the indicated time) but not an abrupt discontinuation without an alternative anti-osteoporotic agent. Another possible explanation could be the reducted physical activity, changes in lifestyle habits, and lower number of falls associated with the lockdown. However, we do not have collected these data to adequately analyze its influence on this outcome.

Regarding non-vertebral fractures, specifically hip fractures, the incidence of this type of fractures was also very low and non-significant across the study groups.

Noteworthy, in the placebo group of the FREEDOM study, the risk of new clinical vertebral fractures was 2.6% (1.6% with 2 or more vertebral fractures), 1.2% for hip fractures, and 8% for non-vertebral fractures (12). Furthermore, in a post-hoc analysis of the FREEDOM study of 1001 patients who discontinued Dmab during the study, 5.6% sustained vertebral fractures and 2.3% non-vertebral fractures (13). The rate of fractures in both studies was quite similar to that observed in our study.

In the FREEDOM extension study, Cosman found in the discontinuation group a crude annualized incidence of vertebral fracture of 11.8% (n = 56) and 7.2% (n = 34) in multiple vertebral fractures [9.5 % (n = 31) in the placebo group for vertebral fractures and 3.7 % (n = 12) for multiple vertebral fracture (14)].

In the COVID-19 era, we should reconsider the management strategies of patients with osteoporosis, highlighting and implementing therapeutic compliance. To achieve this, the methods of providing medical care must also be adapted, either by increasing virtual follow-up consultations or by facilitating a multidisciplinary approach with other health professionals. Although telemedicine reduces costs, waiting times, or trips, it also increases the uncertainty of physicians and patient and a possible medical and legal vulnerability (5).

The study has several limitations. First, the study was conducted in a specific area from northern Spain and included Caucasian people. Therefore, data could not be extrapolated to other geographical areas or ethnicities. Second, data have been reviewed from clinical charts, and the overall time on Dmab or the precise reasons for Dmab withdrawal cannot be well-defined in some cases. Third, the number of fractures was small and the short period of follow-up could be a limitation of the study since the long-term incidence of fractures was not assessed. Fourth, we only have data on clinical vertebral fractures, which required radiology. Finally, we do not have data on Dmab withdrawal during other periods before or after the lockdown.

In conclusion, the lockdown of the Spanish population within the first months of the SARS-CoV-2 pandemic in our health care field of expertise led to almost an 18% rate of Dmab discontinuation due to delayed administration, switch to a different antiosteoporotic agent, or definitive withdrawal without prescribing an alternative therapy. With the limitations inherent to this kind of study design, the interruption of Dmab during this COVID-19 pandemic was not followed by a significant increase in clinical and non-vertebral fractures vs the results of the FREEDOM study. None of the patients sustained multiple vertebral fractures at the 1-year follow-up Despite these data, we consider that current scientific recommendations should be adopted in cases of Dmab withdrawal. In the COVID-19 era, clinicians should conduct more intensive and long-term follow-ups of osteoporotic patients on Dmab to prevent the fractures associated with the discontinuation of this monoclonal antibody.

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Original

Preferences of Spanish-speaking patients for communication of fracture risk due to osteoporosis – A substudy of the Risk Communication in Osteoporosis Study (RICO study)

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Abstract

Introduction: the Risk Communication in Osteoporosis Study (RICO) is an international multicenter study evaluating the preferences of patients with osteoporosis regarding fracture risk communication.

Objective: to compare the results from Spanish-speaking countries participating in the RICO study to determine whether cultural differences influence preferences in fracture risk communication.

Methods: postmenopausal women with varying fracture risks and educational levels were recruited. A structured questionnaire was designed covering various domains, including sociodemographic data, knowledge about the disease and fracture risk, preferences for graphical, written, and/or verbal communication regarding fracture risk, and opinions on therapeutic decisions after understanding the risk.

Results: of the 332 patients included in the international study, 94 were from Spanish-speaking countries: 36 from Mexico, 30 from Argentina, and 28 from Spain. A higher percentage of participants from Spain (78.6 %) and Mexico (86.1 %) had received prior information about their fracture risk compared to the international RICO study average (56 %). Spanish-speaking patients preferred visual forms of communication about fracture risk (e.g., printed materials) but wanted these to be accompanied by verbal explanations from healthcare professionals. Mexican patients displayed different preferences compared to Spanish patients in most presentations.

Conclusions: clearly conveying information about fracture risk while considering the healthcare and cultural differences among patients could improve therapeutic management.

Keywords: Fracture risk. RICO Study. Patient communication.

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**The list of coauthors for the RICO Study is provided at the end.*

Natalia García-Giralt is the senior author of the article, and Xavier Nogués is the first and the corresponding author.

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INTRODUCTION

The shared decision-making model between physician and patient is based on the use of informational tools that enable the patient to better understand their disease. Shared decision-making tools regarding diagnostic tests and/or treatments emerged in the 20th century (1,2), a time when society increasingly demanded information about diseases (3).

The current prevalence of osteoporosis (OP) in postmenopausal women worldwide is estimated at 23.1 %, and in men over 50 years of age, at 11.7 % (4). Projections for 2050, considering the growth of the population over 60 years old, estimate the global number of osteoporotic fractures at 6.26 million hip fractures (5). Thus, OP is an extraordinarily prevalent disease with significant consequences that profoundly impact those affected and will substantially influence healthcare systems in the coming years.

Clear and understandable information from physicians about fracture risk is a critical aspect of the doctor-patient relationship for those diagnosed with OP. Properly communicating this information allows patients to better understand the disease, its consequences, treatment efficacy, and potential side effects. It facilitates shared therapeutic decision-making and positively reinforces treatment adherence (2,6).

Regarding OP, there is a discrepancy between the number of patients with fragility fractures and those receiving treatment to prevent new fractures (7). This suggests that many patients remain at high or very high risk of experiencing another fragility fracture, partly due to insufficient information about the consequences of OP.

However, informing patients about OP and the risk of new fractures in clear and appropriate language is challenging. This complexity arises partly from the wide range of topics to be addressed, including diet, exercise, harmful elements to avoid, and therapeutic options, as well as the lack of established communication tools for conveying such information (8). Additionally, it is uncommon for osteoporotic patients who have already experienced a fracture to receive adequate information about the risk of subsequent fractures, despite the availability of specific prediction tools such as the FRAX risk score (9). This contrasts with conditions like cancer, where patients increasingly share decisions on therapeutic options based on recurrence risk (10).

In this context, the "Risk Communication in Osteoporosis (RICO)" study (11) was developed as an international multicenter study based on a structured survey of women with osteoporosis, with and without fragility fractures, to explore their preferences regarding how to receive information about fracture risk.

The present study presents a subanalysis of the RICO study, analyzing and comparing the results from Spanish-speaking countries—Mexico (MEX), Argentina (ARG), and Spain (SP)—to observe whether cultural differences influence preferences for fracture risk communication.

METHODS

STUDY DESIGN AND OBJECTIVES

The design and methodology of the "Risk Communication in Osteoporosis (RICO)" study were recently published (11). Briefly, we conducted an international study based on a structured survey of women with OP, with and without fragility fractures, varying fracture histories, and different levels of education. The aim was to understand their preferences for receiving information about their risk of fragility fractures. Additionally, the relevance and importance of different communication modalities were evaluated, and a model was proposed tailored to each studied population.

The objective of this substudy is to evaluate the communication preferences of Spanish-speaking patients.

Below, the main features of the study are described.

Study scope

The RICO study was conducted with patients from 11 centers in various countries: the United States, Canada, Argentina, Mexico, Spain, Belgium, the Netherlands, the United Kingdom, and Japan. Participating centers were invited individually by the study coordinators and through patient associations such as the Canadian Osteoporosis Patient Network and the UK Royal Osteoporosis Society.

Inclusion criteria

Each center was required to include 30 postmenopausal women with OP or fracture risk. Each center had to include at least 10 patients with fragility fractures and 10 without prior fractures. Participants were required to have diverse educational backgrounds, with at least 10 patients having higher education and 10 having only primary education. Regarding treatment, at least 10 patients needed to be actively taking pharmacological treatment for osteoporosis, while 10 were not.

Survey design

The methodology for survey design and the preliminary pilot study has been extensively described by Beaudart et al. (11). Briefly, a team of experts developed a questionnaire in English, inviting patient feedback on its design. The finalized questionnaire was structured across multiple domains and translated into several languages: French, Chinese, Japanese, and Spanish, accommodating regional variations for Spain, Latin America, and Central America.

The final questionnaire, included in annex 1 (https://www.revistadeosteoporosisymetabolismomineral.com/anexos/00048-01.pdf), was divided into the following sections: 1) an introduction to the study objective and the collection of sociodemographic data; 2) a section to assess the patients' knowledge and interpretation of fracture risk; 3) a section on patient preferences for receiving information about fracture risk—numerical or written, verbal presentation, or visual formats such as red-yellow-green colors or face icons; and 4) opinions on receiving information about fracture consequences and when to consider starting treatment if fracture risk was presented at 2 or 10 years.

Ethical considerations

The study was approved by Advarra, an Institutional Ethics Committee in the United States, as well as local ethics committees where required. In Spain, approval was granted by the Parc de Salut Mar Research Ethics Committee for Medicinal Products, No. 2022/10299, "Improving Risk Communication in Osteoporosis (RICO)", protocol code 20197347.

Statistical analysis

Statistical analysis was performed using SPSS Statistics 24 (IBM Corporation, Armonk, NY, United States). Differences in patient characteristics and preferences between countries were analyzed using chi-square or Fisher's exact test for categorical/binary variables and one-way ANOVA for quantitative variables. Differences within a single country were analyzed using the chi-square Goodness of Fit test. A p-value < 0.05 was considered significant.

RESULTS

In the global study, a total of 332 women were included, with a mean age of 67.5 ± 8.0 years, 94 of whome were from Spanish-speaking countries: MEX (n = 36), ARG (n = 30), and SP (n = 28). Table I shows the demographic characteristics of the patients. In these three countries, 81.9 % of the participants reported having been diagnosed with OP by their doctors, which was above the 70 % value obtained in the global study.

Table I. Clinical and sociodemographic characteristics of the population included in the study

	All (n = 94)	MEX (n = 36)	ARG(n = 30)	SP (n = 28)	p-value
Interview format					
Online	27 (28.7 %)	26 (72.2 %)	0 (0.0 %)	1 (3.6 %)	p < 0.001
Direct	67 (71.3 %)	10 (27.8 %)	30 (100. %)	27 (96.4 %)	
Age (years)	67.9 ± 8.1	66.5 ± 7.36	68.2 ± 8.55	69.4 ± 8.69	0.007
Education					
Primary	16 (17.0 %)	4 (11.1 %)	2 (6.7 %)	10 (35.7 %)	
Basic Secondary	11 (11.7 %)	6 (16.7 %)	2 (6.7 %)	3 (10.7 %)	
Advanced Secondary	16 (17.0 %)	4 (11.1 %)	9 (30.0 %)	3 (10.7 %)	
Post-Secondary	10 (10.6 %)	2 (5.5 %)	8 (26.6 %)	0 (0.0 %)	
University	41 (43.7 %)	20 (55.6 %)	9 (30.0 %)	12 (42.9 %)	
Diagnosis of OP	77 (81.9 %)	27 (75.0 %)	22 (73.3 %)	28 (100 %)	NDA
Treatment for OP	50 (53.1 %)	14 (38.9 %)	15 (50 %)	21 (75 %)	p < 0.01
History of fracture	38 (40.4 %)	15 (41.7 %)	9 (30.0 %)	14 (50.0 %)	NDA
Previous fractures					
Spine	8	0	1	7	
Hip	6	1	3	2	
Wrist	13	8	1	4	
Humerus	9	4	3	2	
Other	13	6	2	5	

p-value across the different countries using chi-square (or Fisher's exact test) for categorical/binary variables and one-way ANOVA for quantitative variables; OP: osteoporosis; NDA: no data available.

A total of 40.4 % of the women from these three countries reported having had a fracture, with prevalence ranging between 50 % in SP, 41.7 % in MEX, and 30 % in ARG (p = 0.295). In terms of treatment, 75 % of participants in SP were on pharmacological treatment, whereas only 38.9 % in MEX (p < 0.01), and ARG was intermediate at 50 %.

At the start of the interview, participants were asked about their education and specifically their ability with numbers. In both SP and ARG, 46 % of patients reported feeling comfortable with numerical elements, compared to 66.7 % in MEX (Table I).

When participants were asked if they considered it important to receive information about their fracture risk on a scale from 1 to 7, the results were 6.79 ± 0.6 in SP, 6.23 ± 1.3 in ARG, and 6.19 ± 1.8 in MEX, with no significant differences between the three countries.

A total of 78.6 % of participants in SP reported having received previous information about their fracture risk, 46.7 % in ARG, and 86.1 % in MEX, with significant differences between ARG and the other two Spanish-speaking countries (p = 0.007).

GRAPH PREFERENCES

When asked about their preference for receiving information about fracture risk from three proposed color-coded charts (red-yellow-green) (Fig. 1A), 78.6 % of SP patients preferred the information in the form of a red-yellow-green arrow, significantly vs the other 2 options (p = 0.001). On the other hand, participants from MEX and ARG differed from SP, opting for the vertical traffic-light style in 58.3 % and 40 %, respectively (p = 0.001) (Fig. 1B).

