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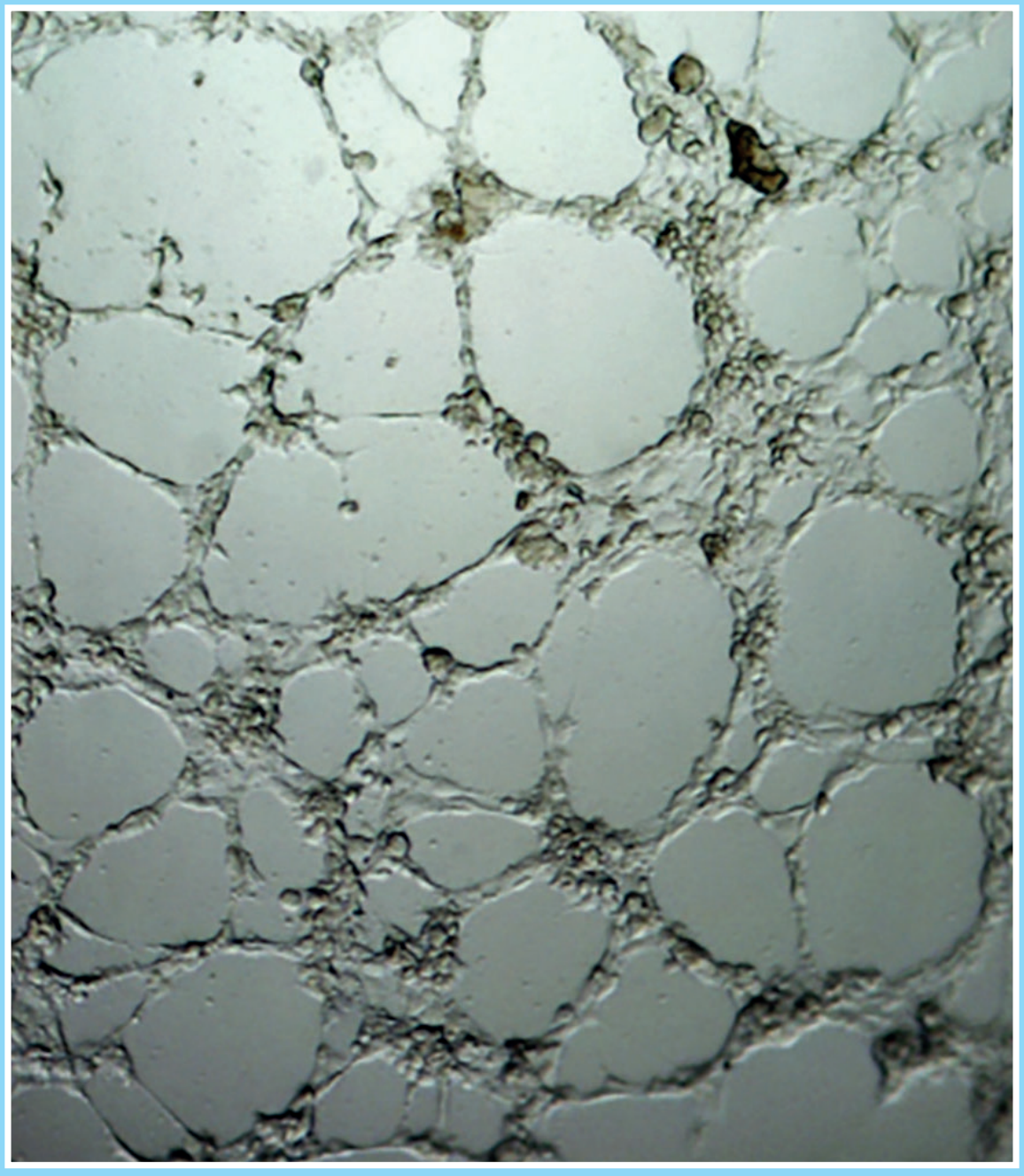

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Oliva-Lozano S, Pulido-Escribano V, Torrecillas-Baena B, Calañas-Continente A, Priego-Capote F, Camacho-Cardenosa M, Casado-Díaz A. Circulating extracellular vesicles affect mesenchymal stromal cell differentiation and angiogenesis. Potential use in bone regeneration. Rev Osteoporos Metab Miner 2025;17(1):19-30.

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Original

Musculoskeletal disorders and bisphosphonates: a disproportionality analysis within the Spanish pharmacovigilance database

M.ª Teresa Yuste, Elisa Escudero, Pedro Marín

Department of Pharmacology. Universidad de Murcia. Murcia, Spain

Abstract

Background: several musculoskeletal adverse effects associated with the use of bisphosphonates have been identified, although their frequency, severity and risk factors are still unknown. The aim of our study is to determine the possible causal relationship between the most widely used bisphosphonates in Spain and the occurrence of musculoskeletal adverse events.

Material and methods: we conducted a retrospective, observational, analytical, case/non-case study using the database of the Spanish Pharmacovigilance System. The bisphosphonates selected were alendronic acid, ibandronic acid and risedronic acid. The adverse reactions studied according to MedDRA terminology were SOC musculoskeletal and connective tissue disorders and PTs myalgia, arthralgia, bone pain, paresthesia, musculoskeletal pain, musculoskeletal stiffness, arthritis, muscle weakness and pain in an extremity.

Results: the ROR values obtained for the SOC were > 1 for all 3 drugs studied. These reactions occur mostly in those over 65 years of age, women and that most of them are classified as serious. For the 9 PTs studied (myalgia, arthralgia, bone pain, paresthesia, musculoskeletal pain, musculoskeletal stiffness, arthritis, muscle weakness and pain in a limb), ROR values > 1 were found for all three drugs, except for the PT paresthesia and PT pain in a limb.

Conclusion: musculoskeletal adverse reactions not listed in the official information have been detected. The information provided by this work could recommend for a re-evaluation and update of the benefit-risk ratio of these drugs.

Keywords:
Alendronic acid.
Bisphosphonates.
Ibandronic acid.
Musculoskeletal adverse effects.
Pharmacovigilance.
Risedronic acid.

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Disclaimer: FEDRA is the Spanish Pharmacovigilance System of Human Medicines (SEFV-H) database and is managed by the Spanish Medicines and Health Products Agency (AEMPS). The information is derived from various sources, and the likelihood of a suspected adverse effect being related to a drug may vary. The authors' findings, discussion and conclusions are their own and do not reflect the position of the Spanish Pharmacovigilance System or AEMPS.

Conflict of interest: the authors declare no conflict of interest.

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Correspondence:
Pedro Marín. Department of Pharmacology. Universidad de Murcia. Campus de Espinardo. 30100 Murcia, Spain
e-mail: pmarin@um.es

INTRODUCTION

Osteoporosis is a bone disorder that increases a person's risk of fracture due to low bone mineral density, impaired bone microarchitecture/mineralization and/or decreased bone strength. It is a silent disease that progresses without symptoms until it shows as a fracture of the hip, spine, proximal humerus, pelvis and/or wrist, which may lead to hospitalization (1). This disease can be caused by several reasons; the main cause is due to hormone depletion, oestrogen depletion in postmenopausal women and androgen depletion in older men. In particular, due to the imbalance in bone remodelling after menopause, osteoclastic activity predominates over osteoblastic activity (2).

The main objective of a pharmacological therapy, in this case, is to reduce the risk of fracture. Drugs to treat osteoporosis are categorized as either antiresorptive (i.e., bisphosphonates, estrogen agonist/antagonists, estrogens, calcitonin, and denosumab) or anabolic (i.e., teriparatide). Antiresorptive drugs primarily decrease the rate of bone resorption while anabolic drugs increase bone formation more than bone resorption does. Bisphosphonates are anti-osteoclastic agents that suppress osteoclastic formation and help to increase or maintain bone mineral density in the long term (1,3). These drugs can be categorized into 2 groups with different molecular modes of action:

1. *Bisphosphonates that do not contain a nitrogen atom in their structure (non-nitrogenous):* these are the simplest and include etidronate and clodronate, among others. They can be metabolically incorporated into non-hydrolysable ATP analogues, which interfere with intracellular ATP-dependent pathways.
2. *Bisphosphonates that contain a nitrogen atom in their structure (nitrogenous):* these are the most potent drugs and include pamidronate, alendronate, risedronate, ibandronate and zoledronate. Although they are not metabolized in the same way as the non-nitrogen bisphosphonates, they inhibit key enzymes of the mevalonate/cholesterol biosynthetic pathway (4). In osteoclasts they inhibit the enzyme farnesyl pyrophosphate synthase (FDPS) a key branch point enzyme in the mevalonate pathway. As a consequence of osteoclast activity inhibition, recruitment and apoptosis, suppression of bone turnover occurs (5).

Oral bisphosphonates such as alendronate, ibandronate or risedronate have been widely used in the treatment and prevention of osteoporosis for 3 decades. Among oral bisphosphonates, alendronate and risedronate have been demonstrated to reduce the rate of hip fractures by approximately 40 %, and all non-vertebral fractures by 20-30 % (6). Initially, bisphosphonates were administered daily. However, nowadays, dosing regimens are weekly in the case of alendronate and risedronate, or monthly for ibandronate, and more recently for risedronate (7).

The variety of indications and the prolonged duration of most bisphosphonate oral treatments have favored the appearance of different adverse reactions. Among the most common ones are those related to the upper digestive tract: nausea, vomiting, erosions, gastric ulcers, oesophagitis, etc. All bisphosphonates are reported to be associated with a complication denominated osteonecrosis of the jaw, defined as the presence of exposed and necrotic bone in the maxillofacial region that does not heal in 8 or more weeks (8). Moreover, bone, joint and muscle pain may be secondary to bisphosphonates therapy. These last adverse reactions have been described as generally infrequent and mild, although severe pain has been reported (9). The onset of musculoskeletal pain may occur years after treatment initiation and does not always resolve with treatment discontinuation (10). A link between bisphosphonate intake and the development of synovitis, including carpal tunnel syndrome, has also been demonstrated (11).

The aims of our study were: a) to study the possible causal association between taking oral bisphosphonates (alendronic acid, ibandronic acid and risedronic acid) and the development of musculoskeletal adverse reactions; b) to determine the reporting frequencies for the variables age, sex and serious of these reactions; c) to identify the different musculoskeletal reactions associated with each bisphosphonate and their reported risks; and d) to analyze the available official information on these musculoskeletal reactions to bisphosphonates and to compare it with the results obtained in our own study.

MATERIALS AND METHODS

DATA MINING

Data required for our study were obtained from the Spanish Pharmacovigilance System's adverse reaction database, FEDRA. FEDRA contains spontaneous reports of adverse reactions made by health care professionals, the pharmaceutical industry and general population from the start of the programme in 1983 to this day. Adverse reactions are subsequently coded in FEDRA according to the terminology of the Medical Dictionary for Regulatory Activities, MedDRA. Through this tool, preferred terms describing the reactions of interest can be identified for searching. This can be done by system organ class (SOC), high level group term (HLGT), high level term (HLT) and preferred term (PT) (12).

FEDRA searches were conducted for the 3 selected bisphosphonates: alendronic acid, ibandronic acid and

risedronic acid. A general search was first performed to identify spontaneous reports with the SOC musculoskeletal and connective tissue disorders, and after more specific searches were conducted with the following selected PTs: myalgia, arthralgia, bone pain, paresthesia, musculoskeletal pain, musculoskeletal stiffness, arthritis, muscle weakness and limb pain. The study of bone necrosis of the jaw has not been addressed in this study nor the occurrence of atypical fractures.

DATA STATISTICAL ANALYSIS

To achieve the proposed endpoints, we conducted an analytical, retrospective, observational using the case/non-case study approach, which is based on the logic of case-control studies (13). The study selects patients with the disease (cases) and compares their exposure to certain risk factors with that of patients without the disease (non-cases). The risk factors associated with a specific disease are thus identified and analyzed; in this case, with an adverse reaction of interest. If risk factors considered are drugs, as in the present study, the role they play in the occurrence of the reaction can then be explored. The strength of the association between the adverse reaction and the bisphosphonate was estimated by calculating a measure of disproportionality, the reporting odds ratio (ROR) with a 95 % confidence interval (CI) and chi-square test with Yates correction. This ROR is based on a 2-by-2 contingency table (ROR = (a/b)/(c/d) = ad/bc) (Table I).

Thus, a = case-exposed; b = non-case-exposed; c = case-non-exposed; and d = non-case-nonexposed. If the ROR value is = 1, there would be no association between the drug and the disease, as the exposure ratio in exposed and unexposed cases would be equal. If the ROR is > 1, then there would be an association; the higher the ROR, the greater the association. If the ROR is < 1, the drug would have a protective effect vs the disease under study (14).

RESULTS

As of 31 October 2023, out of a total of 475,235 reports in the FEDRA database, a total of 371 notifications were identified for alendronic acid in relation to SOC musculoskeletal and connective tissue disorders, accounting for 32.52 % of the notifications for this drug in FEDRA, 248 for ibandronic acid—50 % overall—and 206 for risedronic acid—33.77 % of all notifications (Table II).

The study on disproportionality indicates that the ROR values for this SOC were > 1 for the 3 drugs in the pipeline. Specifically, for alendronic acid, the ROR was 4.2 (3.7-4.8), for ibandronic acid, the ROR was 8.7 (7.3-10.4), and for risedronic acid, the ROR was 4.4 (3.8-5.3) (Table III).