Regarding the graphical options for presenting fracture risk using "happy" or "sad" faces (Fig. 2A), the option with a numerical scale was preferred in SP by 67.9 %, and similarly in ARG with 66.7 %, while in MEX the majority had no preference for any of the options (p = 0.001) (Fig. 2B). Regarding whether the "happy" faces should be placed at the top or bottom, most in SP and MEX had no preference, while ARG preferred the "happy" faces at the top in 61.9 % (p = 0.009). Regarding the graphical representation of the faces in color or black and white, 92.9 % in SP preferred color. MEX and ARG also preferred color, but to a lesser extent (66.7 % and 73.3 %, respectively) (p = 0.006) (Fig. 3A). Regarding the presentation of 10 or 100 faces in the graph, SP preferred the reduced option of 10 faces (57.1 %), as did ARG with 70 %, while in MEX there was no preference (p < 0.001) (Fig. 3B).

Regarding treatment, participants from all three countries preferred that fracture risk be presented both with and without treatment to see the risk reduction effect: 5.8 ± 1.6 on a 1-7 scale for verbal or written communication, 5.93 ± 1.53 for the color chart, and 5.39 ± 1.84 for faces.

When asked about the consequences of having a fracture, participants were shown drawings to select the order of what concerned them most (Fig. 4). SP patients were most concerned about loss of independence (46.4 %), while in MEX, it was the loss of quality of life (36.1 %), and in ARG, it was the possibility of kyphosis (33.3 %) (Table II).

In all three countries, great importance was placed on receiving a verbal explanation of fracture risk supported by graphics: SP 75 %, MEX 86.1 %, and ARG 83.3 %. They also valued receiving printed information on paper: SP 77.5 %, MEX 80.6 %, and ARG 83.3 %.

DISCUSSION

Clinical practice guidelines, such as those from the Spanish Society of Bone and Mineral Metabolism Research (SEIOMM), recommend treatment based on fracture risk (12). It is therefore important to know each patient's specific fracture risk and provide proper information for a shared decision on the most efficient and effective treatment.

In Spanish-speaking countries, particularly in SP and MEX, participants had received information about their fracture risk above the average in the international RICO study, where only 56 % of patients reported receiving information (11). This likely reflects a cultural characteristic of greater verbal communication in Spanish-speaking countries. The questionnaire itself showed the preference in these three countries for receiving information in a graphic and printed form, but also explained verbally by the doctor or nurse. However, the goal of communicating the risk of new fractures to patients with OP should approach 100 %, as demonstrated in the survey. Receiving adequate information was a highly relevant issue for all patients in the study.

An important issue worldwide regarding OP is the difference between the percentage of patients who have had a fracture and those who actually receive treatment to prevent new fractures due to fragility (13). While treatment is recommended after a fracture, recent or past, representing a high or very high fracture risk, the reality in the international RICO study showed that in some countries, such as the United States (California) and Belgium, the percentage of patients not receiving treatment was > 70 %. In SP, this discrepancy was not observed. On the contrary, a higher percentage of patients were on treatment than those with a history of fractures, likely because 100 % of participants were diagnosed with OP.

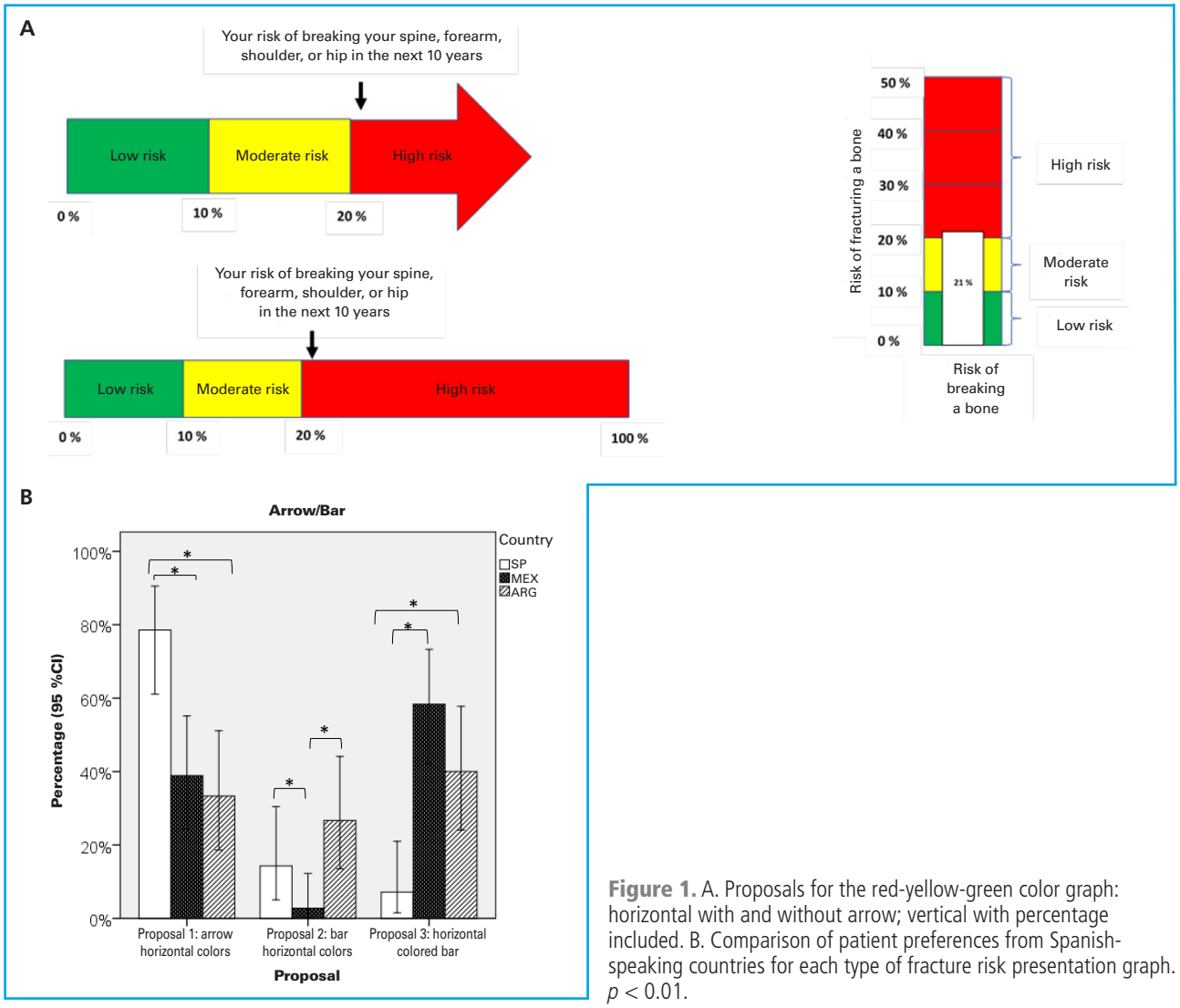

Figure 1. A. Proposals for the red-yellow-green color graph: horizontal with and without arrow; vertical with percentage included. B. Comparison of patient preferences from Spanish-speaking countries for each type of fracture risk presentation graph. $p < 0.01$.

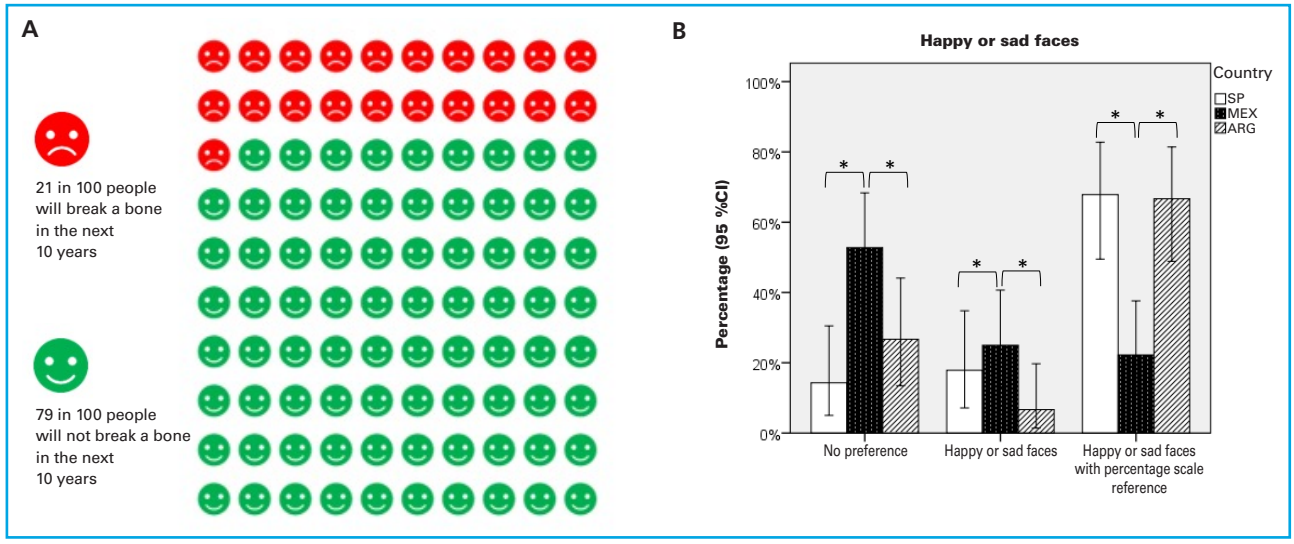

Figure 2. A. Proposal for the "happy" and "sad" faces graph. B. Comparison of patient preferences from Spanish-speaking countries for the type of "happy" or "sad" face graph for fracture risk presentation, with and without a percentage risk reference scale. $p < 0.01$.

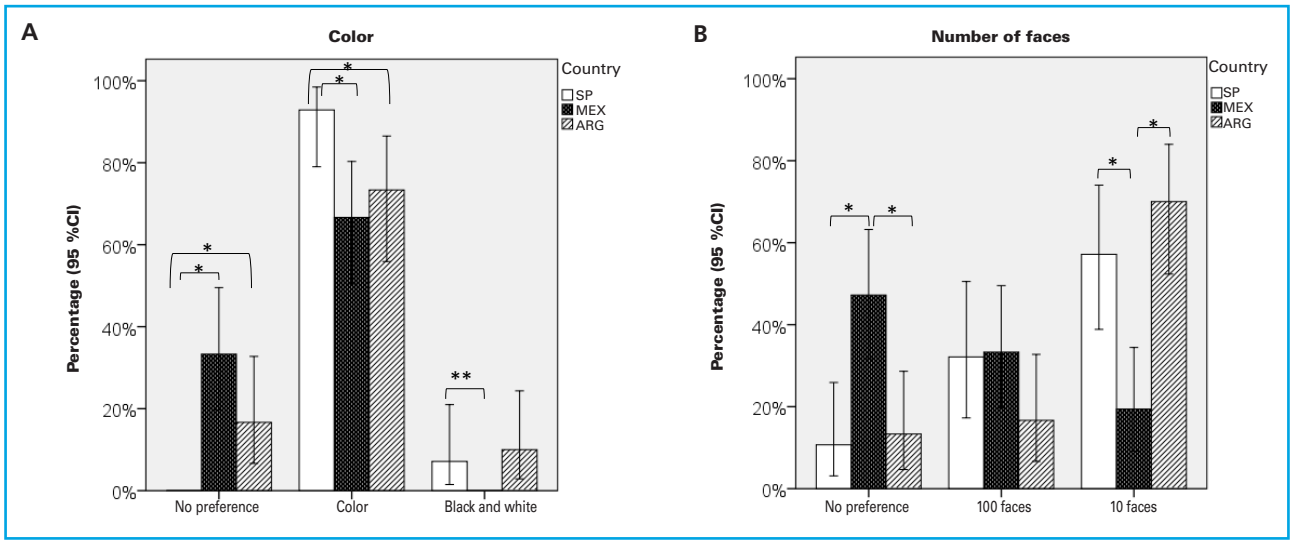

Figure 3. A. Comparison of patient preferences from Spanish-speaking countries for the type of "happy" or "sad" face graph, in color or black and white, for fracture risk presentation. $p < 0.01$. B. Comparison of patient preferences from Spanish-speaking countries for the type of "happy" or "sad" face graph, with 100 or 10 faces for fracture risk presentation. $p < 0.001$.

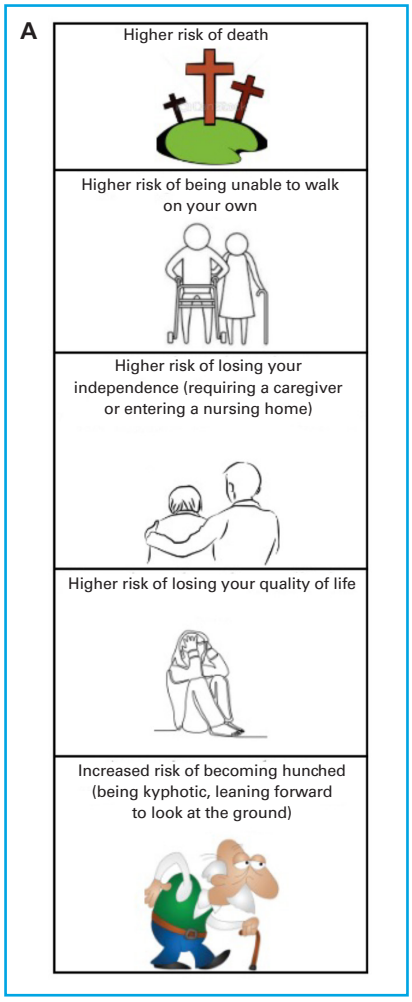

Figure 4. Representative drawings of the consequences of having a fracture.