The analysis of the reports revealed that, for alendronic and ibandronic acid, these reactions mostly occur in patients older than 65 years, and most reports are categorized as serious. For all 3 drugs studied, these reactions occur much more frequently in women (Table II).

In the disproportionality analysis for the 9 studied PTs (myalgia, arthralgia, bone pain, paresthesia, musculoskeletal pain, musculoskeletal stiffness, arthritis, muscle weakness and pain in a limb) across the 3 selected drugs, ROR values > 1 were found for all PTs except for paresthesia with alendronic and risedronic acids, and pain in a limb with alendronic and ibandronic acids (Table IV).

The ROR values for bone pain PT were particularly significant. Alendronic acid had a ROR of 32.4 (24.4-43.0), ibandronic acid had a ROR of 13.9 (7.7-25.6), and risedronic acid had a ROR of 35.1 (24.4-50.5). Additionally, for musculoskeletal stiffness PT, ibandronic acid had a ROR of 15.6 (7.7-31.4), and for arthritis PT, risedronic acid had a ROR of 14.7 (8.4-25.5) (Table III).

Table V compares information on several bisphosphonates marketed in Spain, selected in this study, with the information contained in their package leaflets and technical specifications. Arthritis is not mentioned as such in any of the 3 products; for alendronic acid, a term that could be considered as a synonym, "joint swelling", is mentioned. "Pain in a limb" is not mentioned, nor is "muscle weakness". "Musculoskeletal stiffness", a characteristic and distinct reaction, is mentioned only in the label and package leaflet for ibandronic acid.

Table I. 2 x 2 contingency table

Drug of interest	Adverse reaction of interest	
	Cases	Non-cases
Exposed	a	b
Non-exposed	c	d

a = case-exposed; b = non-case-exposed; c = case-non-exposed; and d = non-case-non-exposed. ROR = (a/b)/(c/d) = ad/bc

Table II. Characteristics of the reported cases of SOC musculoskeletal and connective-tissue disorders in the bisphosphonates studied summited to FEDRA until October 31st, 2023

	Alendronic acid	Ibandronic acid	Risedronic acid
Total reports in FEDRA	1141	496	610
Reports of SOC musculoskeletal and connective-tissue (% of total)	371 (32.52 %)	248 (50 %)	206 (33.77 %)
Age			
Child	0 (0 %)	1 (1 %)	0 (0 %)
Teen	0 (0 %)	0 (0 %)	1 (1 %)
Adult	143 (38 %)	105 (42 %)	106 (51 %)
> 65 years	184 (50 %)	121 (49 %)	88 (43 %)
Unknown	44 (12 %)	21 (8 %)	11 (5 %)
Sex			
Female	345 (93 %)	230 (93 %)	193 (94 %)
Male	20 (5 %)	12 (5 %)	11 (5 %)
Unknown	6 (2 %)	6 (2 %)	2 (1 %)
Serious			
Yes	215 (58 %)	144 (58 %)	69 (33 %)
No	156 (42 %)	104 (42 %)	137 (67 %)

Table III. Disproportionality analysis for bisphosphonates and SOC musculoskeletal and connective-tissue disorders

	Alendronic acid			Ibandronic acid			Risedronic acid		
	n	ROR (95 %CI)	chi-square test	*n*	ROR (95 %CI)	chi-square test	*n*	ROR (95 %CI)	chi-square test
SOC musculoskeletal and connective-tissue disorders	371	4.2 (3.7-4.8)	604.9	248	8.7 (7.3-10.4)	838.8	206	4.4 (3.8-5.3)	359.5

n: number of cases.

Table IV. Disproportionality analysis for bisphosphonates and preferred terms of musculoskeletal adverse reactions selected

	Alendronic acid			Ibandronic acid			Risedronic acid		
	n	ROR (95 %CI)	chi-square test	*n*	ROR (95 %CI)	chi-square test	*n*	ROR (95 %CI)	chi-square test
Myalgia	59	1.1 (0.8-1.4)	0.1	63	2.8 (2.2-3.7)	63.6	57	2.0 (1.5-2.6)	25.2
Arthralgia	65	3.2 (2.5-4.1)	89.5	47	5.5 (4.1-7.5)	152.8	61	5.9 (4.5-7.7)	216.2
Bone pain	54	32.4 (24.4-43.0)	1413	11	13.9 (7.7-25.6)	115.7	32	35.1 (24.4-50.5)	933.1
Paresthesia	14	0.9 (0.6-1.7)	0	11	1.8 (1.0-3.3)	3.2	7	0.9 (0.4-1.9)	0
Musculoskeletal pain	9	2.0 (1.0-3.9)	3.5	10	5.2 (2.8-9.7)	28.9	14	5.9 (3.5-10.1)	50.9

(Continues on next page)

Table IV (cont.). Disproportionality analysis for bisphosphonates and preferred terms of musculoskeletal adverse reactions selected

	Alendronic acid			Ibandronic acid			Risedronic acid		
	n	ROR (95 %CI)	chi-square test	*n*	ROR (95 %CI)	chi-square test	*n*	ROR (95 %CI)	chi-square test
Musculoskeletal stiffness	2	1.6 (0.4-6.6)	0.1	8	15.6 (7.7-31.4)	91.8	3	4.6 (1.5-14.5)	5.3
Arthritis	7	4.1 (1.9-8.7)	13.3	5	6.8 (2.8-16.4)	18.9	13	14.7 (8.4-25.5)	146.1
Muscular weakness	8	1.8 (0.9-3.7)	2.2	4	2.1 (0.8-5.7)	1.4	4	1.7 (0.6-4.6)	0.6
Pain in a limb	8	0.8 (0.4-1.7)	0.1	4	0.9 (0.4-2.6)	0	7	1.4 (0.7-2.9)	0.4

Tabla V. Comparison between the information obtained in this study on 3 bisphosphonates commercially available in Spain and the information included in their technical specifications and leaflets

Reaction	Technical specifications			Leaflet		
	Alendronate	Ibandronate	Risedronate	Alendronate	Ibandronate	Risedronate
Arthritis	X[a]			X[a]		
Arthralgia	X	X		X		
Pain in a limb						
Musculoskeletal pain	X	X	X	X		X
Bone pain	X			X		X
Myalgia	X	X		X		
Paresthesia						
Muscle weakness						
Musculoskeletal stiffness		X[b]			X[b]	

[a]No arthritis as such; instead "joint swelling" is reported. [b]Also known as "muscle cramps".

DISCUSSION

Musculoskeletal reactions, in particular muscle, bone and joint pain, are mentioned as a possibility in the European Medicines Agency (EMA) data for products marketed in Europe; of note, the instances of "severity" or "disability" were rare (15). The FDA reporting mentions 'serious and disabling reactions have been reported' when taking a different approach that excludes rarity. However, the reporting also notes that a similar proportion of musculoskeletal reactions were found in both the alendronic acid and placebo comparison groups during clinical trials (16).

Out of a total of 475,235 reports in the FEDRA database at the time of the study, 49,110 (10.33 %) were identified as SOC reports of musculoskeletal and connective tissue reactions. For alendronic acid, 371 out of 1,141 reports (32.52 %) reported musculoskeletal reactions. Of these, 215 (57.95 %) were considered serious. Out of a total of 496 reports in FEDRA for ibandronic acid, 248 (50 %) had the reaction of interest. Furthermore, more than half of these reactions were considered serious (58.07 %; *n* = 144). For risedronic acid, there was a total of 610 reports, of which 206 (33.77 %) were musculoskeletal reactions. However, a smaller percentage of these reactions were considered serious (33.50 %, *n* = 69). It is important to understand that severity is determined by pharmacovigilance center technicians based on established criteria. Therefore, a life-threatening reaction is typically classified as serious. The disproportionality estimation in FEDRA produced the following ROR values: ROR = 4.2 (3.7-4.8) for all musculoskeletal reactions related to alendronic acid, ROR = 8.7 (7.3-10.4) for ibandronic acid, and ROR = 4.4 (3.8-5.3) for risedronic acid. These results suggest a strong association, but it is important to

consider possible biases. While some musculoskeletal reactions studied may occur in the context of osteoporosis, which is the main indication for bisphosphonates, the fact that they have been reported as suspicious supports a potential causal relationship. The study of disproportionality in the selected PTs found statistically significant ROR values, except for paresthesia and pain in one limb. ROR values are considered statistically significant if they are > 1 and their confidence interval does not contain 1. For the remaining PTs that meet these assumptions, the RORs ranged from 2.0 (1.5-2.6) for the PT myalgia with risedronic acid up to 35.1 (24.4-50.5) for the PT bone pain with risedronic acid.

The ROR values for PT bone pain were significant: alendronic acid had a ROR of 32.4 (24.4-43.0), ibandronic acid had a ROR of 13.9 (7.7-25.6), and risedronic acid had a ROR of 35.1 (24.4-50.5). These high values suggest that the original site of injury is the bone, where bisphosphonates are deposited. Other reactions may be referred reactions depending on the affected bone site. Bone pain is considered to be less common in clinical settings than muscle or joint pain. It is typically described as penetrating, deep, and dull. The patient experiences a pain that is located in the bones and recorded by the physician. This is not a diagnosis based on the patient's symptoms, but rather a felt reaction or symptom. It is likely that reactions such as "pain in a limb", which are listed in the MedDRA dictionary as different entities, may, at least in part, also be referred to as bone pain. Possible mechanisms of bone pain include osteitis, which is produced by acute phase reactions to bisphosphonates and mediated by cytokines (17). Other mechanisms may involve pressure changes in the bone marrow, hypoxia in the bone, and mechanical stimulation of nociceptors (18). Additionally, bisphosphonates, like statins, alter the HMG-CoA and mevalonate pathway. There are documented cases of bone pain in the literature where analytical data, such as elevated sedimentation rate and C-reactive protein, indicate inflammation (19).

On the other hand, risedronic acid showed a strong association with PT arthritis, with a ROR of 14.7 (8.4-25.5). The other bisphosphonates studied also showed ROR values indicative of association: alendronic acid with a ROR of 4.1 (1.9-8.7) and ibandronic acid with a ROR of 6.8 (2.8-16.4). Arthritis is an inflammation of the joints with an immunogenic basis. Drugs could act as haptens and contribute to the development of this type of reaction. The obtained high ROR value suggests a strong association, but it is important to rule out possible reporting biases. Arthritis may occur more frequently in patients with osteoporosis, who are eligible for bisphosphonate therapy, leading to a spurious association between the druf and the reaction. Osteoporosis can be associated with other conditions, including certain joint diseases. However, this does not fully account for all reported cases of suspected joint problems. There is evidence to suggest that reactions such as arthritis may be underreported in association with drug use. Literature contains numerous well-documented cases of arthritis associated with the use of various bisphosphonates, some of which also resulted in positive re-exposure (10). Therefore, the results of the clinical evaluation applied to the presented case series, along with the association data from the disproportionality analysis and literature reports, serve as argumentative sources for establishing causality in the specific combinations of bisphosphonates and musculoskeletal reactions in the absence of specific studies. Finally, the strong association between PT musculoskeletal stiffness and ibandronic acid is noteworthy, with a ROR of 15.5 (7.7-31.4). The technical specifications of the drug reflect this association, but it is not reflected in the technical specifications of risedronic acid, which has this adverse reaction with a ROR of 4.6 (1.5-14.5).

LIMITATIONS

One of the main limitations of this study is underreporting, which refers to the reporting of a small number of suspected adverse reactions relative to the actual number of occurrences (20). Underreporting can impact systems that rely on spontaneous reporting. This issue may arise due to the challenge of linking certain medical conditions with specific drugs. Apart from the difficulty of reporting suspicions, there are various reasons for not reporting. These reasons include the belief that the reaction is already known, laziness, lack of knowledge of the reporting programmes, or fear of being reported. It is important to note that reported information should be objective and free from subjective evaluations. The true rate of musculoskeletal adverse reactions associated with bisphosphonates in the population, as well as any adverse reactions in general, is difficult to determine due to underreporting and lack of information on the actual number of patients treated with these drugs. The information generated through spontaneous reporting only provides a partial view of the situation. Although underreporting does not allow for an accurate estimation of the quantitative magnitude of the problem, it does provide insight into the type of disease produced, its severity, and the clinical and public health repercussions. Additionally, it allows for the identification of possible causal associations, which is particularly relevant in the context of pharmacovigilance. Of note, most regulatory interventions on drug safety have been based on spontaneous reporting data (21). Therefore, these data remain valid.

Bisphosphonates are prescribed based on the presence of osteoporosis, a disease whose symptoms may be considered a confounding factor when evaluating the causal relationship between these drugs and the adverse musculoskeletal reactions studied. Osteoporosis is associated with other rheumatic diseases. Based on this confounding factor, the drug would be prescribed to patients who already have musculoskeletal symptoms, which would later be causally associated with the same symptoms. In other words, the prescription of the drug would be linked to the musculoskeletal symptoms that would later be attributed to the drug. Although associations have

been described, it is difficult to conclude that they are always causal, especially without data on time sequence, withdrawal effects, or response to re-exposure. Clinical data supporting a causal reaction would be valuable.

CONCLUSIONS

Bisphosphonates may cause musculoskeletal adverse reactions that are not listed in the product information for bisphosphonate-containing products. Established reactions such as musculoskeletal stiffness, muscle weakness or arthritis, which are named as such, are not included in the information for use contained in the technical specifications and leaflets. The mandatory information on bisphosphonates in these documents needs updating to include known data on musculoskeletal reactions in a clear and consistent manner.

Bisphosphonates can cause a range of musculoskeletal adverse reactions, being arthritis and arthralgia being the most common ones. A significant proportion of reported musculoskeletal reactions are considered serious. As older individuals tend to have longer exposure to bisphosphonates, any adverse reactions would likely be more prevalent in this age group. Among the most frequently occurring musculoskeletal adverse reactions, bone pain is the reaction that is most strongly associated with bisphosphonates.

The benefit-risk ratio of bisphosphonates should be re-evaluated following new data on their long-term safety and efficacy profile. This work, along with literature reports, provides safety information on bisphosphonates that calls for an update of their benefit-risk ratio. Results obtained support the inclusion of new data in the information on these products. The regulatory authorities—Spanish and European—are responsible for including any new safety information in product information, where appropriate. Health care professionals should establish their own risk-benefit ratio based on new safety knowledge.

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Original

Identification *in silico* of miRNAs and their targets involved in the development of osteoarthritis

Sergio Rafael Carrillo-Patiño[1,2], Fiordaliso Carolina Román-Carraro[3], Juan Fernando Montes-García[2,4], Martha Alicia Ballinas-Verdugo[5], José Eduardo Justo-Frausto[2], Emma López-Espinosa[6], Rogelio Frank Jiménez-Ortega[1,6]

[1]Laboratorio de Genómica del Metabolismo Óseo. Instituto Nacional de Medicina Genómica (INMEGEN). Mexico City. Mexico. [2]Unidad Académica de Ciencias de la Salud. Universidad ETAC campus Coacalco. Coacalco de Berrizobal, Mexico State. Mexico. [3]Departamento de Procesos y Tecnología. Universidad Autónoma Metropolitana. Unidad Cuajimalpa. Mexico City. Mexico. [4]Laboratorio de Genética. Unidad de Morfología y Función. Facultad de Estudios Superiores Iztacala UNAM. Tlalnepantla de Baz, Mexico State. Mexico. [5]Departamento de Inmunología. Instituto Nacional de Cardiología Ignacio Chávez. Mexico City. Mexico. [6]Unidad de Acupuntura Humana Rehabilitatoria. Universidad Estatal del Valle de Ecatepec (UNEVE). Ecatepec de Morelos, Mexico State. Mexico

Abstract

Introduction: osteoarthritis is considered the main cause of joint pain in older people, affecting four core tissues: cartilage, bone, joint capsule, and joint apparatus. In recent years, microRNAs have been described to play a vital role in the development of bone metabolism diseases, including osteoarthritis, since they can have an inhibitory effect or a promoting effect on disease progression.

Objective: through microarray analysis and bioinformatics tools, miRNAs and their potential target genes involved in signaling pathways associated with the development of osteoarthritis are identified.

Methods: the microRNAs were selected through microarray expression analysis from the "Gene Expression Omnibus" database, and through literature search, their target genes were obtained by integrating different databases. This set of genes was compared with a set of differentially expressed genes from expression microarray analysis of samples from patients with osteoarthritis. The shared gene set was subjected to signaling pathway enrichment analysis.

Results: a total of 4 miRNAs were identified, miR-485, miR-940, miR-107, and miR-142-5p, that regulate 185 genes involved in 9 signaling pathways in which *CSF1, CXCL3, FOS, IL6, IL6R, NFATC1, NFKB1, NFKB2, PPARG, THBS1* and *TNF* genes play a crucial role in bone and immune system-associated processes and their deregulation may favor the progression of osteoarthritis.

Conclusions: the microRNAs identified in this study could be used as biomarkers for the timely diagnosis and monitoring of osteoarthritis treatment.

Keywords:
Osteoarthritis. Bone metabolism. MicroRNAs. Bone mineral density. Bioinformatics. Microarrays.

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Correspondence:
Rogelio Frank Jiménez-Ortega. Laboratorio de Genómica del Metabolismo Óseo. Instituto Nacional de Medicina Genómica (INMEGEN). Ciudad de México, México
e-mail: rogeliofrank.jimenez@uneve.edu.mx

INTRODUCTION

Osteoarthritis (OA) is the most prevalent chronic joint disease worldwide. It affects all joint tissues, causes complete joint dysfunction, and causes progressive loss of articular cartilage, which generates damage to other joint structures, such as the subchondral bone and the membrane synovium, leading to chronic disability and decreased quality of life (1). Changes in cartilage can be age-induced. However, cartilage degeneration can occur in response to inappropriate mechanical stress and low-grade systemic inflammation associated with trauma, obesity, and genetic predisposition, which subserve the risk of development and progression of OA (2).

The complex interactions among cartilage, synovium, and subchondral bone significantly impact cartilage function, making it challenging to pinpoint the onset and location of pathological changes. Consequently, it has been suggested that biological factors may trigger temporal and spatial alterations in chondrocytes and cellular components of cartilage, that potentially leading to a pathological state (3). Chondrocytes are derived from mesenchymal progenitors and its function is to synthesize the extracellular matrix and form anlagen cartilage for bone development (4). Chondrogenesis occurs due to the condensation of mesenchymal cells expressing collagens I, III, and V and the differentiation of chondroprogenitor cells with expression of cartilage-specific collagens II, IX, and XI. During limb development, resting chondrocytes can form cartilage at the ends of opposing bones with intermediate interzones formed during cavitation, increase, and then proceed to terminal differentiation towards hypertrophy and apoptosis to allow endochondral ossification so the calcified hypertrophic cartilage is resorbed and replaced by bone (5). Proliferating chondrocytes are under the control of the parathyroid hormone/Indian hedgehog (PTHrP/Ihh) axis and express collagen VI and matrilin 1 (MATN1). The hypertrophic zone is characterized by collagen of vascular endothelial growth (VEGF) and VEGF receptors whose interaction allows non-vascularized and hypoxic tissue to be converted into bone through the activity of osteoclasts (bone-retaining cells) and osteoblasts (bone-forming cells). A similar sequence of events occurs in the postnatal growth plate, leading to rapid skeletal growth (6). These processes depend on a complex regulation through the interaction of transforming growth factor β (TGF-β), bone morphogenic protein (BMP), and the WNT signaling pathway. Therefore, changes to these signaling pathways could lead to the development of OA (7). Recent studies have shown that microRNAs (miRNAs) play an essential role in the appearance and development of different diseases: multiple types of cancer, cardiovascular, metabolic, immune, kidney and bone metabolism diseases (8).

miRNAs are a class of endogenous, small (19-25 nt), non-coding RNAs that negatively regulate gene expression and basic physiological processes such as cell differentiation, growth, proliferation, metabolism, and apoptosis. The miRNA-mediated target gene regulation process begins with the recognition of the pre-miRNA duplex chain through the DICER protein, which is an RNAse III responsible for the elimination of the terminal loop of the pre-miRNA, which together with the argonaute protein (AGO) are part of the RNA-induced silencing complex (RISC). The chains derived from the mature duplex miRNA are loaded into AGO in humans and are ATP-dependent. Overall, the strand with the lowest stability in the 5' position or 5' uracil is preferably loaded into AGO and will be considered as the guide strand. The selection of this chain depends on the union of the first 6-8 nucleotides with the 3'UTR region of the target mRNA (seed region) and the type of AGO protein that is present in the RISC. It has been shown that miRNAs bind to specific sequences, and the base complementarity between the miRNA and its target gene determines the fate of the mRNA. The interaction between the miRNA seed region (2-8 nt) and the 3'UTR of the mRNA is of great importance since perfect complementarity allows the AGO2 protein with exonuclease function to cleave the mRNA at RNA processing proteins, which associate with AGO and function as mRNA storage sites (P bodies). On the other hand, when the binding of the miRNA to the seed region of the mRNA is not perfect, a hairpin is formed between the miRNA and its target gene between the 9th and 10th nucleotides of the miRNA, inducing translation suppression (9). To date, few studies have investigated circulating miRNAs in OA, and findings lack consistency, with the diagnostic value of these miRNAs yet to be elucidated. Therefore, this work aims to identify miRNAs and their potential target genes involved in signaling pathways whose deregulation can lead to the development of OA, through search of existing literature and bioinformatics tools.

MATERIAL AND METHODS

SELECTION OF miRNAs

To select a set of miRNAs involved in the development of OA, microarray files in CEL format were first obtained from studies where changes in miRNA expression profiles in patients with OA were analyzed, which were selected through searching in different databases: PubMed (https://pubmed.ncbi.nlm.nih.gov/) and Gene Expression Omnibus (GEO) (https://www.ncbi.nlm.nih.gov/geo/). Files in ".txt" format were obtained from a study where miRNAs differentially expressed in primary osteoblasts from patients with hip replacement for osteoporosis or OA were identified using the miRCURY LNA microRNA Array, 7th Generation technology (QIAGEN, San Diego, USA) with access No. GSE74209 (10). In a different study using high-throughput seqRNA (DNBSEQ [BGI-Shenzhen, China]), changes in the ex-

pression profiles of ncRNAs from synovial tissue samples of anterior cruciate ligation tears were analyzed, from which the analyzed data of differentially expressed miRNAs were obtained (11). Finally, through a literature search, a set of miRNAs associated with OA was compiled, which are summarized in a review and bioinformatics analysis conducted by Cong et al. 2017 (12). The group of miRNAs selected for this study was selected through a comparative analysis represented in a Venn diagram using the "Bioinformatics & Evolutionary Genomics" tool (https://bioinformatics.psb.ugent.be/webtools/Venn/).

PREDICTION OF POTENTIAL miRNA TARGET GENES

To identify the target genes of selected miRNAs, a search was performed in different databases that use computational algorithms to determine the nucleotide pairing between the 3'UTR region of a target mRNA and the 5' "Seed" region (2-7 nucleotides) of a miRNA. Databases used were miRWalk (http://mirwalk.umm.uni-heidelberg.de/), miRDB (https://mirdb.org/), TargetScan (https://www.targetscan.org/vert_80/), Tools4miRs (https://tools-4mirs.org/software/), and miRTarBase (https://mirtarbase.cuhk.edu.cn/). The target RNAs for each miRNA were selected if they were present in, at least, 3 of the 5 databases used (13).

CANDIDATE GENES SELECTION

To select candidate genes, a search was performed across PubMed and GEO, looking for studies that employed genome-wide analysis technologies to identify OA-related differentially expressed genes (DEG). Files were obtained from a survey that identified differentially expressed genes in a sample of 79 individuals categorized into 3 groups including 20 healthy controls, 26 OA patients, and 33 rheumatoid arthritis (RA) patients through expression microarrays on the GeneChip platform. Human Genome U133A/B from Affymetrix. Files were obtained in CEL format and corresponded to both the control and OA groups. The original files in CEL format were processed to expression values using the Robust Multi-array Averange (RMA) method in the R-BiocMananger environment. Probe-level data were transformed into expression values, followed by background correction and data normalization. The cut-off criteria used to select differentially expressed genes were that they had expression change values < -0.5 and > 0.5 since the change rate is expressed in Log2, which represents that a gene is at least twice as expressed in one condition vs another. A false discovery rate (FDR) < 0.05 was also shown as a cut-off criterion to control the false positive rate. The selection of eligible genes was conducted through a comparative analysis between the genes predicted for each miRNA and the DEG from the microarray analysis. This set of genes was represented through a Venn diagram, ensuring that the shared genes were targets of the miRNAs and were involved in OA.

INTERACTION NETWORK BETWEEN miRNAs AND TARGET GENES

Once the list of genes involved in the signaling pathways of interest was available, an interaction network between miRNAs and target genes was developed using the Cytoscape v3.7.2 software. In Cytoscape, the default damping criterion for setting the dissipation coefficient is the probability of termination (dissipation). This requires a value between 0 and 1, which sets the dissipation directly on average. Therefore, in this study, we used a local clustering index of 0.592, set as an optimal probability value by the same software. These interactions allow the identification of potential miRNAs and candidate genes whose changes in their expression profiles could affect bone metabolism.

RESULTS

IDENTIFICATION OF miRNAs INVOLVED IN THE PATHOGENESIS OF OA

Through the search for miRNA expression data in different databases, 3 groups including a total of 453 differentially expressed miRNAs were identified corresponding to the work where the miRCURY LNA microRNA Array, 7th generation (QIAGEN, San Diego, USA) technology was used) (10), 211 differentially expressed miRNAs where high-throughput Seq-RNA technology was used through the DNBSEQ platform (BGI-Shenzhen, China) (11) and 136 miRNAs from a literature review (12) (Fig. 1A).

miRNA TARGET GENE PREDICTION

The prediction of the potential target genes of the miRNAs (mRNA) was conducted based on their presence in, at least, 3 of the 5 databases used for the analysis, identifying a total of 723 target genes for miR-485, 1030 genes for miR-940, 821 genes for miR-107 and 1133 genes for miR-142-5p, which were unified into a single list, eliminating repeats (Fig. 1B).

ELIGIBLE GENE SELECTION

Data from GEOs with accession No. GSE55235 were analyzed to analyze OA-related GDE. Data were re-

trieved in CEL format from the GeneChip Human Genome U133A/B expression microarray. Differential expression analysis showed 199 downregulated genes and 2123 upregulated genes that met the < -0.5 and > 0.5-Fold-Change cutoff criteria with a *p*-value < 0.05 (Fig. 1C). The list of the GDE from the microarray was compared with the unified list of target genes of the miRNAs through a Venn diagram where it is observed that 379 genes involved in OA are shared and that they are targets of the selected miRNAs (Fig. 1D). The genes recovered from this analysis were used to identify the signaling pathways involved in the development of OA.