Table II. Importance of potential consequences of having a fracture for Spanish-speaking participants

Consequence	MEX	ARG	SP
Death	19.4 %	20 %	25 %
Inability to walk	25 %	16.7 %	3.6 %
Loss of independence	16.7 %	16.7 %	46.4 %
Loss of quality of life	36.1 %	13.3 %	17.9 %
Kyphosis	2.8 %	33.3 %	7.1 %

In MEX or ARG, this figure was around 70 %. Despite having a lower incidence of fragility fractures vs countries in Northern Europe (13,14), SP still has a higher proportion of patients with OP who are susceptible to treatment vs those receiving treatment. According to the recent Scope 2021 study, SP is the country with the most significant increase in this aspect compared to other European countries (14).

Regarding preferences for the graphical presentation of fracture risk information, it is noteworthy that SP participants preferred a combination of color arrows and percentage, while MEX and ARG preferred a simpler traffic light option without arrows or percentages.

Of note that in most cases, MEX patients' preferences were quite different from those of SP patients. On the other hand, ARG patients had preferences that were very similar to those of the Spanish participants. This demonstrates that each country has its own peculiarities, independent of language and geographical proximity.

It is clear, however, that visual communication methods are greatly appreciated by all patients, especially when using icons that are easy to interpret.

Decision support tools for osteoporosis have been developed over the years (15). Recently, a Dutch group developed a validated decision aid tool specifically designed for Fracture Liaison Services (16). This tool includes the assessment and communication of fracture risk through a brochure explaining the disease of OP, its consequences, dietary advice, lifestyle, and treatments. This documentation, which is given to patients, also provides general fracture risk information using broken bone figures, similar to the "happy" faces presented in the RICO study.

Information about OP in the form of brochures or digital formats is common in primary care and specialized care; however, specific information about fracture risk is less common (17). FRAX is the most widely used tool, but it is not designed for patient communication and is often difficult to understand (18). Therefore, it is important to develop new patient support tools that offer visual aids to help them understand their risk of future fractures. It is also crucial to explain to patients how much this risk will be reduced with the proposed treatment to achieve greater therapeutic adherence (19).

The RICO study has limitations. On the one hand, it was conducted during the COVID-19 pandemic, which meant that some interviews with participants were held virtually, potentially influencing some responses. In SP, all interviews were conducted after the period of strictest restrictions and were held in person, while in MEX and ARG, the interviews were mixed. Moreover, the inclusion of patients already diagnosed with OP may have influenced results such as treatment, as many of them were likely more sensitized to receive treatment to prevent further fractures. Another limitation is the scope of the study, which, being international, included participants from different cultures and, in particular, different health care systems. Among Spanish-speaking countries, the public system with medication funding in ES differs from that in MEX, where coverage is not universal, and in ARG, where private healthcare predominates over public. However, this would not affect issues such as knowledge of the disease, fracture risk, or consequences. It could, however, influence the decision not to receive medication until the patient sees that their risk is very high to avoid high financial spending.

CONCLUSIONS

The RICO study demonstrates that there is a deficit in transmitting information to OP patients about their fracture risk. Spanish-speaking patients showed different preferences in presenting the graphs. In particular, MEX patients showed preferences opposite to those of SP patients. The results suggest that healthcare professionals need to make an effort to find effective communication methods about fracture risk that are adapted to the cultural idiosyncrasies of the population being treated. Establishing effective doctor-patient communication tools could increase treatment acceptance and likely improve the quality of life for patients with OP.

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Original

The effect of falls on perceived health in the general population and its clinical consequences

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Resumen

Introduction and objective: the objective was to assess the effect that falls have on the perceived quality of life and its clinical consequences.

Material and methods: 324 men and women > 50 years old who underwent dorso-lumbar radiology and bone mineral density (BMD) repeated the same tests after 4 years and performed biochemistry, history of falls and the SF-36 quality of life questionnaire. The cohort was followed up for an additional 4 years to determine the incidence of osteoporotic fracture.

Results: falls were more frequent in women than in men (34.9 % vs 14.7 %, $p < 0.001$). Except for role limitations due to physical and emotional problems, 6 of the other 8 dimensions of the SF-36 quality of life questionnaire were significantly affected by the presence of falls. The linear regression analysis showed that the perception of general health, vitality, mental health and total score were associated with falls. In women, pain was significantly affected in the last 4 weeks; perception of general health; vitality; mental health and global score. In men, no dimension was affected. Logistic regression analysis adjusted for age, sex and BMI showed that falls were associated with the incidence of osteoporotic fracture [odds ratio (OR) = 3.56, 95 % confidence interval (95 % CI) = (1.04 -12,21)].

Conclusions: falls affected quality of life in women in relation to vitality, pain and general health, but also mental health that includes depression, anxiety, emotional and behavioral control. In men, perceived health was not affected by the presence of falls. Preventing falls in both sexes could prevent an increase in the incidence of osteoporotic fracture.

Keywords: Falls. Quality of life. Fracture.

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INTRODUCTION

A fall is defined as an involuntary or accidental descent to the ground or another lower level. In the United States, falls are the leading cause of accidental death and the 7th leading cause of death in people older than 65 years (1).

In people aged 65 or older, falls are the leading cause of hospitalization-related injuries and mortality (2). Therefore, fall-related injuries cause a substantial economic burden (3). However, doctors often do not detect falls in a patient who presents no injuries, or because the specific search for this event is not usually included in the medical history and physical examination. On the other hand, many elderly people are reluctant to report a fall because they attribute it to the normal aging process or because they fear that their knowledge might limit their future activities. Although we know that many elderly people fall, falls are not part of the normal aging process.

Falls in the elderly are common and lead to fractures and other serious health consequences (4,5). In the elderly population, the risk of fracture increases exponentially, not only with the decrease in bone mineral density (BMD) but also due to the increase in falls. Several studies have shown that a history of falls is associated with a higher probability of future falls and a greater risk of fractures (6-10). The increase in life expectancy in the Western world will lead, unless preventive measures are implemented, to a growth in expenses associated with osteoporotic fractures. Therefore, preventing some of the factors that most contribute to their occurrence, such as falls, will have a significant financial and social health impact.

On the other hand, the fear of falling is another important factor that can mediate the relationship between physical and cognitive factors, depressive symptoms, and falls. The fear of falling has been linked to symptoms of depression and anxiety, and a relationship has been established between fear of falling and avoidance of activities that affect strength and physical capacity (11,12), which may mark the onset of disability in elderly individuals.

Therefore, the aim of this study was, first, to assess the effect that falls have on perceived quality of life through the SF-36 health questionnaire, and, second, to analyze whether the presence of falls could have clinical consequences through an increase in the incidence of osteoporotic fractures.

MATERIAL AND METHODS

As part of the European multicenter study designed to assess the prevalence and incidence of vertebral fractures (European Vertebral and Prospective Osteoporosis Study – EVOS-EPOS) (13), a total of 308 men and 316 women over 50 years of age were randomly selected from the municipal registry of Oviedo (Asturias, Spain). The protocol referenced by this study involved completing a questionnaire on osteoporosis-related risk factors, 2 lateral thoraco-lumbar X-rays, and the collection of anthropometric measurements such as height and weight to determine the body mass index (BMI). In addition, more than 80 % of the participants underwent BMD tests for lumbar spine and femoral neck. All subjects treated at the Clinical Management Unit of Bone Metabolism at Hospital Central Universitario de Asturias were ambulatory or, if necessary, with the assistance of another person, and 99 % lived in their own homes.

Four years after the baseline study, participants were invited to repeat the same tests as the baseline study, in addition to a biochemical study, the SF-36 health questionnaire, and a fall history in the last 12 months. Of the 404 subjects who attended the second cross-sectional study, 324 men and women (80.2 %) agreed to undergo all the previously mentioned tests.

This cohort was prospectively followed for another 4 years through two postal questionnaires to investigate the incidence of non-vertebral osteoporotic fractures.

SF-36 HEALTH QUESTIONNAIRE

The 324 participants who completed all the tests in the second cross-sectional study were administered the SF-36 health questionnaire through a personal interview. The questionnaire was conducted by a person trained in administering questionnaires.

The SF-36 consists of 36 items, 35 of which are categorized into 8 dimensions: physical functioning: 10 items; role limitations due to physical problems: 4 items; pain: 2 items; general health perception: 5 items; vitality: 4 items; social functioning: 2 items; role limitations due to emotional problems: 3 items; and mental health: 5 items. Each of the items in each dimension has scores that are transformed into a scale ranging from 0 (worst health status) to 100 (best health status). The Spanish version of the SF-36 health questionnaire was validated a few years ago by Alonso et al. (14).

BIOCHEMICAL ANALYSIS

In the second cross-sectional study, a blood sample was drawn from each subject. Once the serum was separated, the samples were stored frozen at -80 °C

until quantification. Among other measurements, estimated glomerular filtration rate, total alkaline phosphatase, and tartrate-resistant acid phosphatase were measured using an autoanalyzer (Hitachi Mod. 717, Ratigen, Germany).

Serum levels of calcidiol (25OHD) were determined by prior extraction with acetonitrile (IDS, Ltd., Bolton, United Kingdom). Intact PTH levels and total osteocalcin were measured by radioimmunoassay (Nichols Institute, San Juan Capistrano, CA, United States).

DENSITOMETRIC EVALUATION

BMD was measured using a Hologic® QDR-1000 DXA densitometer (Hologic Inc., Waltham, MA, United States). In all cases, antero-posterior lumbar spine (L2-L4) and right femur BMD were analyzed. The coefficients of variation (CV) were 1.2 % and 1.9 %, respectively (15). Quality control was performed daily using a lumbar spine phantom, yielding a CV of 0.0 ± 0.1 %. In the fourth year, BMD was determined in the same areas as the first study, and the percentage change between the two measurements was used to evaluate changes in BMD at the two anatomical locations.

INCIDENCE OF OSTEOPOROTIC FRACTURE

After attending the second cross-sectional study, all participants were sent postal questionnaires at an interval of 2 years regarding any fractures they had experienced during that period. All osteoporotic fractures, excluding those of the skull and extremities (hands and feet) due to doubts about their osteoporotic cause, were confirmed by X-ray. The percentage of people who participated in these two postal follow-ups (excluding deaths) was 82.4 % and 81.3 %, respectively.

All studies were conducted following the principles outlined in the Helsinki Declaration and were formally approved by the Clinical Trials Committee of the Principality of Asturias.

STATISTICAL ANALYSIS

Data analysis was performed using SPSS version 25.0 for Windows. Quantitative variables were analyzed using the Student's t-test. Qualitative variables were analyzed using chi-square test.

To analyze the effect of falls on perceived health at the multivariate level, linear regression adjusted for age, sex, and BMI was used for the entire study population or separately by sex. Similarly, to analyze the effect of falls on the subsequent occurrence of incident osteoporotic fractures in both sexes, logistic regression was used, adjusting for age, sex, and BMI, as the dependent variable was dichotomous.

RESULTS

The mean age and BMI of those who fell were similar to those who did not fall (Table I). No changes were found in the degree of renal function, measured by estimated glomerular filtration rate, between those who fell and those who did not (Table I). Bone and mineral metabolism markers (calcidiol, PTH, osteocalcin, total alkaline phosphatase, and tartrate-resistant acid phosphatase) were similar between those who fell and those who did not (Table I). Regarding falls, 25 % of participants (n = 81) reported having fallen at least once over the past 12 months (Table I), more frequently in women than in men (34.9 % vs 14.7 %, $p < 0.001$). A total of 28 % of the falls (n = 23) occurred at home. There were no changes in the percentage change in BMD at the lumbar spine and femoral neck between the two cross-sectional surveys and the presence of falls of the past year (Table I).

In the univariate analysis of the entire cohort, 6 of the 8 dimensions of the SF-36 health questionnaire, as well as the global score, were significantly affected by the presence of falls, except for role limitations due to physical problems and emotional problems. The most affected dimensions were: vitality and mental health ($p < 0.001$ in both cases) (Table II).

The linear regression analysis adjusted for age, BMI, and sex showed that the perception of general health (β = -0.140, p = 0.014), vitality (β = -0.158, p = 0.004), mental health (β = -0.130, p = 0.016), and total score (β = -0.138, p = 0.009) were associated with falls (Table III).

By sex, in women, pain over the past 4 weeks (β = -0.231, p = 0.004); perception of general health (β = -0.183, p = 0.020); vitality (β = -0.291, $p < 0.001$); mental health (β = -0.162, p = 0.042); and the global score (β = -0.203, p = 0.015) were significantly affected (Table IV). In men, none of the dimensions of the SF-36 health questionnaire were affected. The number of falls did not change these results.

In neither sex, the changes in BMD at the lumbar spine and femoral neck that occurred between the 2 cross-sectional surveys were associated with the presence of falls. In men, the changes in BMD were very similar between those who fell (lumbar spine: 1.12 ± 4.71; femoral neck: 0.98 ± 4.71) and those who did not (lumbar spine: 1.09 ± 4.47; femoral neck: 0.97 ± 5.73, $p > 0.05$ in both cases).