OA-RELATED SIGNALING PATHWAYS

The genes shared between microarrays and target genes were analyzed for signaling pathways using the KEGG tool in the ShinyGO software. This tool identifies the signaling pathways associated with a given set of genes by referencing an online database of genomes, enzymatic pathways, and cellular biomolecules, as well as their specific variants in different organisms. The analysis identified nine signaling pathways related to OA development (Table I). An interaction network between these pathways was generated (Fig. 2), and an enrichment analysis of the involved genes revealed 20 OA-related diseases (Fig. 3).

INTERACTION NETWORK BETWEEN TARGET GENES AND miRNAs

From the 185 genes identified in the OA-related signaling pathways, an interaction network was generated along with the 4 selected miRNAs (Fig. 4). From this interaction network, a total of 12 genes were selected that play an essential role in bone metabolism and that, based on literature review, are associated with the development of OA: Colony Stimulating Factor 1 (CSF1), C-X-C Motif Chemokine Ligand 3 (CXCL3), Fos Proto-Oncogene, AP-1 Transcription Factor Subunit (FOS), Interleukin 6 (IL6), Interleukin 6 Receptor (IL6R), KRAS Proto-Oncogene, GTPase (KRAS), Nuclear Factor Of Activated T Cells 1 (NFATC1), Nuclear Factor Kappa B Subunit 1 (NFKB1), Nuclear Factor Kappa B Subunit 2 (NFKB2), Peroxisome Proliferator Activated Receptor Gamma (PPARG), Thrombospondin 1 (THBS1), and Tumor Necrosis Factor (TNF).

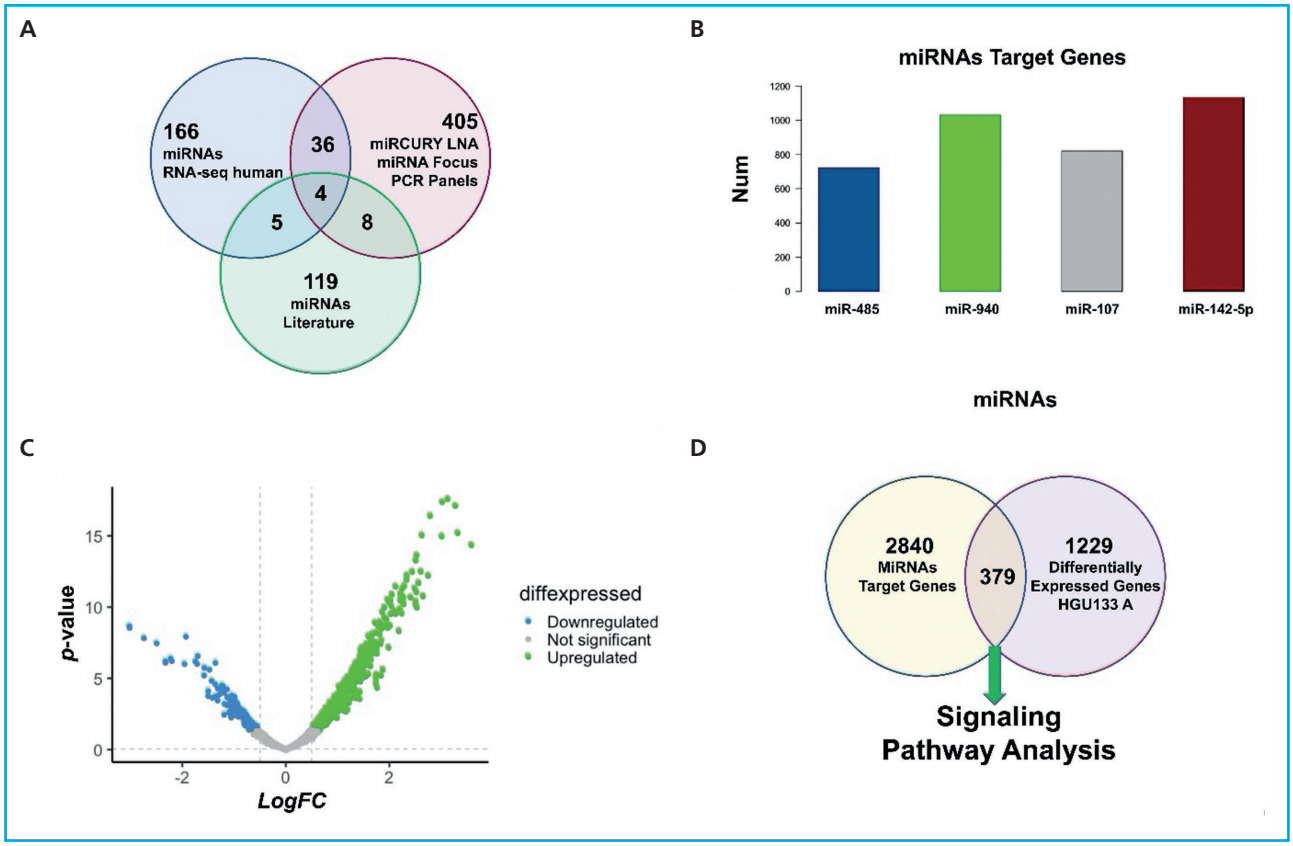

Figure 1. Analysis of miRNA selection and potential target genes. A. Venn diagram between groups of miRNAs from seqRNA, microarrays, and systematic literature review. B. No. of target genes present in, at least, 3 databases identified for each miRNA. C. Volcano diagram of differentially expressed genes from the HGU133A/B expression microarray analysis. D. Venn diagram between the group of target genes of each miRNA and the differentially expressed genes from the HGU133A/B microarray analysis.

Table I. Osteoarthritis-related signaling pathways

Pathways	nGenes	Total pathway genes	Enrichment FDR
PI3K-Akt signaling pathway	70	354	3.81E-14
MAPK signaling pathway	57	294	1.45E-11
TNF signaling pathway	40	212	1.20E-16
FoxO signaling pathway	35	131	3.05E-11
Osteoclast differentiation	32	200	9.18E-10
JAK-STAT signaling pathway	28	232	1.84E-05
Rheumatoid arthritis	27	231	8.65E-10
NF-kappa B signaling pathway	25	126	1.79E-07
AMPK signaling pathway	23	156	2.18E-05

FDR: false discovery rate. nGenes: no. of genes.

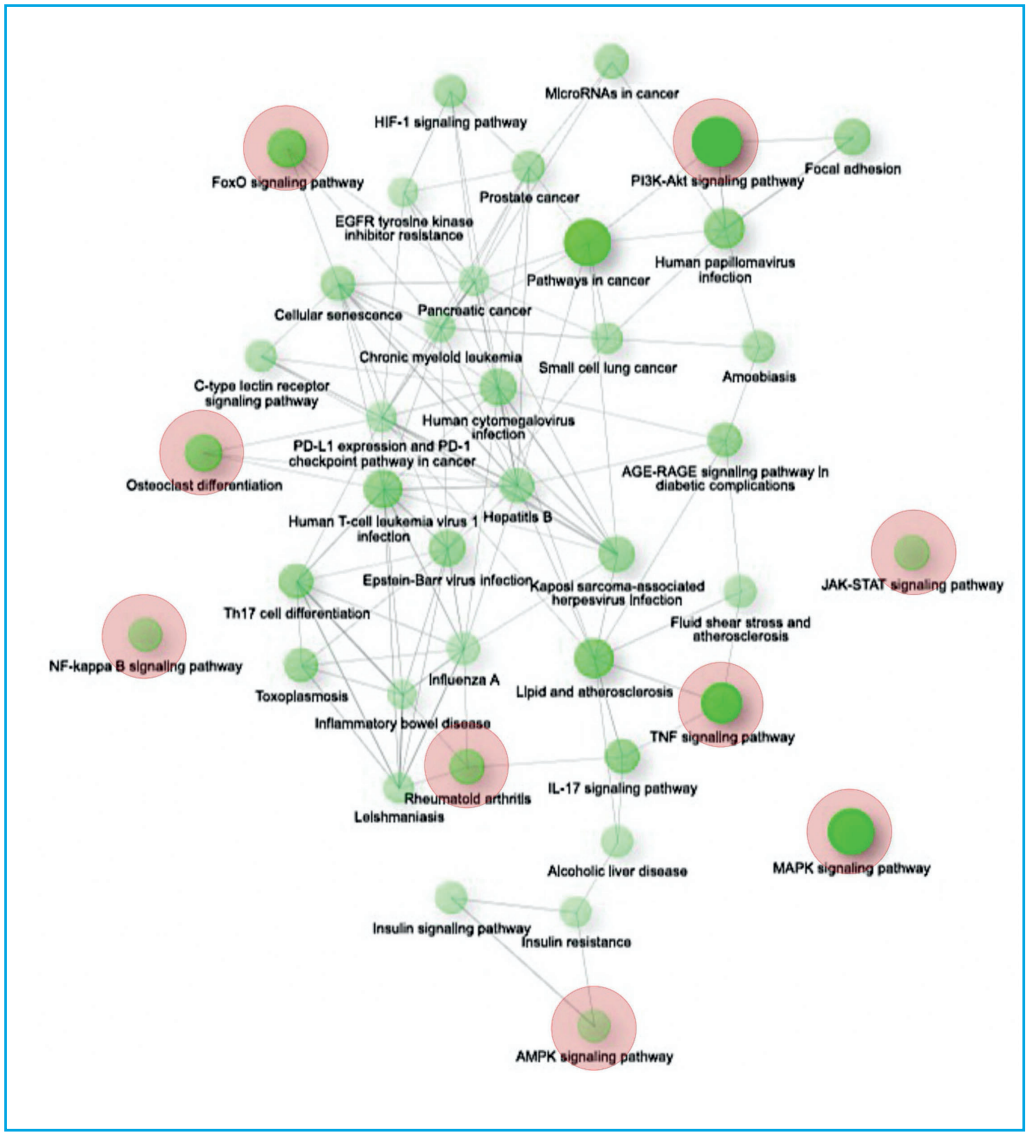

Figure 2. Analysis of interaction networks between the selected signaling pathways. Pathways involved in the development and progression of OA are highlighted in red. PI3K-Akt signaling pathway, MAPK pathway, TNF signaling, FOX signaling pathway, osteoclast differentiation, JAK-STAT signaling pathway, rheumatoid arthritis, NF-kappa B signaling pathway, and AMPK signaling pathway.

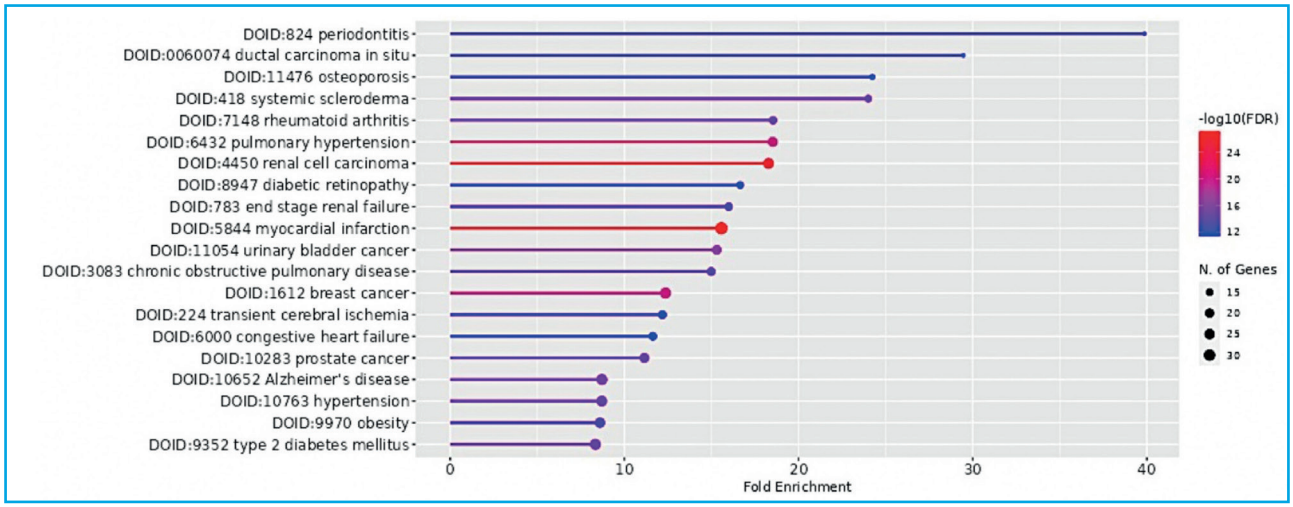

Figure 3. Chart of OA-related conditions. The different comorbidities associated with the development and progression of OA are shown.