Table I. Demographic. anthropometric. and clinical variables in the cohort of men and women who fell and those who did not over the past 12 months

Variables	Falls (*n* = 81)	No falls (*n* = 243)	*p*-value
Age (years)	69 ± 9	68 ± 8	0.195
BMI (kg/m^2)	29 ± 5	28 ± 4	0.149
Female sex n (%)	58 (71.6)	108 (44.4)	< 0.001
Estimated glomerular filtration rate (eGFR) (mL/min)	64 ± 17	67 ± 17	0.179
Calcidiol (ng/mL)	18 ± 11	17 ± 9	0.322
PTH (pg/mL)	52 ± 20	53 ± 24	0.720
Osteocalcin (ng/mL)	5.9 ± 2.4	6.0 ± 2.3	0.832
Total alkaline phosphatase (U/L)	175 ± 50	176 ± 75	0.887
Tartrate-resistant acid phosphatase (U/L)	2.0 ± 0.7	2.1 ± 0.7	0.554
% change in lumbar spine BMD	0.10 ± 4.81	-0.39 ± 5.07	0.497
% change in femoral neck BMD	-0.25 ± 6.17	0.87 ± 5.37	0.158

Table II. SF-36 health questionnaire dimension scores in men and women who fell or did not fall over the past 12 months

Variables	Falls (*n* = 81)	No falls (*n* = 243)	*p*-value
Dimension 1 (physical function)	*76 ± 21*	*82 ± 21*	*0.015*
Dimension 2 (physical role limitations)	71 ± 45	80 ± 40	0.124
Dimension 3 (pain over the past 4 weeks)	*71 ± 19*	*78 ± 19*	*0.004*
Dimension 4 (general health perception)	*55 ± 25*	*65 ± 24*	*0.003*
Dimension 5 (vitality)	*57 ± 28*	*70 ± 33*	*< 0.001*
Dimension 6 (social functioning)	*85 ± 26*	*91 ± 21*	*0.044*
Dimension 7 (emotional role limitations)	73 ± 45	79 ± 41	0.253
Dimension 8 (mental health)	*62 ± 23*	*73 ± 23*	*< 0.001*
Overall score	*66 ± 18*	*75 ± 19*	*< 0.001*

Table III. Linear regression analysis, adjusted for age, BMI, and sex, of the different SF-36 health questionnaire dimensions in the cohort of men and women based on the presence of falls over the past year

Variables	Standardized beta coefficient (*n* = 10)	*p*-value
Dimension 1 (physical function)	-0.061	0.274
Dimension 2 (physical role limitations)	-0.047	0.398
Dimension 3 (pain over the past 4 weeks)	-0.098	0.080
Dimension 4 (general health perception)	*-0.140*	*0.014*
Dimension 5 (vitality)	*-0.158*	*0.004*
Dimension 6 (social functioning)	-0.049	0.381
Dimension 7 (emotional role limitations)	0.002	0.977
Dimension 8 (mental health)	*-0.130*	*0.016*
Overall score	*-0.138*	*0.009*

Table IV. Linear regression analysis, adjusted for age and BMI, of the different SF-36 health questionnaire dimensions separated by sex based on the presence of falls over the past year

Gender	Variables	Standardized Beta coefficient (n = 10)	p-value
Women	Dimension 1 (physical function)	-0.024	0.745
	Dimension 2 (physical role limitations)	-0.028	0.725
	Dimension 3 (pain over the past 4 weeks)	*-0.231*	*0.004*
	Dimension 4 (general health perception)	*-0.183*	*0.020*
	Dimension 5 (vitality)	*-0.291*	*< 0.001*
	Dimension 6 (social functioning)	-0.057	0.469
	Dimension 7 (emotional role limitations)	-0.040	0.618
	Dimension 8 (mental health)	*-0.162*	*0.042*
	Overall score	*-0.203*	*0.015*
Men	Dimension 1 (physical function)	-0.062	0.431
	Dimension 2 (physical role limitations)	-0.040	0.622
	Dimension 3 (pain over the past 4 weeks)	0.062	0.445
	Dimension 4 (general health perception)	-0.045	0.779
	Dimension 5 (vitality)	0.067	0.396
	Dimension 6 (social functioning)	0.016	0.836
	Dimension 7 (emotional role limitations)	0.087	0.273
	Dimension 8 (mental health)	-0.088	0.276
	Overall score	-0.025	0.752

In women, while the changes reported in BMD at the lumbar level were very similar between those who fell and those who did not (-0.59 ± 4.91 vs -0.49 ± 5.37, p = 0.916), at the femoral neck, there was a trend, though not significant, toward greater losses in those women who fell compared to those who did not (-0.71 ± 6.32 vs 0.74 ± 6.10, p = 0.185).

At the 4-year follow-up, a total of 15 participants (10 women and 5 men) reported 16 incident non-vertebral fractures (6 hip fractures, 6 distal forearm fractures, and 4 fractures at other locations). A total of 8.6 % (n = 7) of those who had fallen over the past year fractured at the 4-year follow-up period vs 2.9 % (n = 7) of incident osteoporotic fractures that occurred in those who had not fallen (p = 0.035). The logistic regression analysis adjusted for age, BMI, and sex showed that having had previous falls was associated with an increased incidence of osteoporotic fracture [odds ratio (OR), 3.56, 95 % confidence interval (95 %CI, 1.04-12.21)].

DISCUSSION

Falls were more common in women than in men. Falls affected women's quality of life in dimensions related to vitality, pain, and general health, but also mental health, which includes depression, anxiety, emotional control, and behavioral issues. In men, on the other hand, none of the dimensions of the SF-36 health questionnaire were significantly affected. The changes in BMD at the lumbar spine and femoral neck that occurred between the two cross-sectional surveys analyzed were not associated with the presence of falls in either sex. However, it is interesting to highlight that the presence of falls in this population was associated with a 3.5-fold increase in the incidence of non-vertebral osteoporotic fractures.

As we have observed, consistent with the literature, falls were much more common in women than in men (16). Although the reasons that may explain this sex difference remain unknown, several theories related to lifestyle differences, greater longevity, frailty, and even genetic factors could explain this gender-related behavior (17). Approximately 30 % of people aged 65 or older living at home and 50 % of residents in nursing homes fall more than once a year (4,18,19). In our case, this percentage in people older than 54 years, 99 % of whom lived at home, was slightly lower than 30 %, possibly due to the age difference in the cohort studied.

Falls are common in the elderly, and a positive correlation between depression and falls has been found in some studies (20,21). In a meta-analysis, the authors

showed that depression was a predictor of falls (22). Moreover, the Hendrich II fall risk model, which is an assessment tool to predict fall risk in patients, identified depression as one of the eight risk factors analyzed (23). Falls and depressive symptoms, so common in the elderly, represent a significant burden on the health care system and society (24).

The relationship between depressive symptoms and falls is complex. Depressive symptoms in the elderly have been associated with a series of known fall risk factors, including cognitive impairment, slow walking speed, poor balance, slow reaction time, and lack of strength. Some authors consider it likely that depressive symptoms are linked to physical problems and cognitive decline, which could lead to an increased risk of falling (22). Furthermore, frailty has been shown to be related to worse cognitive function, worse physical function, poorer self-perceived health, and problems with mobility and activities of daily living, all of which have also been linked to falls (25), although it is unclear whether depressive symptoms are the cause or the result of falls, or vice versa.

In our case, we observed a greater effect of falls on perceived health in women, an effect that we did not observe in men. Some authors have suggested a gender effect on quality of life, which is observed in cohorts older than ours [> 75 years (26)].

Fear of falling is another important factor that may mediate the relationship between physical and cognitive factors, depressive symptoms, and falls. Fear of falling has been associated with depressive and anxiety symptoms, and a relationship has been established between fear of falling and avoiding activities that affect strength and physical capacity (11,12).

Individuals with fear of falling have significantly lower scores in social functioning, mental functioning, and role limitation due to emotional problems. This reduction in quality of life is said to be associated with a decrease in the amount of social interaction the individual experiences, leading to less contact with friends and family, social isolation, depression, and anxiety (27).

The effect of falls and emotional problems has been described in other works, although no other dimensions were affected (17). In our case, this dimension was not significantly affected. We cannot dismiss the fact that in people aged 65 or older, a prevalence of mild depressive symptoms is estimated at 15 % in those living at home, with this proportion increasing in those living in nursing homes for the elderly (28-30). In our study, 99 % of participants lived in their own homes, so emotional limitations in behavior were probably less frequent compared to those living in nursing homes, where they often feel abandoned by their families, which likely has a significant emotional impact. However, we must remember that this study was conducted nearly 30 years ago, and the perception of the emotional well-being of the elderly may have changed over the years.

It is well known that the aging population is accelerating, especially in the Western world, and our country is one of the most aging countries in the region. Falls and their consequences are becoming more common. Therefore, preventing and treating falls is a challenge from a socio-health perspective. Significant efforts are needed to make sure that older adults are not afraid to report their falls so that effective measures can be implemented to prevent the loss of functional capacity and quality of life for the elderly (31-34).

Another interesting aspect to highlight is the presence of falls as an important predisposing risk factor for fractures. A study by Kim et al. (35) shows that, in older adults, a fall within a 4-month period carries a high risk of fracture during the following year, regardless of the type of fall (35). In our case, having had at least one previous fall increased the incidence of osteoporotic fracture by 3.5 times. Similar results have been observed in other longitudinal studies, some with a stronger association (36), while others with a much weaker one (37). Some authors have even been unable to observe the effect of falls as a risk factor for imminent fractures, probably due to the short observation period of just 1 year (38), while our study had a 4-year follow-up. Nevertheless, our results on the presence of fractures (8.6 %) at the 4-year follow-up in those who fell were proportionally lower, but similar to those found by other authors in a Spanish study with only a 1-year follow-up (2.3 %), but with an older population (19).

Several drugs used to treat depression have been shown to increase the risk of falls and fractures, and some also have a direct effect on bone, potentially increasing the risk of fracture in the event of a fall (39,40). However, only 2 studies identified antidepressants as a predictor of subsequent falls, an effect that was lost in multivariate analysis (41,42). Other studies have reported the use of psychotropic or psychoactive drugs as predictors of falls (22).

One recent meta-analysis shows that the risk of falls increasing the likelihood of fractures is independent of BMD (43), something similar to what we have observed in our cohort in both sexes at the lumbar spine and femoral neck. These results reinforce the importance of controlling and preventing falls as a major predisposing factor for fractures, regardless of BMD levels.

Our study has limitations, but also strengths. Regarding the former, the fact that the SF-36 quality of life questionnaire was not self-administered but conducted by an interviewer could have biased some of the participants' responses. It is likely that recalling

the fall history in elderly individuals may not be entirely reliable, but this is an inherent limitation of epidemiological studies. Another limitation could be the small number of incident osteoporotic fractures occurring during the 4-year period. It is worth mentioning that this study was part of a European multicenter study that, in its guidelines, did not include quality of life studies. In our case, trying to take advantage of the possibility of having a large population cohort followed prospectively, we analyzed some specific aspects not contemplated in the European study clinical practice guidelines. We must also remember that the study was conducted nearly 30 years ago, and some of the results obtained may be influenced by the time that has passed.

As for strengths, the participation rates of more than 80 % in the postal follow-ups conducted up to 8 years after the baseline study support the representativeness of the analyzed sample. Furthermore, this study was prospective rather than cross-sectional, which strengthens the validity of the results found and their higher degree of association. Former studies from this cohort have shown how falls were also associated with losses in functional capacity, which in some way supports the consideration of the results of this study (44).

In conclusion, we can highlight that preventing falls in women could lead to better health perception and greater vitality, preventing depression, anxiety, as well as better emotional and behavioral control. However, these results should be properly confirmed in a specific fall study. Falls were not associated in either sex with a greater loss of BMD, either at the lumbar spine or femoral neck, but falls could have significant clinical consequences with an increase in the incidence of osteoporotic fractures.

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Original

Exploring NF-κB silencing biosafety in mesenchymal stem cells as a possible strategy for osteogenic augmentation

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Abstract

Introduction: the decrease in oestrogen levels after menopause increases inflammation, activating the NF-κB pathway in mesenchymal stem cells (MSCs). This pathway has a marked anti-osteogenic effect and plays critical roles in cellular function. Our hypothesis is that silencing key genes in the canonical and non-canonical NF-κB pathways in endogenous MSCs using the Aptamer-Lipid Nanoparticle-Gapmer system previously designed by our group could be a viable strategy for the treatment of osteoporosis. However, it is essential to verify that such silencing does not compromise basic cellular functions, given the multiple roles of NF-κB in regulating immune responses, apoptosis, and cellular homeostasis.

Materials and methods: the murine MSC line C3H10T1/2 was used to assess the silencing of key genes (Ikkα, Ikkβ, Nemo, and Nik) in the canonical and non-canonical NF-κB pathways using GapmeRs. Gene expression levels were measured post-silencing and compared with those achieved using commercial inhibitors (BMS-345541 and MLN120B). Additionally, basic cellular function assays, including proliferation, chemotaxis, cell migration, and viability, were conducted to evaluate the safety of NF-κB silencing.

Results: silencing Ikkα, Ikkβ, Nemo, and Nik resulted in a significant reduction in gene expression in vitro. While NF-κB activation with lipopolysaccharide (LPS) significantly increased the expression of target genes such as IL-6 and NFKB1A, this increase was blocked after gene silencing, reaching levels comparable to those achieved with commercial inhibitors. Cellular function assays showed no significant changes in proliferation, chemotaxis, cell migration, or viability following silencing.

Conclusions: NF-κB pathway silencing using GapmeRs does not negatively impact basic cellular functions, suggesting that this approach is safe and efficient. These findings support its potential clinical application in promoting the osteogenic differentiation of MSCs for osteoporosis treatment.