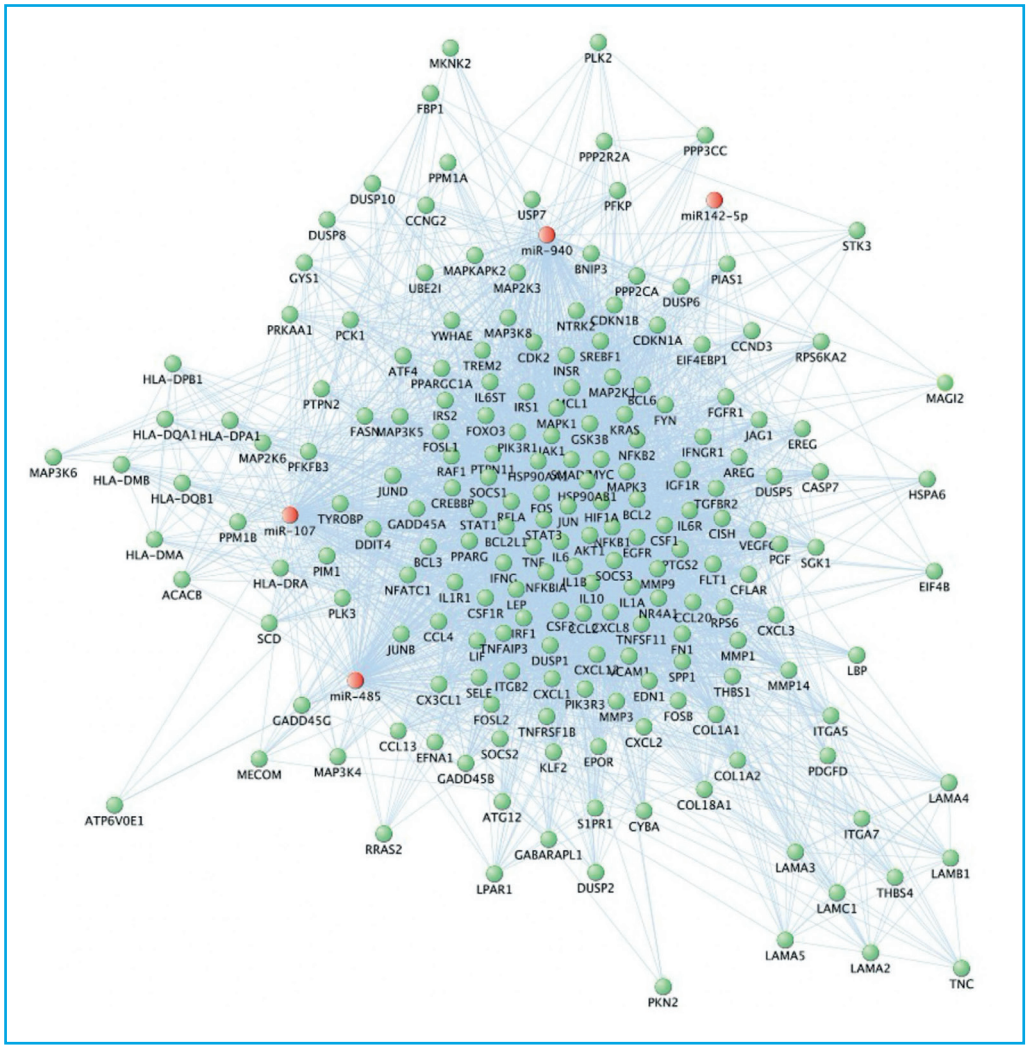

Figure 4. Interaction network between miRNA and target genes. Genes marked in red were selected for their participation in OA-related signaling pathways. They are targets of miRNAs miR-485, miR-940, miR-107, and miR-142-5p and present interaction with multiple signaling pathways.

The expression profile of this set of genes was represented through a heat map showing the downregulated and upregulated genes in OA (Fig. 5)

DISCUSSION

Our study presents an *in-silico* analysis focused on evaluating the expression signatures of human miRNAs involved in the regulation of genes that participate in different signaling pathways whose alterations can lead to the development of OA. Based on the bioinformatics search, 4 miRNAs involved in OA were identified: miR-485/miR-142 is down-regulated, and miR-940/miR-107 is up-regulated. MiR-485 has been associated with the development of OA through the inhibition of the Notch2 and NF-kB signaling pathways, promoting chondrocyte proliferation in OA and inhibiting apoptosis (14). MiR-142 has a protective effect against OA by competing with the lncRNA XIST that regulates chondrocyte growth and apoptosis (15). MiR-940 regulates the expression of genes such as MyD88, which induces a level of inflammation and simultaneously stimulates the NF-kB signaling pathway mechanism (16). MiR-107 affects cartilage matrix degradation in the pathogenesis of OA through the regulation of caspase 1, positively regulating chondrocyte proliferation (17). However, although these miRNAs have been linked to the development of OA, their role as potential biomarkers in bone metabolism and related diseases is yet to be elucidated.

Our analysis revealed a total of 9 bone metabolism-related signaling pathways whose dysregulation is associated with the development of OA. PI3K-Akt signaling pathway involves different molecules that regulate diverse biological processes. In cartilage, it regulates synovial inflammation, subchondral bone sclerosis, extracellular matrix homeostasis, chondrocyte proliferation, apoptosis, autophagy, and inflammation (18). MAPK pathway transmits extracellular signals to cells through a cascade reaction involving kinases in articular chondrocytes and inducing phosphorylation cascades. These stimuli include inflammatory factors, cytokines in the joint fluid, changes in osmotic pressure, and changes in biological stress (19). TNF signaling is tightly regulated by post-translational ubiquitination, an essential mechanism for the regulation of many biological processes. The role of inflammatory factors such as IL-1, TNF, and caspase-8/3 are involved in chondrocyte apoptosis, leading to further degenerative changes in cartilage (20). FOX signaling pathway is related to cell fate and promotes chondrocyte homeostasis (21). Osteoclast differentiation is a biological process responsible for the resorption of bone tissue, its role is well established in average bone turnover. However, osteoclasts play key roles in other diseases, such as progressive joint destruction. It has been reported that the degradation of the cartilage and osteochondral junction compartments of the joint is carried out by the action of osteoclast-derived metalloproteinases (MMPs) so that changes to the differentiation pathway of these cells could be constitutively activated, leading to the resorption of cartilage tissue, and favoring the development of OA (22). JAK-STAT signaling pathway is responsible for regulating cellular responses to cytokines such as IL-6 and epidermal growth factor (EGF) and biological processes such as cell proliferation, cell differentiation, and apoptosis. One study suggests that *CXCL8* and *CXCL11* may be involved in apoptosis and inhibit primary chondrocyte

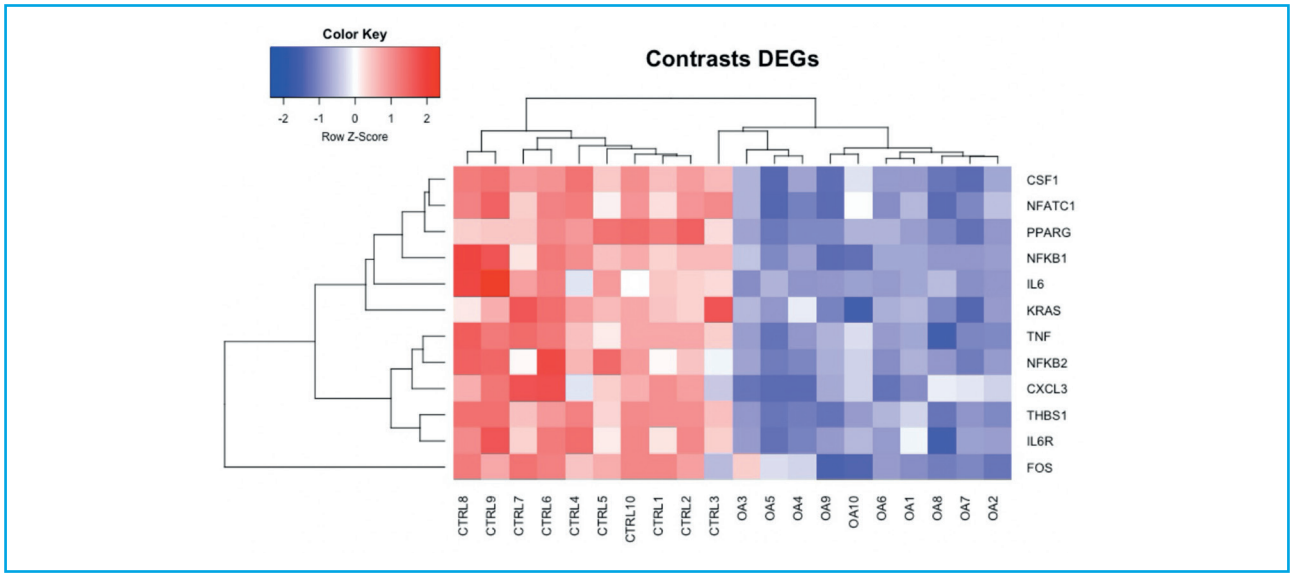

Figure 5. Differential expression analysis. Heatmap showing the expression profiles of genes involved in the development and progression of OA. Down-regulated genes are shown in blue, and up-regulated genes in red.

proliferation by regulating the expression of phosphorylated *STAT3*, leading to the development of OA (23). Rheumatoid arthritis is a disease that affects the joints and induces inflammation, which causes thickening of the tissues surrounding the joints, resulting in joint failure and pain (24). The TNF-kappa B signaling pathway regulates the expression of proinflammatory genes. It has been reported that this signaling pathway regulates the activation of osteoclast differentiation, activates the inflammatory response, and promotes the expression of catabolic factors such as MMPs that induce the destruction of articular cartilage (25). The AMPK signaling pathway plays a role in regulating growth and reprogramming metabolism. AMPK proteins are essential mediators of AMPK signaling activities and could provide energy for the inflammatory reactions that promote the development of OA (26).

Interestingly, we have observed that the miRNAs identified in this study, as well as their potential target genes involved in the described signaling pathways, play a key role in the activation and differentiation of osteoclasts. The *CSF1* gene encodes an essential cytokine for osteoclastogenesis that promotes the proliferation, survival, and differentiation of monocytes/macrophages and is regulated by miR-485, miR-940, and miR-107. Its negative regulation inhibits the formation of mature osteoclasts. However, when miR-485 is deregulated, it could allow the expression of *CSF1* and, therefore, the differentiation of osteoclasts (27,28). On the other hand, the *CXCL3* gene can recruit and activate various immune cells such as monocytes/macrophages, neutrophils, T cells, natural killer (NK) cells, fibroblasts, and endothelial cells involved in the pathogenesis of OA (29). This gene is regulated by miR-485 and miR-940; these miRNAs could play a vital role in the recruitment of cells such as monocytes, which have a fundamental role in the progression of OA, given their participation in inflammatory responses and their ability to differentiate into osteoclasts (30). The *NFkB1*/*NFkB2* genes are precursors of NF-κB, which, along with *FOS,* are transcription factors that are activated in immune cells and activated in osteoclast precursors. These genes are regulated by miR-485, while the FOS gene is regulated by miR-107, so these miRNAs could play a key role in regulating the differentiation of osteoclasts capable of degrading cartilage in OA. Another cytokine involved is IL6, which is present in elevated levels of synovial fluid of individuals with a confirmed clinical diagnosis of OA, and its mechanism of action has been shown to involve its ability to interact with its receptor IL6R. This interaction significantly suppresses the synthesis of neutrophil gelatinase-associated lipocalin (NGAL) in the immortalized human chondrocyte line, C28/I2 (31). Keeping this in mind, here, we report that NGAL regulates the activity of matrix metalloproteinase-9 (MMP-9), whose activity is crucial in OA for the destruction of articular cartilage (32). MiR-485, miR-940, and miR-107 could regulate the expression of *IL6*, while *IL6R* is targeted by miR-485, miR-940, miR-107, and miR-142-5p so that these miRNAs could play a vital role in the secretion of MMPs by osteoclasts in individuals with OA. *KRAS* gene is a small GTPase that functions as a signal transducer from cell surface receptors activated by extracellular stimuli to various well-regulated cytoplasmic signaling networks, such as mitogen-activated protein kinase (MAPK) and phosphoinositide 3-kinase (PI3K). Although the role of *KRAS* in bone metabolism remains unclear, studies in mice have shown that mutations in this gene are associated with an increase in the number of osteoclasts and, therefore, in bone resorption (33). *KRAS* is targeted by miR-485, miR-940, miR-107, and miR-142-5p so that these miRNAs could be involved in the activation of osteoclast differentiation. The *NFATC1* gene plays the role of the master regulator of osteoclast differentiation transcription. Its activation allows the differentiation of cells of the monocyte/macrophage lineage after stimulation by the two essential cytokines, CSF1 and RANKL. This gene is the target of miR-485, so deregulation of this miRNA could promote osteoclast differentiation and increase cartilage and bone tissue resorption. *PPARG* is a gene that regulates chondrocyte apoptosis in individuals with OA through the caspase-3-dependent mitochondrial pathway, and PPARG-mediated autophagy activation alleviates inflammation in rheumatoid arthritis (34). MiR-485, miR-940, and miR-107 regulate this gene, and these miRNAs might play a role in regulating chondrocyte cell death. The THBS1 gene is involved in chondrogenesis; its primary known function is its antiangiogenic and anti-inflammatory effect in several models, mainly in cancers and heart diseases. *THBS1* exerts an antiproliferative role in T lymphocytes, exerting an anti-inflammatory effect, which demonstrates that this gene has a chondroprotective effect (35). Such gene is targeted by miR-485, miR-940, miR-107, and miR-142-5p, so the regulation mediated by these miRNAs could be associated with the development of OA. TNF is a proinflammatory cytokine and, together with other cytokines, is a catabolic factor for cartilage; this cytokine promotes the release of matrix metalloproteinases (MMPs) from synovial fibroblasts, resulting in cartilage destruction, and inhibits chondrogenesis through the nuclear factor-kB (NF-kB) pathway by downregulating SOX production (36). MiR-485, miR-940, and miR-107 regulate a TNF, which means that the function of these miRNAs could be related to cartilage formation and maintenance.