Keywords: Mesenchymal stem cells. Osteogenic differentiation. Osteoporosis. NF-κB. Cellular therapy.

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INTRODUCTION

Osteoporosis, a condition commonly associated with advanced age, is characterized by a reduction in bone mass density and a concomitant deterioration of bone microstructural integrity, leading to an increased susceptibility to fractures (1). The overall prevalence of osteoporosis results in more than 10 million fragility fractures annually, a number that is expected to rise in the coming years due to the aging global population (2). Importantly, osteoporosis-related fragility fractures linked are associated with a significant reduction in quality of life and increased disability.

Currently, there are 2 different approaches to manage osteoporosis: antiresorptive and osteoanabolic drugs. Antiresorptive agents block the osteoclast-mediated bone resorption process. Typical antiresorptive agents are monoclonal antibodies such as Denosumab—targeting RANKL—an activator of pre-osteoclast maturation or bisphosphonates such as alendronate, which induce osteoclast apoptosis. On the other hand, drugs such as teriparatide, a truncated PTH derivative (3), or abaloparatide, a PTH analog (4) have an osteoanabolic function, stimulating osteoblasts proliferation. Both antiresorptive and osteoanabolic drugs have important adverse effects associated to prolonged used, something difficult to avoid in a chronic disease such as osteoporosis (3,5). No new agents had come to market in the last few years except for romosozumab, an anti-sclerostin antibody with a dual antiresorptive and osteoanabolic action (6). However, although treatment with romosozumab dramatically reduces the occurrence of fragility fractures in patients with postmenopausal osteoporosis (7), clinical trials showed an increased cardiovascular risk in patients on romosozumab (7). In addition, the cost of treatment per patient with romosozumab is significantly higher than that of other anti-osteoporotic drugs, something that would negatively impact the national health system. Hence, an emergency clinical need exists to develop cost-effective and long-term safe osteoporosis treatments.

In recent years, there has been increasing interest in using mesenchymal stem cells (MSCs)-based approaches to improve bone regeneration (8). MSCs are multipotent cells with self-renewal capacity, which makes them promising candidates for therapeutic applications in regenerative medicine (9). Importantly, MSCs from osteoporotic individuals seem to have an increased adipogenic capacity at the expense of their osteogenic potential. Therefore, we hypothesize that the enhancement of this osteogenic capacity in MSCs would be a valid approach for osteoporosis treatment. We propose to increase this osteogenic potential of MSCs by silencing key genes with anti-osteogenic activities.

The Wnt/β-catenin (10) and bone morphogenetic proteins (BMP) signaling (11) pathways play major roles in bone formation. The activation of these pathways drives the transcription of genes, such us alkaline phosphatase (*ALPL*), osteocalcin (*BGLAP*) and the Runt-related transcription factor 2 (*RUNX2*), which regulate osteoblast differentiation, extracellular matrix synthesis, and bone mineralization. Previous works from our group have shown that silencing inhibitors of the BMP (*Smurf1*) or the Wnt/b-catenin (*Sfrp1*) pathways lead to a significant increase in the osteogenic capacity of MSCs *in vitro* in MSCs from osteoporotic patients (12,13). Moreover, we designed a system to specifically silence those genes at the endogenous MSCs (14). Our system is based on the use of a particular type of lock nucleic acid-antisense oligonucleotides (LNA-ASOs), known as GapmeRs. These molecules consist of a single-stranded deoxyribonucleotide typically composed by 14-20 bp, which specifically binds to its mRNA target creating a DNA/RNA hybrid duplex that will be then recognized by the RNAseH leading to the degradation of the targeted mRNA (15). The GapmeR construct includes a central DNA sequence flanked by 2 RNA sequences modified for heightened resistance to endonucleases, ensuring optimal efficacy and durability (13). The silencing produced by the GapmeRs is transient and does not cause permanent changes to the DNA, thus reducing possible unwanted side effects. This method is clinically safe enabling the translation of this treatment into the routine clinical practice (16). In fact, some treatments currently used in clinical practice are based in the use of ASOs (17), such as eteplirsen for treatment of Duchenne muscular dystrophy (DMD) (18), or nusinersen for treatment of spinal muscular atrophy (19). To specifically deliver the GapmeRs to endogenous MSCs, these molecules are encapsulated within hybrid nanoparticles and specifically directed to the BM-MSCs using aptamers that recognize these cells. We have shown that silencing *Sfrp1 in vivo*, at the level of the endogenous MSCs, using this system is a viable approach to increase bone mass in an osteoporotic mouse model. Targeting this inhibitor of the Wnt/b-catenin *in vivo* using this gapmeR-nanoparticles-aptamer system increases bone mass in osteoporotic mice by up to 30 % (12,14). Our current aim is to increase bone-formation efficiency of our system. For this purpose, in this work we tested the feasibility of targeting another signaling pathway that has an anti-osteogenic activity, the NF-kB pathway, with the notion that the simultaneous silencing of *Sfrp1* and a putative target from the NF-kB pathway will significantly increase the previous percentage.

The NF-kB signaling pathway includes 3 integral components: the NF-kB dimer, the IKK complex, and the IκB protein (15). Although the NF-kB dimer is constitutively present in the cytosol of cells it is kept in an inactive state by the action of the IkB proteins. IkB proteins have a domain called the destruction box, which is rich in serine residues. When the cell receives a signal activating the NF-kB pathway, serine residues in the destruction box domain are phosphorylated by the IKK

protein kinases, thus inducing the degradation of the IkB protein and the release of the NF-kB dimer. Once released, this dimer can translocate to the nucleus and regulate the expression of target genes (15). The NF-kB pathway exhibits 2 distinct modalities known as the canonical and alternative pathways. In the canonical route, the IKK complex is composed by *Ikkα*, *Ikkβ*, and *Nemo*. *Ikkβ* serves as the primary kinase responsible for phosphorylating the serine residues within the IkBα destruction box. The phosphorylation of IkBα leads to the release of the NF-kβ dimer, which translocates into the nucleus (15). In contrast, the non-canonical or alternative route depends on *Nik*, which is a constitutively active but normally degraded protein. When the cell receives an activation signal from the NF-kB pathway, the degradation of *Nik* stops and *Nik* phosphorylates the *Ikkα* protein, thus leading to a subsequent release of the NF-kB dimer, which again translocates into the nucleus (15). The NF-kB canonical and no canonical pathways are involved in many processes, such as cell proliferation and survival, DNA damage repair, and immunity (20). NF-kB inactivation has been associated with an increased osteoblast activity and bone formation (21) suggesting that inactivation of this pathway could be a likely approach for further enhancing bone formation in our model (22).

The NF-kB signalling pathway plays a pivotal role in regulating various basic cellular functions, including viability, migration, chemotaxis, and proliferation, particularly in mesenchymal stem cells (MSCs). NF-kB is a family of transcription factors that control the expression of genes involved in immune and inflammatory responses, as well as cell survival, differentiation, and apoptosis (23). In MSCs, NF-kB activation is crucial for maintaining cellular viability by regulating anti-apoptotic genes and promoting resistance to stress-induced cell death. Moreover, NF-kB influences the migratory and chemotactic capabilities of MSCs, which are essential for their therapeutic potential in tissue repair and regeneration. This pathway modulates the expression of chemokines and adhesion molecules, thereby guiding MSCs migration towards injury sites (24). NF-kB also plays a significant role in cell proliferation by regulating the expression of cyclins and other cell cycle-related proteins, ensuring proper cell cycle progression and proliferation. These multifaceted roles underscore the importance of NF-κB signalling in the functional regulation of MSCs and its potential implications in clinical applications. Besides its implication in bone formation and basic cellular functions, the NF-kB pathway plays a crucial role in the regulation of inflammation, which indirectly impacts bone formation. Ageing and, in women, the decline in estrogen levels after menopause, lead to a significant increase of inflammation in the bone marrow (BM) microenvironment, resulting in the establishment of a hostile environment. This inflammatory environment prevents the differentiation of MSCs into osteoblasts promoting their adipocytic differentiation (25). The activation of the NF-kB pathway in BM MSCs directly contributes to the establishment of this hostile microenvironment. Once this pathway has been activated in MSCs, these cells would produce a set of pro-inflammatory cytokines, that, in turn, exacerbate inflammation, further hindering bone regeneration.

We propose that silencing key genes of the canonical and/or non-canonical NF-kB pathways in MSCs would increase their osteogenic capacity, thus increasing the efficiency of our previous model based on the sole silencing of *Sfrp1*. Importantly, this approach would not only enhance osteogenic differentiation but could also modulate the composition of the MSCs secretome, thus reducing the presence of pro-inflammatory factors. This immunomodulatory effect would create a permissive BM microenvironment that would facilitate bone regeneration. Both strategies aim to effectively increase bone mass, offering a promising treatment approach for diseases characterized by reduced bone mass, including osteoporosis.

MATERIALS AND METHODS

CELL CULTURE

C3H10T1/2 (Clone 8, Ref. CCL-226, ATCC, Manassas, VA, United States), an immortalized murine MSCs line was cultured in Dulbecco's Modified Eagle's Medium (DMEM, Invitrogen, Waltham, MA, USA) supplemented with 10 % Fetal Bovine Serum (FBS) and 1 % penicillin-streptomycin. Cell passage was performed using TrypLE Express (Ref. 12604-013, Gibco, Thermo Fisher Scientific, Waltham, MA United States).

GapmeR DESING

Antisense LNA GapmeRs were purchased from Exiqon (Qiagen, Venlo, The Netherlands). A non-specific GapmeR Negative Control A (Ref. 339515 LG00000002-DDA) was used as a control GapmeR, and specific GapmeRs were used to target *Ikkα* (Ref. LG00824583-DDA), *IKKβ* (Ref. LG00824663-DDA), *Nemo* (Ref. LG00824633) and *Nik* (Ref. LG00824353).

CELL TRANSFECTION

Lipofection was performed in a 24-well plate using Dharmafect (Ref. T-2001-01, Dharmacon, Horizon Discovery, Cambridge, United Kingdom), following the manufacturer's instructions for use. Cells were seeded at 12,500 cells/cm^2 24 hours before transfection. Two hours before transfection, cells were washed twice

with PBS 1X and culture medium was replaced with Opti-MEM (Ref. 31985047, Gibco, Thermo Fisher Scientific, Walthman, MA, United States). GapmeR concentration used was 20nM. Transfection was performed following the manufacturer's instructions for use. After incubation at 37 °C for 24 hours, an equal volume of culture medium was added. Finally, 48 hours after transfection, the medium was removed and the cells were washed once with PBS, and culture medium was added.

RNA EXTRACTION AND CONVERSION TO cDNA

Cells were washed twice with PBS prior to collect the mRNA using TRIzol reagent (Invitrogen, Walthman, MA, United States). After TRIzol addition, the plate surface was scrapped to maximize the material collected. RNA was extracted following the manufacturer's instructions for use. mRNA retrotranscription was performed with the PrimeScript RT Reagent Kit (RR037A, Takara Bio Inc, Shiga, Japan) following the manufacturer's instructions for use. The resulting cDNA was diluted 4 times with ddH_2O to perform gene expression analysis by semi-quantitative PCR.

GENE EXPRESSION ANALYSIS

Gene expression levels were measured using semi-quantitative PCRs TaqMan assays (Applied Biosystems, Waltham, MA, United States). To test gene silencing, the following TaqMan assays were used: Mm00432529_m1 (*Ikk*α), Mm01222247_m1 (*Ikk*β), Mm00494927_m1 (*Nemo*), Mm00444166_m1 (*Nik*). To analyze gene expression of the NF-kβ pathway target genes, the following TaqMan assays were used: Mm00446190_m1 (*Il-6*) and *NFkB1A* (Mm00477798_m1). For normalization, we used the mouse housekeeping GAPDH gene (Assay Mm99999-915_G1).

FLOW CYTOMETRY AND APOPTOSIS ASSAY

FITC Annexin V Apoptosis Detection Kit I (556547, BD Bioscience, San Diego, CA, United States) was used following the manufacturer's instructions for use. Forty-eight hours after transfection, 100,000 cells per condition were transferred into 2 flow cytometry tubes and washed twice with 1× PBS at 4 °C. The cells were, then, resuspended in 100 µL of Binding Buffer and 2.5 µL of Annexin V-FITC labelled antibody was added to 1 tube for each condition. After 30 minutes incubation in the dark, cells were washed twice with 1X PBS at 4 °C and resuspended in 100 µL of Binding Buffer. Finally, 2.5 µL of 7-amino-actinomycin D (7-AAD) was added to each tube just before flow cytometry.

CELL PROLIFERATION ASSAY

Cells were transfected in 24-well plates as previously explained and harvested 48 h after transfection. Afterwards, different cell numbers (100, 200, 300 and 400 cells per well) were seeded onto a 96-well plate in triplicates and allowed to attach overnight. Then, cells were allowed to proliferate for 1, 4, 6, 8, 10 and 12 days. To determinate cells viability at different timeframes, culture media was substituted by complete DMEM (10 % FBS and 1 % penicillin-streptomycin) containing 0.5 mg/mL of 3-(4.5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and cells were incubated for 4 hours. Then, the media was changed to 100 µL of 2-propanol and incubated at 37ºC for 10 minutes. Finally, absorbance at 570 nm was measured with a plate lector Eon (BioTek, Winooski, VT, United States). The results are represented as the increase in cell number vs the previous day.