Based on bioinformatics analysis and literature search on the role of miRNAs and their potential target genes involved in the development of OA, we propose a model that represents the role of the genes involved in the identified signaling pathways and their miRNA-induced regulation (Figure 6). On the other hand, changes to the expression profiles of miRNAs and target genes identified in this study are also related to other diseases that may be risk factors promoting the development of OA. Recent studies from

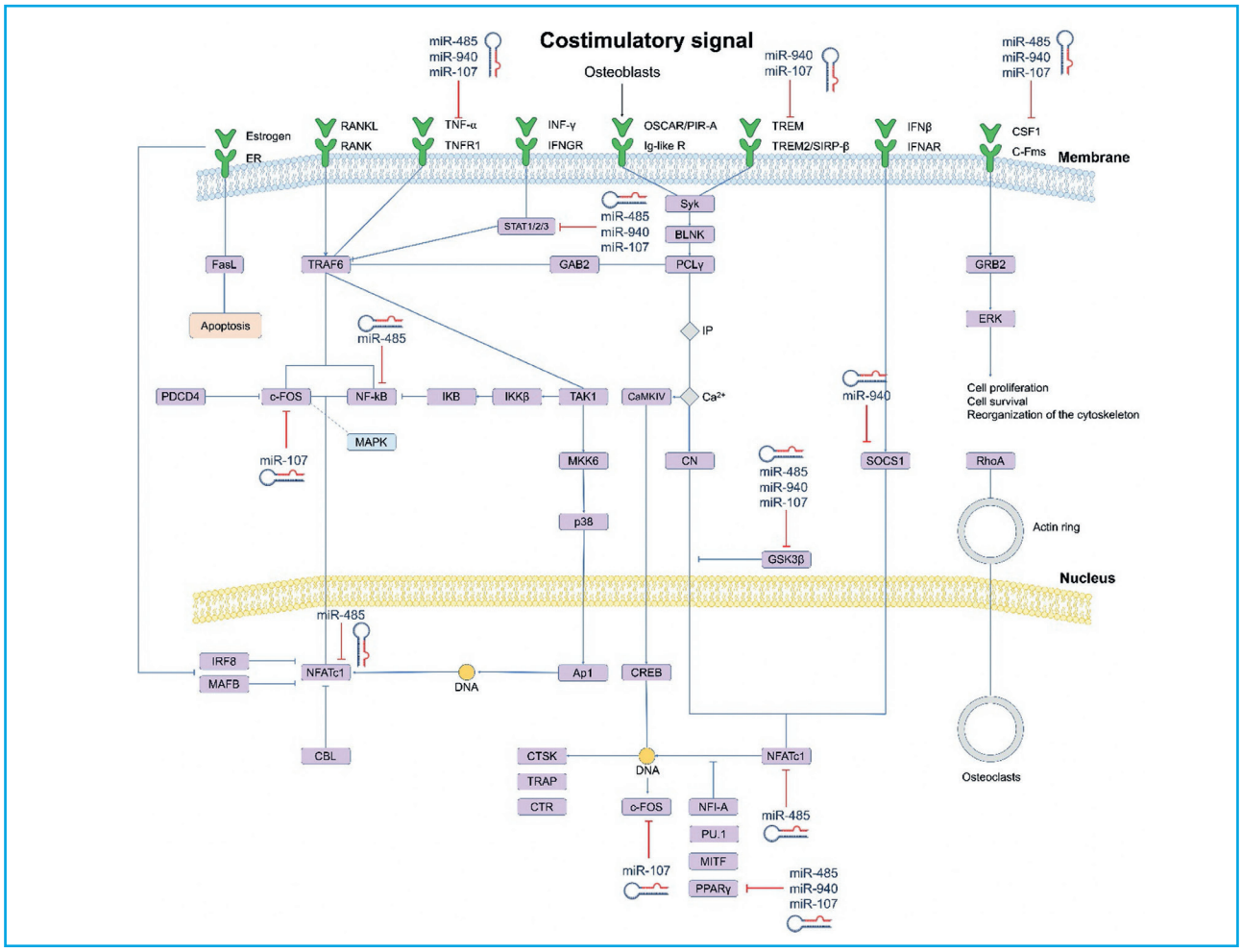

Figure 6. Schematic of the signaling networks involved in OA development and their miRNA-induced regulation. It is shown that miRNAs directly (solid lines) or indirectly (dashed lines) inhibit vital genes and transcription factors in osteoclast differentiation.

Finnish population suggest that periodontitis and osteoarthritis are related in a bidirectional pattern (37). Other studies have analyzed the relationship between osteoporosis and OA, where the role of common and divergent factors has been identified, leading to new findings on the role of BMD. It has been reported that the relationship between BMD and OA depends on the stage, definition, location, and way in which BMD is measured, suggesting that OA should be further specified in terms of bone involvement. Therefore, the osteoporotic and erosive phenotypes would be candidates for bone-targeting drugs. At the same time, the bone-forming subtype, which refers to bone-forming tumors that can be benign or malignant and are characterized by abnormal proliferation of bone cells, could be studied (38).

Cases of osteoarticular signs are commonly present in patients with systemic sclerosis and have a significant impact on the patient's quality of life (39). A study analyzed the risk of mortality and cardiovascular morbidity in patients with OA. Authors compared the rate and prevalence of hypertension between rheumatoid arthritis and OA. Their results showed no inter-group differences in the rate or prevalence of hypertension. Only patients with rheumatoid arthritis with long-term remission had a marginally lower prevalence of hypertension (40). In obesity, OA is related to excessive joint loading with impaired biomechanical patterns along with hormonal and cytokine deregulation. In OA, weight loss can bring clinically significant improvements in pain and delay the progression of structural joint damage. On the other hand, the coexistence of type 2 diabetes *mellitus* in patients with OA has been associated with the development and progression of the disease. Furthermore, DM is associated with a higher degree of osteoarthritic pain. Numerous risk factors are common to both DM and OA, such as, obesity, hypertension, and dyslipidemia (41). Finally, this work presents strengths and weaknesses. Of note, the identification of new therapeutic targets and signaling pathways involved in joint metabolism is essential to elucidate the mechanisms that lead to the development of OA and thus propose new molecules

that can be used as potential biomarkers for drug monitoring or early detection of the disease. The use of standardized methods for identifying miRNA target genes while conducting microarray analysis enhances the reproducibility of results. Additionally, by utilizing data from patient samples analyzed through various technologies, the study ensures a robust association of the selected miRNAs with OA. These methodological strengths support the reliability and validity of the findings, providing a solid foundation for future research. However, the study also has limitations. Results may not be generalizable due to potential variability in the samples analyzed, impacted by factors such as diet, lifestyle, environmental conditions, and genetic differences among populations. Additionally, while bioinformatics methods are consistent across reports, variations in the number of samples, platforms used, and specific analysis techniques can lead to differing outcomes. Therefore, biological validation assays are necessary to confirm the bioinformatics predictions. Furthermore, we consider that the expression of these miRNAs could be analyzed in different biological fluids, such as plasma, serum, urine, and saliva, to better support their use as potential noninvasive biomarkers for the early detection of OA.

CONCLUSIONS

miRNAs play an essential role in the pathogenesis of OA. Deregulation of miR-485/miR-142, as well as upregulation of miR-940/miR-107, affects different pathways involved in the pathogenesis of this disease, increasing the expression of enzymes that degrade the cartilage of articular chondrocytes, decreasing the production of matrix components or facilitating the apoptosis of these cells. In addition, miRNAs also participate in the production of proinflammatory cytokines and the induction of joint inflammation, and in pathways associated with OA progression. Given the critical role of miRNAs in the development of this disease, these molecules could be proposed as potential biomarkers for the early detection of OA. However, further studies are needed to validate the specificity and sensitivity of these molecules across different populations.

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Original

Circulating extracellular vesicles affect mesenchymal stromal cell differentiation and angiogenesis. Potential use in bone regeneration

Sara Oliva-Lozano[1], Victoria Pulido-Escribano[1], Bárbara Torrecillas-Baena[1], Alfonso Calañas-Continente[1,2], Feliciano Priego-Capote[3,4], Marta Camacho-Cardenosa[1], Antonio Casado-Díaz[1,2,4]

[1]Instituto Maimónides de Investigación Biomédica de Córdoba (IMIBIC). Córdoba, Spain. [2]Unit of Clinical Management of Endocrinology and Nutrition. Hospital Universitario Reina Sofía. Córdoba, Spain. [3]Department of Analytics Chemistry. Rabanales Campus. Universidad de Córdoba. Córdoba, Spain. [4]Centro de Investigación Biomédica en Red de Fragilidad y Envejecimiento Saludable (CIBERFES). Córdoba, Spain

Abstract

Introduction: the use of extracellular vesicles (EVs) has a high potential in regenerative medicine. Although mainly those derived from mesenchymal stromal cells (MSC) have been studied, circulating EVs from umbilical cord blood (UCBEV) or from healthy young adults (peEV) also contain factors that can favor tissue regeneration. This study evaluates the effect of UCBEV and peEV on MSC differentiation to osteoblasts and adipocytes, and endothelial cell angiogenesis.

Material and methods: MSC cultures were treated with UCBEV and peEV during differentiation into osteoblasts or adipocytes. The expression of osteoblastic or adipogenic genes was studied. Mineralization and lipid droplet formation were quantified. Umbilical cord vein endothelial cells (HUVEC) were evaluated in angiogenesis assays.

Results: UCBEV and peEV did not affect MSC viability, but peEV increased HUVEC viability. In osteoblasts, collagen type I alpha 1 (COL1A1) expression was increased by peEV, but mineralization was not affected. In adipocytes, adipose triglyceride lipase (ATGL) and fatty acid-binding protein 4 (FABP4) expression was inhibited, and lipid droplet formation was decreased with both types of EV. In HUVEC, UCBEV and peEV induced angiogenesis.

Conclusion: the results suggest that both types of EVs, from abundant sources, without major ethical issues and easy to isolate, have high potential in regenerative medicine applied to bone, inhibiting bone marrow adiposity and favoring angiogenesis.

Keywords:
Mesenchymal stromal cells. Adipocytes. Osteoblasts. Circulating extracellular vesicles. Exosomes. Angiogenesis.

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Ethical aspects: This study has been conducted in compliance with the Declaration of Helsinki. All participants provided written informed consent prior to their inclusion in the study.

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Correspondence:
Antonio Casado-Díaz. Unit of Clinical Management of Endocrinology and Nutrition. Hospital Universitario Reina Sofía. Edificio IMIBIC. Menéndez Pidal, s/n. 14004 Córdoba, Spain
e-mail: bb1cadia@uco.es

INTRODUCTION

The bone system depends on a balance between bone formation and resorption. When this balance is disrupted by trauma in the form of bone fractures, this tissue has the ability to self-renew (1). In this healing process, various cell types, extracellular matrices, and signaling molecules are involved (2), across the three main phases that occur: the inflammatory phase, the reparative phase, and the remodeling phase. In the reparative phase, osteoprogenitor cells and undifferentiated mesenchymal cells are induced to differentiate into osteoblasts, rather than into other cell types such as adipocytes (3). Furthermore, the formation of new blood vessels, which supply oxygen and nutrients necessary for bone formation, is crucial in the regenerative process (2). However, in some cases, healing delays, non-union fractures, or bone diseases (osteoporosis, osteonecrosis, or cancer) can occur. These conditions present significant morbidity and substantially reduce the activity and quality of life of patients suffering from them. Therefore, new therapeutic strategies are needed to reduce prolonged immobilization or repeated surgical interventions. These could also pose a significant cost to healthcare systems and society (4).

Recent advances in tissue engineering have proposed the combination of different cell types with synthetic biomaterials as an alternative to bone grafts. Specifically, mesenchymal stem cells (MSC) derived from bone marrow have been proposed for the treatment of various pathologies due to their differentiation, regenerative, and immunomodulatory abilities (5). However, although they can be obtained from third-party donors, the possibility of the persistence of implanted MSCs in recipient patients or the formation of ectopic tissues has hindered their application in bone injury healing (6). Additionally, other limitations for their application include the difficulty of maintaining optimal potency and viability during cell expansion and the method of administration to the patient (7). Recent studies have revealed that the potential of MSCs in tissue regeneration is linked to their paracrine activity, which partly depends on extracellular vesicles (EVs) derived from the secretome. Thus, the use of these EVs in regenerative medicine has been proposed as a cell-free therapeutic strategy (8).

EVs play an intercellular communication role and contain a wide variety of biologically active molecules, such as proteins, lipids, and different types of nucleic acids that can be relevant in the inflammatory response and tissue regeneration through signals transmitted to recipient cells (9). Depending on their size, EVs can be classified as microvesicles, exosomes, and apoptotic bodies. Specifically, exosomes range from 40 to 100 nm, have an endosomal origin, and are released by exocytosis from multivesicular bodies through plasma membranes (7). Thus, they can be administered intravenously and circulate through bodily fluids, such as blood, urine, or saliva (10). These exosomes seem to transmit the therapeutic effects of the originating cell while overcoming the limitations associated with the use of cells in regenerative medicine (7,9). They can be isolated from multiple bodily fluids such as semen, blood, urine, saliva, breast milk, amniotic fluid, ascitic or cerebrospinal fluid, and bile (11). The content of these circulating EVs depends on the organism's state, making them a source of biomarkers and factors that can even be used for therapeutic applications as an alternative to MSC-derived EVs. Indeed, using MSC-derived EVs involves manipulating *in vitro* cell cultures, which may cause loss of MSC properties and genetic instability when performed outside their natural niche (12). This can be partially avoided using stable and immortalized MSC lines obtained by genetic manipulation, for example, inducing the expression of human telomerase reverse transcriptase (hTERT). This procedure has produced MSCs with high proliferation and expansion capacity, maintaining their immunomodulatory, differentiation, and regenerative properties (13,14). Although various animal studies have shown that over time, immortalized MSCs do not transform into tumor cells, and therefore conclude that they can be considered safe for potential clinical use, these studies have certain limitations. Among them are the lack of clinical trials and the still unknown possibility of immortalized MSCs accumulating unwanted mutations after prolonged expansion periods. Therefore, it has been suggested that the use of these cells, both in cell therapy and in cell-free therapy based on the use of EVs, should be subject to strict controls during culture (15). This increases the complexity of the procedure due to the need to maintain stable MSC lines and the design of bioreactors for large-scale EV production. Therefore, it is interesting to study other alternative sources of regenerative EVs, such as circulating EVs in blood, which can be obtained without the need to expand and maintain cells in culture (16). Moreover, the amount of EVs obtained from plasma can be between 10 and more than 100 times higher than that obtained from cell cultures (17). One potential source is umbilical cord blood. It has a composition similar to that of adult bone marrow, but unlike this, it also contains a series of immunosuppressive cells, allowing it to reduce levels of inflammatory cytokines (18). In humans, it has been observed that human umbilical cord blood cell-derived extracellular vesicles (UCBEV) vs peripheral blood-derived derived extracellular vesicles from adults (peEV) have higher expression of miRNAs involved in pregnancy, leukemia suppression, inflammation inhibition, cell mobility, and nervous system development, as well as factors related to embryonic development (19,20), suggesting a high regenerative potential. As for peEV, several studies have shown their therapeu-

tic potential for the treatment of ischemic processes and wound healing (21). Thus, the objective of this study was to evaluate the potential effects of UCBEV and peEV on endothelial cell angiogenesis and MSC osteoblastic and adipogenic differentiation.

MATERIAL AND METHODS

EXTRACTION OF EXTRACELLULAR VESICLES FROM BLOOD PLASMA

After signing informed consent, healthy women without chronic pathologies and of adult age (between 26 and 31 years) underwent a single blood extraction. The umbilical cord blood was donated by mothers who met the same inclusion criteria as the healthy adults and also signed their participation consent.

Blood samples were centrifuged at 3000 rpm for 10 minutes to obtain plasma. One milliliter of plasma was passed through PURE-EVs size-exclusion chromatography columns (HansaBioMed Life Sciences Ltd.) using phosphate-buffered saline (PBS) as the vehicle. The 3 milliliters in which the EVs eluted were concentrated by ultrafiltration with 10 MWCO concentrators (Vivaspin 6 centrifugal concentrator, Sartorius) until a volume of 300-350 µl was obtained, which was stored at -20 °C until use. The concentration and size of the exosomes obtained from each sample were determined with a nanoparticle tracking analyzer (Nanosight NS300) based on "Nanoparticle Tracking Analysis" (NTA) technology at the University Institute of Nanochemistry (IUNAN) at the Universidad de Córdoba.

CHARACTERIZATION OF EVS BY WESTERN BLOT

EVs were characterized by Western blot. For this, 10 µg of each sample were loaded into an 8-16 % acrylamide gel (nUView Tris-Glycine Precast Gels, NuSeP) under denaturing conditions and separated by electrophoresis using the "Mini-Protean" system (Bio-Rad). Then, the proteins were transferred to a polyvinylidene fluoride (PVDF) membrane (Bio-Rad) using the Trans-Blot Transfer System (Bio-Rad). The membrane was blocked with a 5 % skim milk solution in TTBS buffer (20 nM Tril-CL pH 7.6, 150 mM NaCl, 0.05 % Tween) for 1 hour at room temperature and incubated overnight at 4 °C with primary antibodies anti-CD81 (25kDa, 1:500; ref.: 10630D), CD9 (25kDa, 1:1000; ref.: 10626D), and anti-CD63 (30-60 kDa, 1:1000; ref.: 10628D), all in 1 % milk with TTBS. Subsequently, the membrane was washed with TTBS and incubated for 1 hour with the secondary anti-mouse antibody (1:4000; ref.: 32430), in 2 % milk with TTBS. All antibodies used were from Invitrogen, ThermoFisher Scientific. Detection was performed using the chemiluminescent substrate Clarity Western ECL Substrate (Bio-Rad), and the images were acquired with the ChemiDoc™ XRS+ system (Bio-Rad) using the Image Lab 6.1 software from the same manufacturer.

MSC AND HUVEC CELL CULTURES

The MSCs used were isolated from cryopreserved mononuclear cells (Stemcell Technologies, Cologne, Germany) according to the protocol previously described by our group (22). They were expanded in minimum essential alpha medium (αMEM) from Biowest (Nuaillé, France), containing 2 mM of ultraglutamine (Biowest), 10 % fetal bovine serum (FBS; Gibco-Thermo Fisher Scientific), 100 U of penicillin, 0.1 mg of streptomycin/mL, and 1 ng/mL of fibroblast growth factor (FGF-2) from Sigma-Aldrich (Saint Louis, MO, USA). Umbilical vein endothelial cells (HUVEC) from Lonza (Basel, Switzerland) were grown in endothelial basal medium (EBM), with supplements and growth factors, known as endothelial growth medium (EGM) from Lonza. This contained 10 % FBS, hydrocortisone, gentamicin, human epidermal growth factor (hEGF), and bovine brain extract.

Both MSC and HUVEC cultures were incubated at 37 °C with 95 % humidity and 5 % CO_2. When they reached 90 % confluence, the cells were lifted with trypsin-EDTA (Gibco) and seeded in 12 (P12), 24 (P24), or 96-well (P96) plates for different experiments.

CELL VIABILITY ASSAY

Cell viability was determined using the 3-(4,5-dimethylthiazol-2)-2,5-diphenyl tetrazolium bromide (MTT; Sigma-Aldrich) assay. MSCs and HUVECs were seeded in P96 plates at a density of 4000 and 8000 cells per well, respectively, in the corresponding culture medium for each cell type, as described earlier. After 24 hours, the medium was replaced with medium containing exosome-free FBS, supplemented with different concentrations of UCBEV or peEV (10×10^6, 20×10^6, 40×10^6, 80×10^6, and 160×10^6 particles/mL). After 72 hours, the medium was removed, and 50 µL per well of Dulbecco's modified Eagle medium (DMEM) without phenol red, supplemented with 1 mg of MTT/mL (both from Sigma-Aldrich), was added. After 2 hours of incubation, the medium was removed, and the formazan crystals produced were dissolved in isopropanol. The resulting solution's absorbance was measured at 570 nm, with the absorbance at 650 nm used as a reference, using a BioTek Instruments PowerWave XS microplate spectrophotometer (Winooski, VT, USA).

CELL MIGRATION ASSAY

Migration was studied using the Scratch Assay in P24 plates. In confluent cultures, a cell-free zone was generated using the tip of a P200 pipette, and different concentrations of UCBEV or peEV (10×10^6 and 160×10^6 particles/mL) were added to the medium. Cultures were maintained for up to 24 hours, and images were taken at different times using the IncuCyte Zoom Imaging System from Sartorius. Images at 18 and 15 hours of migration for MSC and HUVEC, respectively, were analyzed with ImageJ V1.53f51 software (NIH; Bethesda, MD, USA). The times were selected because, after these times, the cell-free zone was fully occupied, making it impossible to identify differences between treatments. Migration was quantified relative to the percentage of the initial area not occupied by cells.

DIFFERENTIATION OF MSCs INTO OSTEOBLASTS AND ADIPOCYTES

In MSC cultures at 60-80 % confluence, differentiation to osteoblasts or adipocytes was induced. Osteoblastic differentiation was maintained for 21 days in medium supplemented with 10 nM dexamethasone, 0.2 mM ascorbic acid, and 10 mM β-glycerol phosphate (Sigma-Aldrich), in the presence or absence of the different EV concentrations tested (10×10^6 and 160×10^6 particles/mL). On the other hand, differentiation into adipocytes was induced with 500 nM dexamethasone, 0.5 mM isobutylmethylxanthine, and 50 µM indomethacin (all from Sigma-Aldrich), maintained for 14 days, in the presence or absence of the different EV concentrations.

CYTOCHEMICAL STAINING

Alizarin red staining at 21 days of osteoblastic differentiation was used to visualize and quantify extracellular matrix mineralization. Cultures in P12 plates were fixed with 3.7 % formaldehyde for 10 minutes and then stained with 40 mM alizarin red in water, pH 4.15 (Sigma-Aldrich) for 10 minutes. The wells were then washed several times with 60 % isopropanol, dried, and visualized under an optical microscope. Alizarin red deposits were measured after elution with 10 % acetic acid, neutralization with 10 % ammonium hydroxide, and quantification by spectrophotometry at 405 nm absorbance of the resulting solution.

The formation of fat vesicles in cultures induced to differentiate into adipocytes was evaluated by Oil Red O staining. For this, cells were fixed with 3.7 % formaldehyde for 15 minutes, washed with 60 % isopropanol in water, and stained for 15-20 minutes with a 0.3 % Oil Red O solution (weight/volume) in 60 % isopropanol. The cells were then washed twice with distilled water, stained with hematoxylin, and images were taken, at least nine per well, using an optical microscope. Fat vesicle staining was quantified with ImageJ software (NIH), and values were normalized with the number of cells per image. Lipid accumulation in the cultures was expressed as: (Oil Red O stained area / number of cells).

RNA ISOLATION AND GENE EXPRESSION QUANTIFICATION

Samples for RNA isolation and subsequent analysis of adipogenesis and osteoblastogenesis marker genes were taken from MSC cultures 10 days after induction to differentiate into osteoblasts or adipocytes. RNA was isolated following the manufacturer's instructions using the NZY total RNA isolation kit (NZYTech, Lisbon, Portugal) and quantified with a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific). Then, 900 ng were retrotranscribed using the iScript cDNA synthesis kit from Bio-Rad (Hercules, CA, USA). Quantitative real-time polymerase chain reaction (RT-qPCR) tests were carried out in a CFX96 Connect (Bio-Rad). Each reaction contained 1 µL of cDNA, 10 pmol of each primer pair (Table I), and SensiFAST Sybr No-Rox Mix from Bioline (London, UK). The PCR amplification program included a cycle at 95 °C for 2 minutes (DNA denaturation and polymerase activation) and 44 amplification cycles: 95 °C for 5 seconds (DNA denaturation) and 65 °C for 30 seconds (hybridization and extension). Results were analyzed using the CFX Maestro V 2.3 software (Bio-Rad) to obtain threshold cycles (Ct). The POLR2A gene, encoding RNA polymerase II subunit A, was used as a constitutive gene, and relative expression vs control samples was expressed as arbitrary units calculated using the $2^{-(\Delta\Delta Ct)}$ method, where $\Delta\Delta Ct = \Delta Ct$ (sample) – ΔCt (control sample); and ΔCt (sample) = Ct (gene of interest sample) – Ct (constitutive gene sample), and ΔCt (control sample) = Ct (gene of interest control sample) – Ct (constitutive gene control sample).

ANGIOGENESIS ASSAY IN HUVEC

To evaluate the effect of UCBEV and peEV on angiogenesis in HUVEC, a tube formation assay in Matrigel was performed. HUVEC cells were pretreated for 24 hours with different concentrations of UCBEV or peEV (10×10^6 and 160×10^6 particles/mL) in EGM medium + 10 % FBS without exosomes. For the angiogenesis assay, 10 µL of reduced growth factor Matrigel (Corning, NY, USA) at 4 °C was added to P96 microplates from Greiner Bio-One (Kremsmunster, Austria), allowed to gel at room temperature. Then, from each HUVEC culture pretreated with the different EVs, 15,000 cells per well were added, resuspended in 70 µl of EBM + 2 % FBS without exosomes, supplemented with the corresponding type and concentration of EV. The cells were maintained under culture conditions for 4 hours at 37 °C and 5 % CO_2.

Table I. Primers used for QRT-PCR

Gene	Sequence (5'-->3')	Product (bp)
Polymerase (RNA; DNA directed) II polypeptide A (POLR2A)	TTTTGGTGACGACTTGAACTGC CCATCTTGTCCACCACCTCTTC	125
Runt-related transcription factor 2 (RUNX2)	TGGTTAATCTCCGCAGGTCAC ACTGTGCTGAAGAGGCTGTTTG	143
Osterix (SP7)	AGCCAGAAGCTGTGAAACCTC AGCTGCAAGCTCTCCATAACC	163
Integrin-binding sialoprotein (IBSP)	AGGGCAGTAGTGACTCATCCG CGTCCTCTCCATAGCCCAGTGTTG	171
Collagen, type I, alpha 1 (COL1A1)	CGCTGGCCCCAAAGGATCTCCTG GGGGTCCGGGAACACCTCGCTC	263
Peroxisome proliferator activated receptor gamma 2 (PPARG2)	GCGATTCCTTCACTGATACACTG GAGTGGGAGTGGTCTTCCATTAC	136
Patatin Like Phospholipase Domain Containing 2 (ATGL)	CCAACACCAGCATCCAGTTCA ATCCCTGCTTGCACATCTCTC	102
Lipoprotein lipase (LPL)	AAGAAGCAGCAAAATGTACCTGAAG CCTGATTGGTATGGGTTTCACTC	113
Fatty-acid-binding protein 4 (FABP4)	TCAGTGTGAATGGGGATGTGAT TCTGCACATGTACCAGGACACC	162

After this period, images were taken using an optical microscope and analyzed with the Angiogenesis Analyzer extension of ImageJ software. As a negative control of angiogenesis, cultures maintained in unsupplemented medium without EVs were used, and as a positive control, the medium was supplemented with 30 ng/mL of the angiogenesis inducer fibroblast growth factor 2 (FGF-2).