CELL MIGRATION ASSAY

Cell migration capacity after transfection with the GapmeRs was analyzed by a wound healing assay. For this, C3H10T1/2 cells were seeded at high confluence in a 6-well plate (25,000 cells/cm^2) and then transfected following the standard protocol. Wounds were performed using a 0.1-20 µL pipette tip to produce wounds 300 µm wide. In a Nikon Eclipse Ti (Nikon Instruments Inc. Melville, NY, United States), pictures of the 6 different fields were taken every 3 hours for a total of 15 hours. The live cell microscope maintained the cells at 37 °C under normoxic conditions. Finally, we measured the area of the wound every 3 hours with the NIS-Elements software (Nikon Instruments Inc., Melville, NY, United States), and determine the average wound area per time and condition.

TRANSWELL MIGRATION ASSAY

A total of 7000 transfected cells resuspended in 200 µL of serum-free media were seeded per upper chamber of a 6.5 mm Transwell with 8 µm Pore Polycarbonate Membrane Insert (3422, Corning, Somerville, Massachusetts, United States). Cells were incubated with 100 ng/mL Stromal cell-Derived Factor-1 (SDF-1, Ref. 10118-HNAE, Sino Biological Inc., Houston, Texas, United States) in 600 µL of serum-free media in the lower chamber for 24 hours at 37 °C. After incubation, to avoid background signal, cells that did not migrate through the membrane were eliminated by washing the upper chamber twice with 1× PBS and cleaning it with a cotton swap. Finally, cells were stained with 600 µL 1.01 µg/mL DAPI (Ref. 62248, ThermoFisher Scientific, Rockforf, United States) in 1X PBS for 10 minutes and kept in 1× PBS until

4-5 fields per well at 10× pictures were taken in a fluorescence or phase contrast microscope. Graph represents average cell number per well.

STATISTICAL ANALYSIS

Error bars on graphs represent the standard error of the mean value (SEM). Statistical significance was calculated using the Students' t test. Significance was always set at $p < 0.05$.

RESULTS

NF-κB PATHWAY INACTIVATION IN THE MSCs CELL LINE C3H10T1/2

We hypothesized that silencing key genes in the NF-kB pathway may led to a reduced overall activity of the pathway, thus increasing the osteogenic potential of MSCs. To put this hypothesis to the test, we first needed to efficiently silence these genes and confirm the inactivation of the pathway in the murine MSCs cell line C3H10T1/2. We selected 4 key components of the canonical and non-canonical pathways: *Ikk*α, *Ikk*β, *Nemo* and *Nik* and designed specific GapmeRs to achieve gene silencing. The expression of the targeted genes was assessed by qPCR 48 hours after transfection with these GapmeRs (Fig. 1A). Transfection with a non-specific gapmeR was used as a control in all the experiments (GpmR Ctrl). We observed a statistically significant silencing of 61.2 % of *Ikk*α, 62.1 %of *Ikk*β, 64.5 % of *Nemo* and 43 % of *Nik* vs control.

Once we confirmed the silencing of the different targets, we needed to make sure that those levels of silencing led to a significant inhibition of the NF-kB pathway. For this purpose, we needed to establish a method to monitor the activation of the NF-kB signaling pathway. Activation of this pathway in MSCs *in vitro* is generally achieved through incubation with lipopolysaccharide (LPS) (26). To determine the optimal concentration of LPS for pathway activation in our experimental settings, we measured the activation of the pathway in response to different concentrations of LPS by evaluating the increase in *Il-6* expression levels, a direct target of this pathway (27). We conducted a screening in untransfected cells using 3 different concentrations of LPS (0.5 µg/mL, 1 µg/mL, and 50 µg/mL) (Fig. 1B). Our results indicate that a concentration of 1 µg/mL LPS leads to a significant increase in *Il-6* expression levels and thus, is optimal for NF-kβ pathway activation. This amount of LPS, maintained in the culture media for 18 hours, resulted in a 1.5-fold increase in *Il-6* expression.

Simultaneously, we determined the appropriate concentration of commercial inhibitors of the NF-kB pathway (BMS-345541 and MLN120B) that effectively block its activation without inducing toxicity in the C3H10T1/2 cell line (Fig. 1C). These molecules would be used as controls of pathway inhibition in our experiments. Two different concentrations of each inhibitor were tested, 10 µM and 20 µM in cells cultured in the presence of 1 mg/mL LPS. With both inhibitors a concentration of 10 µM was found to significantly inhibit the surge in *Il-6* expression in response to LPS. In the case of the BMS-345541 inhibitor, a higher reduction in *Il-6* expression was detected when the concentration used was 20 µM due to the toxic effect of this condition that would lead to a significant increase in cell death.

Once the appropriate concentrations of LPS and inhibitors were determined, we were set to verify that the inhibition of the different components of the NF-kB pathway with the specific GapmeRs did indeed lead to a reduction in the activity of the pathway. Therefore, we compared the expression of *Il-6* in transfected cells grown in LPS to that of cells transfected with the control GapmeR grown in the same inductive conditions. The decrease in the level of pathway activity achieved by the silencing of each of the genes was compared to that achieved by the commercial inhibitors BMS-345541 and MLN120B (Fig. 2A). The results show that cells transfected with *Ikk*α, *Nemo* and *Nik* GapmeRs did not respond to LPS by increasing *Il-6* expression confirming that the silencing of those genes greatly impacts the activity of the pathway. Results obtained with cells where *IKK*β had been silenced where somewhat different. The graph shows that *Il-6* expression significantly increases in cells where *Ikk*β has been silenced vs the gpmR control group without LPS. Since it has been described that *Ikk*β silencing could have an effect on the expression of negative regulators of the pathway, such as IkBa, which sequester NF-κB in the cytoplasm, affecting both basal and stimulated activity, we decided to analyze the expression of an alternative target of the NF-κB pathway, the *NFkB1A* (28) gene (Fig. 2B) to provide insights into whether the observed effects on *Il-6* are specific to its regulatory mechanisms or reflect a broader alteration in NF-kB signaling. In this case, the pattern of *NFkB1A*, unlike *Il-6*, would support the negative effect of *Ikk*β silencing on general NF-kB activity.

EVALUATION OF THE BIOSAFETY OF SILENCING NF-κB GENES IN THE MURINE MSCs CELL LINE C3H10T1/2

Since the NF-kB pathway regulates various cellular responses, including cell proliferation, migration, and survival, we decided to analyze the effect of

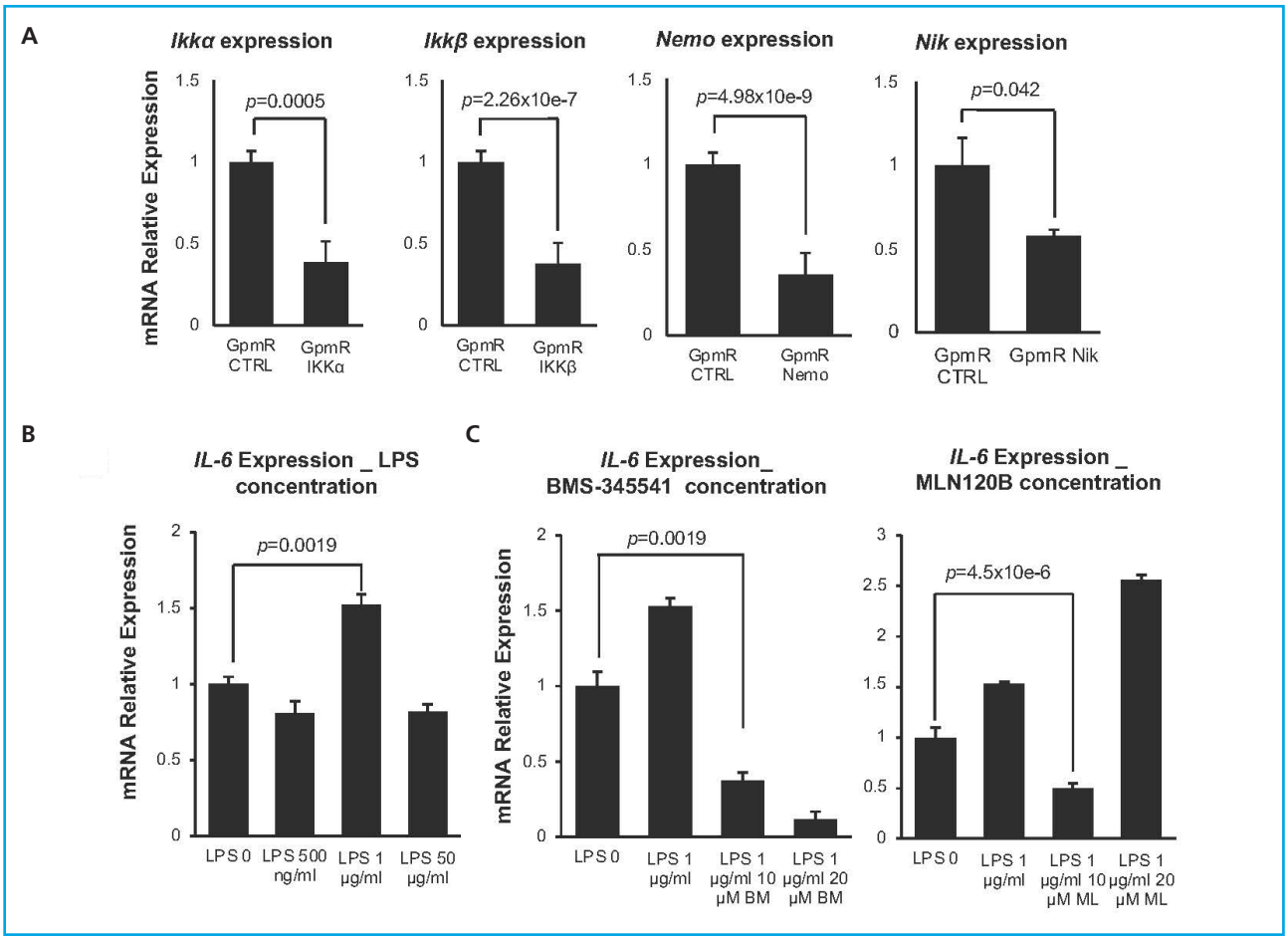

Figure 1. Silencing of NF-kβ pathway in the C3H10T1/2. A. Relative expression of targeted genes. Expression was measured 48 hours after transfection with specific GapmeRs. Absolute values were normalized vs those obtained after transfection with a control GapmeR ($n = 4$). B. Relative expression of *Il-6* after treatment with different concentrations of LPS. Expression was measured 18 hours after growing C3H10T1/2 with LPS 0.5, 1 and 50 µg/mL to activate NFkβ pathway ($n = 3$). C. Relative expression of *Il-6* with commercial inhibitors BMS-345541 and MLN120B in the presence of 1 µg/ml LPS ($n = 3$). Bars show standard deviation of mean values. *$p < 0,05$; **$p < 0,005$).

Ikkα, *Ikkβ*, *Nemo* and *Nik* silencing these basic cellular functions.

To analyze the proliferation of the transfected cells, we performed an MTT assay (Fig. 3A). Results are represented as the increase in cell number vs the previous day. No significant differences were observed between the silenced cells and control, except for *Ikkβ* silenced cells at the D8/D4 point exhibiting a significant increase in proliferation. Nevertheless, this increase was resolved at the last day, a point at which proliferation levels were comparable to those of the control.

CXCL12/CXCR4 signaling is involved in the chemotaxis and homing of stem cells (29). Since one of the known targets of the NF-kB pathway in other cells is C-X-C chemokine receptor type 4 (CXCR4) (30), the receptor for the stromal cell derived factor 1 (SDF-1 α) (27), we decided to analyze if silencing the different genes could affect the capacity of transfected cells to respond to this chemotactic agent (Fig. 3B). Therefore, we performed a chemotaxis analysis using a transwell migration assay. Forty-eight hours after transfection, cells were added to the upper side of a 0.8 transwell membrane and exposed to medium containing SDF-1 on the lower chamber of the transwell. Cells were allowed to migrate towards the lower part of the transwell membrane for 24 hours. Cells that had migrated and were attached to the lower part of the transwell membrane were stained with DAPI and detected using fluorescence microscopy. The results are normalized to gapmeR control-transfected cells exposed to SDF-1. The negative control (CTRL corresponding to cells not exposed to SDF-1α) showed a significantly lower migration than that of cells exposed to SDF-1α (SDF-1α). However, no significant differences were found between the chemotactic response to SDF-1α of the cells where the different genes of the NF-kB pathway have been silenced and non-transfected cells.

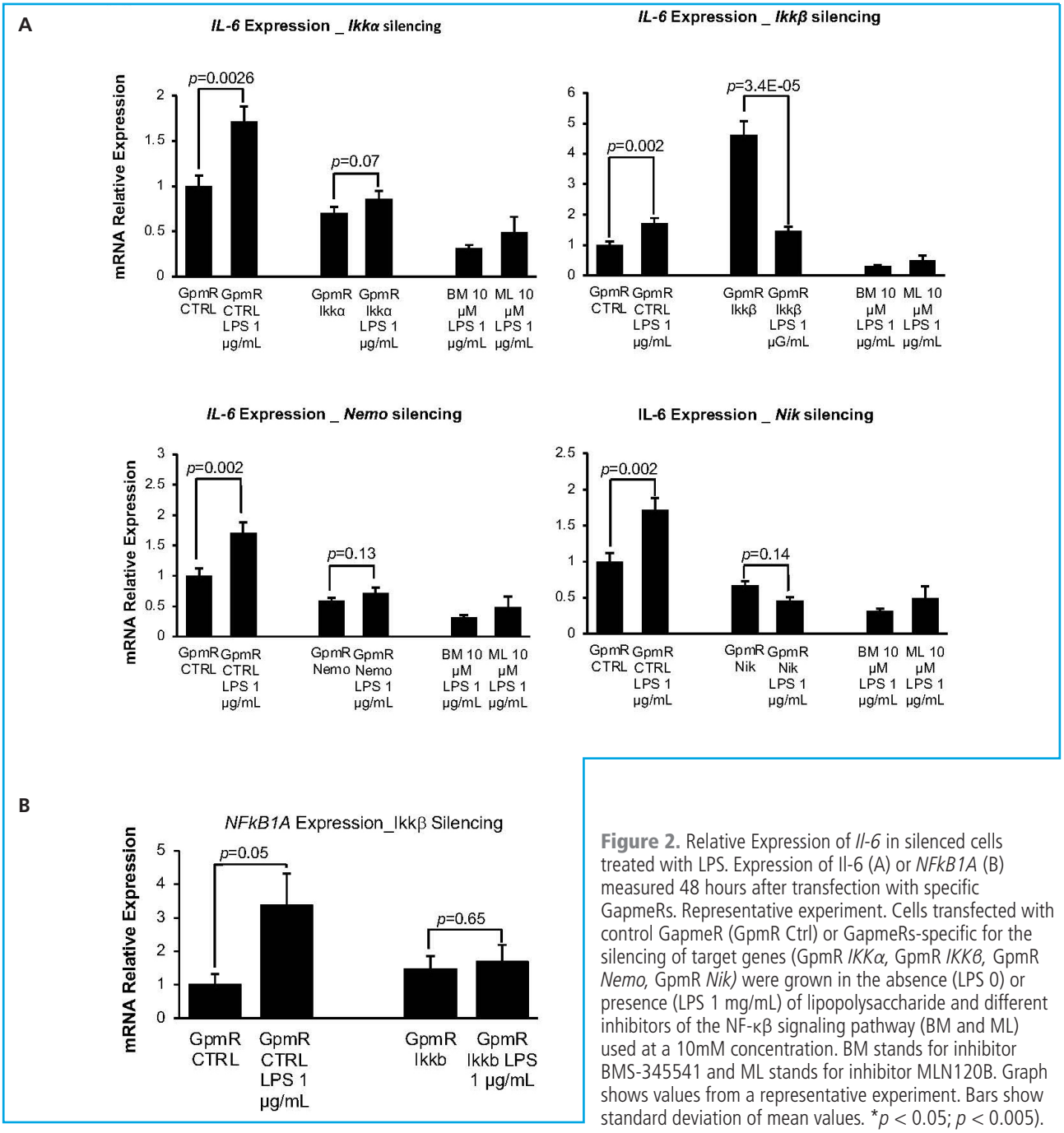

Figure 2. Relative Expression of *Il-6* in silenced cells treated with LPS. Expression of Il-6 (A) or *NFkB1A* (B) measured 48 hours after transfection with specific GapmeRs. Representative experiment. Cells transfected with control GapmeR (GpmR Ctrl) or GapmeRs-specific for the silencing of target genes (GpmR *IKKα,* GpmR *IKKβ,* GpmR *Nemo,* GpmR *Nik)* were grown in the absence (LPS 0) or presence (LPS 1 mg/mL) of lipopolysaccharide and different inhibitors of the NF-κβ signaling pathway (BM and ML) used at a 10mM concentration. BM stands for inhibitor BMS-345541 and ML stands for inhibitor MLN120B. Graph shows values from a representative experiment. Bars show standard deviation of mean values. *$p < 0.05$; $p < 0.005$).

Finally, to analyze the effect of *Ikk*α, *Ikk*β, *Nemo* and *Nik* silencing on MSCs viability, we performed an Annexin/7-AAD assay (Fig. 3C) 48 hours after transfection, using annexin V (AnnV) and 7-amino-actinomycin D (7-AAD). AnnV marks cells undergoing apoptosis, as it binds to phosphatidylserine when this phospholipid translocates to the outer part of the cell membrane, one of the early signs of apoptosis. Conversely, 7-AAD marks cells in late apoptosis, as it binds to DNA but cannot penetrate intact cell membranes, thus only entering the cells when cellular integrity is compromised. We can detect viable cells (Ann-/7-AAD-), early apoptotic cells (Ann+/7-AAD-), late apoptotic cells (Ann+/7-AAD+), and dead cells (Ann-/7-AAD+). Although a tendency to an increase of apoptotic cells was observed upon transfection with the gapmeR for the Nemo silencing, this difference was no significant, suggesting that the silencing of genes from the NF-kB signaling pathway has no effect on cell viability.

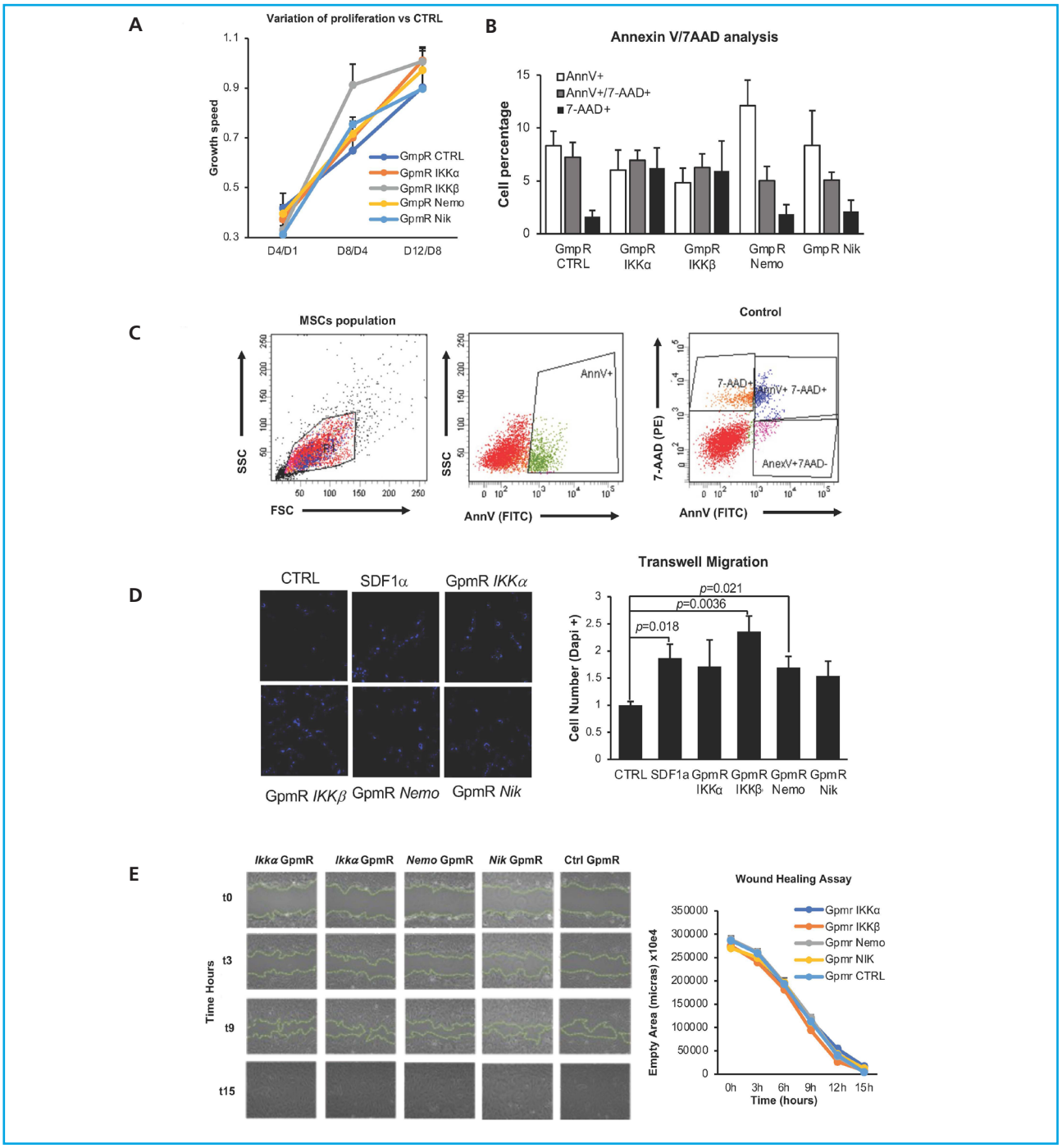

Figure 3. Evaluation of the biosafety of MSCs C3H10T1/2 cell line expressing low levels of NF-κβ key genes. A. Effect of target inhibition on cell proliferation. Results from the MTT analysis to measure the effect of target gene inhibition on cell proliferation ability. The results are expressed as the difference of each endpoint relative to the previous one ($n = 2$). B. Effect of target inhibition over the response to chemotactic agents. C3H10T1/2 migration capacity in response to SDF-1α. To the left, representative images of transfected cells that migrated through the transwell membrane stained with DAPI (magnification 10×). To the right, quantification of migrating cells. The number of migrating cells in each case is normalized vs the number of cells transfected with the control GpmR migrating in the absence of SDF-1α. Representative result ($n = 5$). C. Effect of target genes inhibition over MSCs viability. Results are expressed as the percentage of cells marked with AnnV and/or 7-AAD. AnnV+ are early apoptotic cells, AnnV+/7-AAD+ are late apoptotic cells, and 7-AAD+ are necrotic cells ($n = 3$). For all experiments, graph express the mean values of 5 or 3 experiments, as indicated. Bars show standard deviation of the mean values. *$p < 0.05$; **$p < 0.005$). D. Wound healing assay performed 48 h after transfection. E. Right. representative pictures at time 0, 3, 9, 15 hours of control group and cells transfected with specific GapmeRs for *Ikkα, Ikkβ,* Nemo and *Nik.* Areas lacking cells are outlined in green. Left. Mean values of the quantification of the wound area (in pixels) performed every 3 hours.

DISCUSSION

MSCs-based approaches had been proposed as an alternative to improve bone regeneration. We have previously managed a 30 % increase in the osteogenic potential of osteoporotic MSCs by silencing *Sfrp1*, an inhibitor of the Wnt/β-catenin signaling pathway (14). We hypothesize that it would be possible to further increase this osteogenic potential by silencing key genes of the NF-kB canonical and non-canonical pathways. This hypothesis originates from the previously explained role of the pathway in reducing bone mass, both directly through the inhibition of osteoblastogenesis and indirectly through the increase of inflammation levels in the BM microenvironment.

Regarding its anti-osteogenic activity, the NF-kB signaling pathway influences osteogenesis through different mechanism. NF-kB binds to the promoter of *Smurf1* and *Smurf2,* both inhibitors or the BMP pathway, and increases their transcription (22). In addition, NF-kB inhibits the activity of Fos-related antigen 1 (Fra-1) (31). This protein, part of the activator protein-1 (AP-1) family, regulates the expression of various genes involved in osteoblast differentiation and bone matrix production. In relation to the indirect influence of NF-kB in bone formation, this pathway plays a major role in establishing the BM pro-inflammatory microenvironment. Loss of estrogen increases the activity of Th17 (32) cells, and, consequently, proinflammatory cytokines such as Interleukine-6 (IL-6), tumor necrosis factor alpha (TNFα), interleukine-17 (IL-17) and receptor activator of NF-kβ (RANKL) (33). These molecules work by activating the NF-kβ signaling pathway, thus perpetuating the activation of Th17 lymphocytes by producing cell survival factors and pro-inflammatory cytokines. In addition, the activation of the NF-kβ pathway inhibits the anti-inflammatory activity of B lymphocytes. B cells reduce the pro-inflammatory environment and increase bone formation producing osteoprotegerin (OPG) (33), while T cells have a pro-inflammatory action (34). These pro-inflammatory cytokines also inhibit the Wnt/β-catenin pathway by increasing the expression of sclerostin and decreasing the synthesis of *RUNX2*, *IBSP* and *BGLAP* (35). All this combined results in major differentiation of MSCs into adipocytes, decreasing their differentiation into osteoblasts, thereby compromising bone homeostasis.

To silence the selected target genes of the NF-kB pathway we designed a specific gapmeR for each target gene which binds to the mRNA molecule by inducing its degradation by the RNase H. The silencing caused by this system is transient and does not cause permanent changes to the DNA, thereby ensuring its safety. Silencing target genes with GapmeRs was confirmed by qPCR. The expression of target genes is significantly reduced after transfection vs the control GapmeRs, indicating an efficient silencing of those genes. Although the different expression of the targeted genes between control cells and those transfected with the correspondent GapmeR were rarely > 65 %, this reduction in gene expression is enough to produce substantial changes in cellular behavior, protein production, and overall phenotype. For instance, in therapeutic contexts, even the partial knockdown of a gene involved in a disease can result in meaningful clinical improvements (36,37).

Once the efficiency of GapmeRs in silencing gene expression was evaluated, we needed to perform an essay to make sure that the inhibition of target genes effectively reduced NF-kB signaling. IL-6 serves as a marker of pro-inflammatory activity, showing clear responses to pathway activation levels (27). *Ikk*α, *Ikk*β, *Nemo* and *Nik* targeting showed a statistically significant reduction of *Il-6* expression upon activation with LPS 1 µg/mL for 18 hours vs control (Fig. 2A), meaning that NF-kB is effectively inhibited even in the presence of an inducer. This underscores the fact that a small percentage of gene silencing could have substantial effects on cell phenotype, particularly in the case of *Nik* silencing where a significant decrease of *Il-6* expression is achieved. Interestingly, we found that cells where *Ikk*β had been silenced showed a significant increase in *Il-6* expression without LPS. However, expression levels of *NFKBIA*—another direct target of the NF-kB pathway—did follow the expected expression pattern and were downregulated in *Ikk*β-silenced cells. The differences in the effects of *Ikk*β silencing on *Il-6* and *NFkBIA* (IκBα) expression highlight the complex nature of NF-kB signaling regulation. When *Ikk*β is silenced, the canonical NF-kB pathway is inhibited, as evidenced by the decreased expression of NFKBIA. However, the increased *Il-6* expression could be due to compensatory mechanisms such as the activation of the MAPK/ERK or JNK pathways, which can independently regulate IL-6 production (38). Moreover, the NF-κB pathway involves intricate feedback loops, and silencing *IKK*β might disrupt these loops, leading to an unexpected upregulation of *Il-6* as a compensatory response (39).

NF-kB pathway is not only involved in inflammation but in other cell functions such as cell proliferation. Therefore, we needed to make sure that the changes introduced in the MSCs did not affect basic cell functions. Although the results of our MTT assays showed a significant difference in *Ikk*β-silenced cells at the midpoint. Of note, this difference disappears at the endpoint when its proliferation matches the control. This suggests that this difference found at midpoint is not due to a true increase in their proliferation capacity. No other significant differences were found. Therefore, these results suggest that the silencing target genes does not affect the cells proliferative capabilities.

On the other hand, reducing NF-kB pathway activity by silencing target genes could reduce CXCR4 expres-

sion affecting the MSCs behavior in the bone marrow. However, the results of our transwell migration assay eliminated this possibility in our system since no statistically significant differences were found between the different conditions. These results suggest that NF-kB silencing is not affecting the ability of the transfected cells to respond to chemotactic agents. In fact, MSC chemotaxis could be regulated by other signaling pathways that are not affected by the silencing of NF-kB components. For instance, chemokine receptors such as CXCR4 are also regulated by the PI3K/Akt and MAPK/ERK signalling pathways, which might maintain chemotactic responses independently of NF-kB (40).

To be able to use a pro-regenerative approach involving silencing of any of the targeted genes *in vivo*, we also needed to guarantee the safety of this change and check that silencing of the targeted genes was not touching the cells viability. Therefore, we conducted a flow cytometry analysis with AnnV and 7-AAD. Our results show no significant differences at this level between control and the silenced cells. However, *Nemo*-silenced cells seem to have more apoptotic cells, and *Ikk*α and *Ikk*β-silenced cells seem to have more percentage of necrotic cells. However, none of these differences were significant confirming that silencing targeted genes does not affect the cells viability.

Our study demonstrates that targeted silencing of key genes in the NF-kB pathway could be used as an approach to enhance the osteogenic potential of MSCs without compromising their viability, proliferative capabilities, or chemotactic behavior. The significant reduction in *Il-6* expression upon silencing key components of the pathway underscores the critical role of the NF-kB pathway in modulating inflammatory responses within the BM microenvironment. Moreover, the transient nature of gene silencing via GapmeRs ensures the safety and reversibility of changes, making this approach viable for potential clinical applications.

The combination of silencing genes from the NF-kB signalling pathway with the previously proven silencing of *Sfrp1* in endogenous MSCs (12,14) could synergistically enhance the pro-osteogenic properties of osteoporotic MSCs. Our previous research has shown that silencing Sfrp1—an inhibitor of the Wnt/β-catenin signalling pathway—leads to a 30 % increase in osteogenic potential (14). Therefore, combining the silencing of Sfrp1 with key genes from the NF-kB signalling pathway may further amplify the regenerative effects, offering a robust strategy to fight osteoporosis and other bone-related conditions. Future research should focus on *in vivo* studies to validate these findings and explore the long-term effects of combined pathway modulation on bone homeostasis and regeneration. Additionally, investigating the interplay between NF-kB and other signalling pathways involved in MSC differentiation and function could provide deeper insights into optimizing regenerative strategies.

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Revista de Osteoporosis y Metabolismo Mineral

Artículo Especial

The most interesting papers on vitamin D published in 2023

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Abstract

We have chosen the most important articles related to vitamin D published in 2023. We chose manuscripts with clinical importance on the non-classical actions of this hormone, and its use in the prevention or treatment of non-bone diseases. We will analyze the genetics and the presence of an onco-suppressor gene to explain why supplementation with vitamin D can be effective in some subjects and not in others, the lower appearance of autoimmune diseases in a randomized controlled study, the use of vitamin D in women with diabetes gestation, the possible programming of stems cells in embryos and the risk of type 2 diabetes mellitus, the relationship with Parkinson's disease, the increase in levels with supplementation related to basal body weight, and the relationship with uterine fibroids (leiomyomas or fibroids).

Keywords:
Vitamin D.
Colecalciferol.
25OH Vit D.

This is a highly subjective selection of papers that caught my attention published from January 1st 2023 through January 31st, 2024.

Although, for years, it has been known why vitamin D deficiency is clearly associated with the presence of non-bone diseases—also in longitudinal studies—and the appearance of these conditions, the first studies ever reported on supplementation vs placebo did not prove beneficial in these conditions (cancer, diabetes, hypertension, cardiovascular disease, etc.). It quickly became clear to me that many publications were being conducted with low doses, using short regimens, or with "sufficient" populations of 25OHD.

After delving into these articles, interesting topics emerged:

1. Some beneficial effects of supplementation occur "in many" but not "in all" patients due to genetics. Studies have been published (in my opinion with very little impact) showing benefits in subgroups of people with some genetic polymorphisms and not in others. The first paper was published years ago by Baron in England, United Kingdom (1). He published that in subjects with colonic polyps, supplementation with vitamin D did not prevent the appearance of new polyps. However, the next year, when they genetically studied that population, they found that polyps had been prevented in the subgroup that

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had the rs7968585 polymorphism among individuals with the AA genotype (26 %) in the vitamin D receptor (VDR) gene. It is in this subgroup that the supplementation of vitamin D was able to reduce the appearance of new polyps by 64 % (2).
The first new study on this subject is of that same style. It has a first "negative" part and a second, new "positive" part. This is the AMATERASU study conducted by Urashima et al. (3), which, in 2019, showed that administering 2000 IU/d for 3.5 years vs placebo did not prevent recurrences or improve survival in Japanese subjects with digestive cancers. Now, it is postulated that the key to achieving a certain "extraosseous" benefits is not the dose administered but to what extent 25OHD levels were reached. In the subgroup with < 20 ng/mL at baseline, a mean of 36 ng/mL was reached. In the subgroup with between 20 and 40 ng/mL at baseline, they reached a mean of 45 ng/mL. In the analysis of subgroup study, lower recurrence and higher survival were demonstrated... but in which subgroup? In those who had very little vitamin D (< 20 ng/mL) or in those who had enough for the IOM criterion (> 20 ng/mL)? The answer is that it was prevented in the subgroup that had more 25OHD (HR, 0.44), which are those that reached a mean value > 40 ng/mL. The authors highlighted this finding with caution. At that time, it was not postulated that perhaps 40 mg/mL could be a cut-off value to achieve to prevent or alleviate some non-classic conditions. In this study, polymorphisms of the VDR receptor and the transporter protein were analyzed, and no relationship with benefit was ever found. The new study by this group—whose lead author is now Kanno (4)—studied how many of these subjects were "immunoreactive to the onco-suppressor protein P53", defining them by having serum antibodies against P53 (+) or an accumulation of that protein in more than 99 % of cancer cells. The hypothesis was that supplementing with vitamin D can increase the anti-cancer immunoreaction only in a subgroup, and results proved it: in the P53 (+) subgroup, relapse or death occurred in 9/56 subjects (16.7 %) vs 14/26 with placebo (53.8 %), while no differences were reported in the P53 (-). In my opinion this manuscript did not achieve the impact it deserves. It is clear that the answer to 25OHD in some non-classical diseases—cancer in this case—may depend on genetics or the presence of certain onco-suppressive proteins.

2. Another re-analysis: supplementing with vitamin D prevented the appearance of autoimmune diseases in the vital study. In 2021, Hanh published that in the 5.3-year follow-up the appearance of an autoimmune disease was confirmed in fewer supplemented patients vs the placebo group (HR, 0.61; 0.43-0.86), concluding that it reduced them by 25 % to 30 %—especially rheumatoid arthritis—and after 2 years of supplementation (5).
What is new about this topic?: Well, 2 years later (at the end of the 7th year), Costenbader published that prevention was no longer significant (patients no longer had supplementation) yet the condition appeared: psoriasis was prevented (HR, 0.61; 0.38-0.98). The other finding is that categorizing them by their BMI, those whose BMI was < 25 kg/m^2 maintained prevention (HR, 0.75; 0.59-0.95). Of note, the main limitation of this study, which did not prevent any positive results: participants were allowed the voluntary use of vitamin supplements (6).

3. Vitamin D supplementation improves outcomes in women with gestational diabetes. A meta-analysis (7) that analyzed 20 RCTs concluded that those supplemented increased their HDL-C levels, decreased their LDL-C and triglyceride levels, and reduced the risk of premature birth, hyperbilirubinemia and neonatal hospitalization. Of note what doses these women (mostly from Iran and China) received. Another topic is the prevention of gestational diabetes, which we have analyzed with other "outcomes" in a review (8).

4. A work as fascinating as it is complex is "Embryonic vitamin D deficiency programs hematopoietic stem cells to induce type 2 diabetes mellitus". The authors study in mice the epigenetic mechanisms that influence the risk of intrauterine diabetes associated with vitamin D deficiency. Studies in rodents confirm the relationship among intrauterine vitamin D deficiency, inflammation, hepatic steatosis, excess adiposity and insulin resistance (IR), suggesting it induces epigenetic programming. It has been suggested that the VDR receptor plays a role in programming the immune system during embryogenesis. In these studies, vitamin D-deficient fetal hematopoietic stem cells (HSCs) were transplanted into the uterus of vitamin D-sufficient mice inducing diabetes. These studies show that the deficit epigenetically suppresses Jarid2 expression and activates the Mef2/PGC1 step in HSCs, which results in adipose infiltration into macrophages, which secrete miR106-5p, which in turn promotes IR. Vitamin D-deficient monocytes in the human cord have, these studies state, the above-mentioned changes, causing IR. The authors conclude that this vitamin D deficiency during development has enough epigenetic consequences on the immune system to trigger IR (9).

5. Vitamin D and Parkinson's disease (PD). Two meta-analyses from 2017 and 2018 confirmed the association between 25OHD deficiency and PD risk and severity (10,11). What is new is the appearance of a meta-analysis on supplementation. It only includes 5 articles (4 on PD and 1 on restless legs syndrome). Some studies showed improvement, especially 1 from Poland where 4000 IU/d were ad-

ministered only for 3 months to patients with BMI < 25; 5000 IU/d with BMI = 25-30 and 6000 IU/d with BMI > 30. Another study with a 4-month regimen of 10,000 IU/d only seemed to improve balance in young participants, while the remaining studies which administered 1000 IU/d and 1200 IU/d found modest improvements. The authors conclude that screening for vitamin D deficiency and supplementation might be necessary in patients with PD (12).

6. A Vital sub-study showed that the increase in 25OHD observed with 2000 IU/d supplementation was lower in obese and overweight subjects. What was the extent of this 25OHD increase based on their respective BMI?

BMI	*n*	< 18.5	18.5-24.9	25.0-29.9	30.0-34.9	> 35.0	
25OHD ng/mL	16.375	32.6	32.4	30.6	29.0	28.0	$p < 0.001$

This may partly explain higher or lower responses to health objectives that were found in other megatrials (13).

7. Vitamin D and uterine fibroids, more commonly called leiomyomas, or fibromas. This year (14) Okoro that 75 women with uterine leiomyoma had 25OHD levels of 15.26 ± 4.96 ng/mL while the 75 controls had 25OHD levels of 22.45 ± 6.93 ng/mL ($p < 0.001$). Additionally, there was a negative correlation between fibroid volume and 25OH Vit D ($p < 0.001$). In 2013 reports came on the negative correlation between 25OHD and fibroid weight (15) and, in 1997, on the fact that black women had less vitamin D and greater fibroid masses (16).
In 2023 Combs conducted an interesting meta-analysis and concluded that 14 studies confirmed the correlation between 25OHD and the presence of fibroids. A different question is if vitamin D supplementation could reduce the size of leiomyomas? Four out of 5 studies found that compared to placebo, leiomyomas did not grow or shrink. Of note, 2 studies were conducted for as long as 2 months, another 2 for as long as 3 months, and 1 study for as long as 1 year. All participants in all studies were supplemented with 50,000 IU/week, except for the 1-year study in which participants received 50,000 IU/month for 2 months, followed by 2000 IU/day (17).
Finally, a Chinese study conducted after this meta-analysis compared 25 women with fibroids supplemented with a 3-month regimen of 1600 IU/day vs placebo and found a decrease in size with vitamin D. What is striking is that this increase was not very significant (from 10.45 up to 17.14 ng/mL), which are values that stand far away from for the bone health target of 30 ng/mL and even further from the targets suggested for non-classical actions (40 ng/mL) (18).

In conclusion, these are arbitrarily selected studies that, nonetheless, allow us to keep in mind the possibility that vitamin D supplementation can prevent or alleviate multiple non-bone conditions.

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