STATISTICAL ANALYSIS

Data are expressed as the mean ± standard error of the mean (mean ± SEM). In all experiments, the number of replicates for each parameter studied was at least 3. Comparison between the different treatments was performed using the ANOVA test to detect significant changes, followed by a Tukey test to identify significant differences between pairs of treatments. Significant changes were considered for $p < 0.05$.

RESULTS

CHARACTERIZATION OF CIRCULATING EVs FROM UMBILICAL CORD BLOOD OR ADULT PLASMA

The size analysis of the UCBEV and peEV by Nanoparticle Tracking Analysis shows that most of the EVs obtained have a diameter ranging from 50 to 150 nm (Figs. 1 A and B). The concentration of particles per ml of umbilical cord plasma ranged from 0.7×10^{11} to 1.3×10^{11}, and for adult blood plasma, it ranged from 3.2×10^{11} to 1.1×10^{12}. On the other hand, the characterization of the protein expression of the markers CD81, CD9, and CD63, by Western blot, indicated that both types of EVs express these markers (Fig. 1C). These results show how, after processing the plasma samples from umbilical cord blood or adult blood, circulating EVs were obtained.

EFFECT OF UCBEV AND peEV ON CELL VIABILITY OF MSCs AND HUVECs

To evaluate the effect of UCBEV and peEV on the cell viability of MSC cultures, the cells were treated with different concentrations of EVs (10×10^6, 20×10^6, 40×10^6, 80×10^6, and 160×10^6 particles/mL) for 48 hours. The results showed that the viability was not significantly affected by any of the doses and types of EVs (Fig. 2A). Based on these results, in HUVEC cultures, the effect of the lowest and highest concentration of EVs used in the study on MSC viability (10×10^6 and 160×10^6 particles/mL) was evaluated. In this case, a significant increase in viability was observed when the cells were treated with the smaller concentration (10×10^6) of particles/mL of peEV (Fig. 2B).

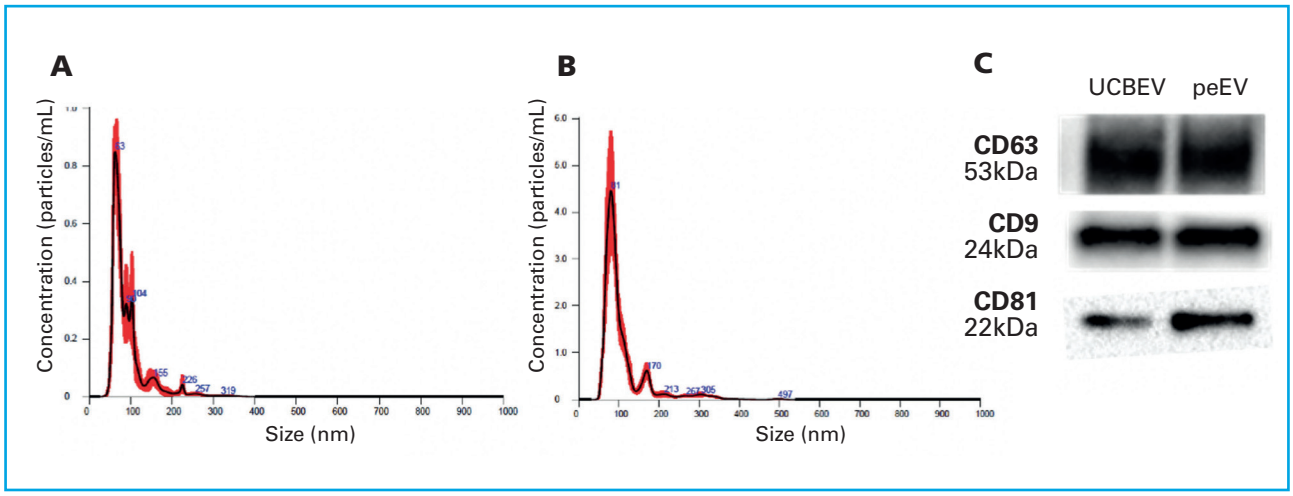

Figure 1. Quantification and characterization of circulating exosomes isolated from umbilical cord blood plasma (UCBEV) or peripheral blood plasma from healthy adults (peEV). A and B. Show the analysis of particle size distribution obtained from UCBEV and peEV, respectively. C. Shows the protein expression via Western blot of EV markers CD63, CD9, and CD81 in UCBEV and peEV.

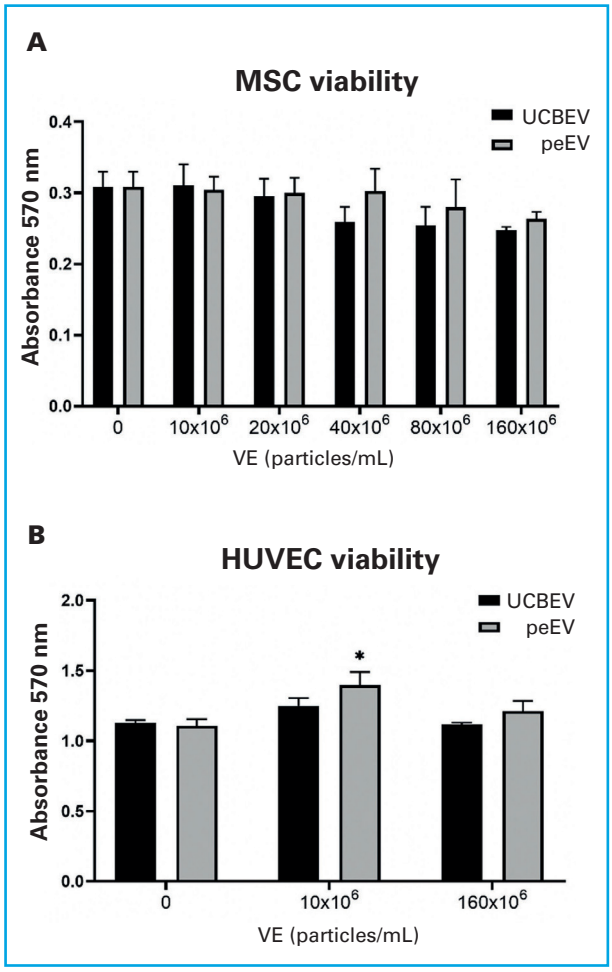

Figure 2. Quantification of cell viability in MSC (A) and HUVEC (B) treated for 72 hours with different concentrations of circulating UCBEV or peEV. *$p < 0.05$ compared to untreated cultures (control).

EFFECT OF UCBEV AND peEV ON MSC AND HUVEC MIGRATION

In the cell migration assay, the presence of 10×10^6 or 160×10^6 particles/mL of both types of EVs (UCBEV and peEV) reduced migration in MSC and HUVEC cultures, after 18 hours or 15 hours, respectively (Fig. 3). The decrease was more pronounced in the presence of peEV (Fig. 3).

EFFECT OF UCBEV AND peEV ON MSC DIFFERENTIATION

Osteogenic differentiation

The results of extracellular matrix mineralization after 21 days of osteogenic differentiation in MSCs induced to differentiate into osteoblasts in the presence of different concentrations of UCBEV and peEV, show that none of the concentrations used affected this mineralization (Figs. 4 A and B).

After 10 days of osteoblastic differentiation, the expression of osteoblastic marker genes, such as the *transcription factor RUNX2, osterix* (SP7), the integrin-binding sialoprotein (IBSP), and type I collagen alpha-1 (COL1A1), was also studied in these cultures. These genes code for two transcription factors responsible for osteogenic differentiation and proteins of the extracellular matrix. The results shown in figure 4C indicate that, like mineralization, treatment with the different types of EVs did not produce significant changes in the expression of these osteoblastic genes. Only in the expression of COL1A1, an increase was observed in cultures treated with the highest concentration of EVs (Fig. 4C).

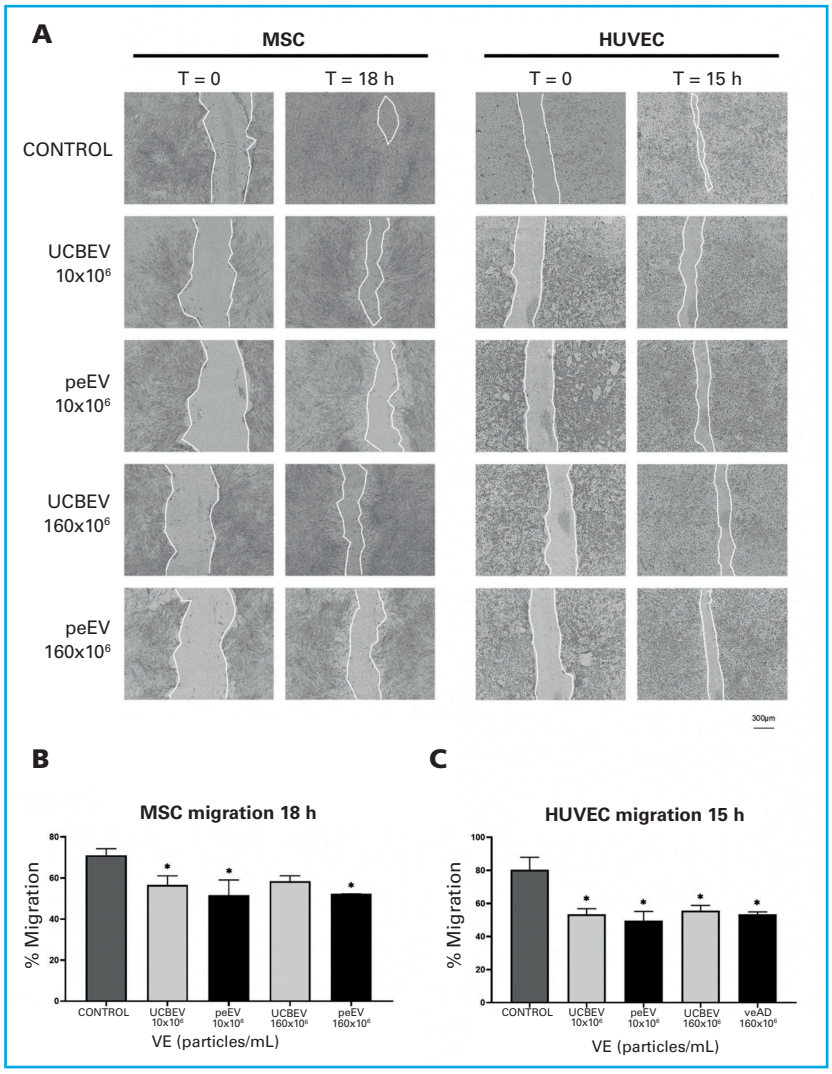

Figure 3. Effect of UCBEV and peEV on cell migration. A. Representative images of MSC or HUVEC cultures at time 0, after creating a cell-free line on the plate, and at 18 or 15 hours of culture, respectively, in the presence or absence of different concentrations of UCBEV or peEV. B. Quantification of the migration area percentage in MSC after 18 hours of treatment. C. Same as B, after 15 hours with HUVEC. *$p < 0.05$ compared to untreated cultures (control).

Adipogenic differentiation

In MSCs differentiated into adipocytes, the results of the Oil Red O staining analysis showed that the presence of UCBEV or peEV in the adipogenic medium reduced the formation of fat vesicles compared to the untreated cultures (Fig. 5B). Regarding the expression of the gene coding for the main transcription factor responsible for adipogenic differentiation, peroxisome proliferator-activated receptor gamma 2 (PPARG2), no significant differences were observed between the different treatments after 10 days of adipogenic differentiation. Changes were observed in the expression of genes involved in fatty acid metabolism, such as adipose triglyceride lipase (ATGL) and fatty acid-binding protein 4 (FABP4). The mRNA levels of ATGL decreased in cultures treated with 160 × 10^6 particles/mL of UCBEV, and the gene expression of FABP4 was inhibited with both concentrations of peEV used (Fig. 5C). The decrease in the expression of the genes ATGL and FABP4 may be related to the reduction in fat vesicle formation and different mechanisms of action of UCBEV and peEV on MSC cultures differentiated into adipocytes.

STUDY OF THE EFFECT OF UCBEV AND peEV ON ANGIOGENESIS

The results of the quantification of the total length of segments and tubular structures indicate that all EV treatments increased angiogenesis vs untreated HUVECs (Control -). This increase was more significant with UCBEV treatments (Figs. 6 B and C).

DISCUSSION

Regenerative medicine applied to bone has great potential for the treatment of pathologies such as osteoporosis, osteoarthritis, osteonecrosis, and trau-

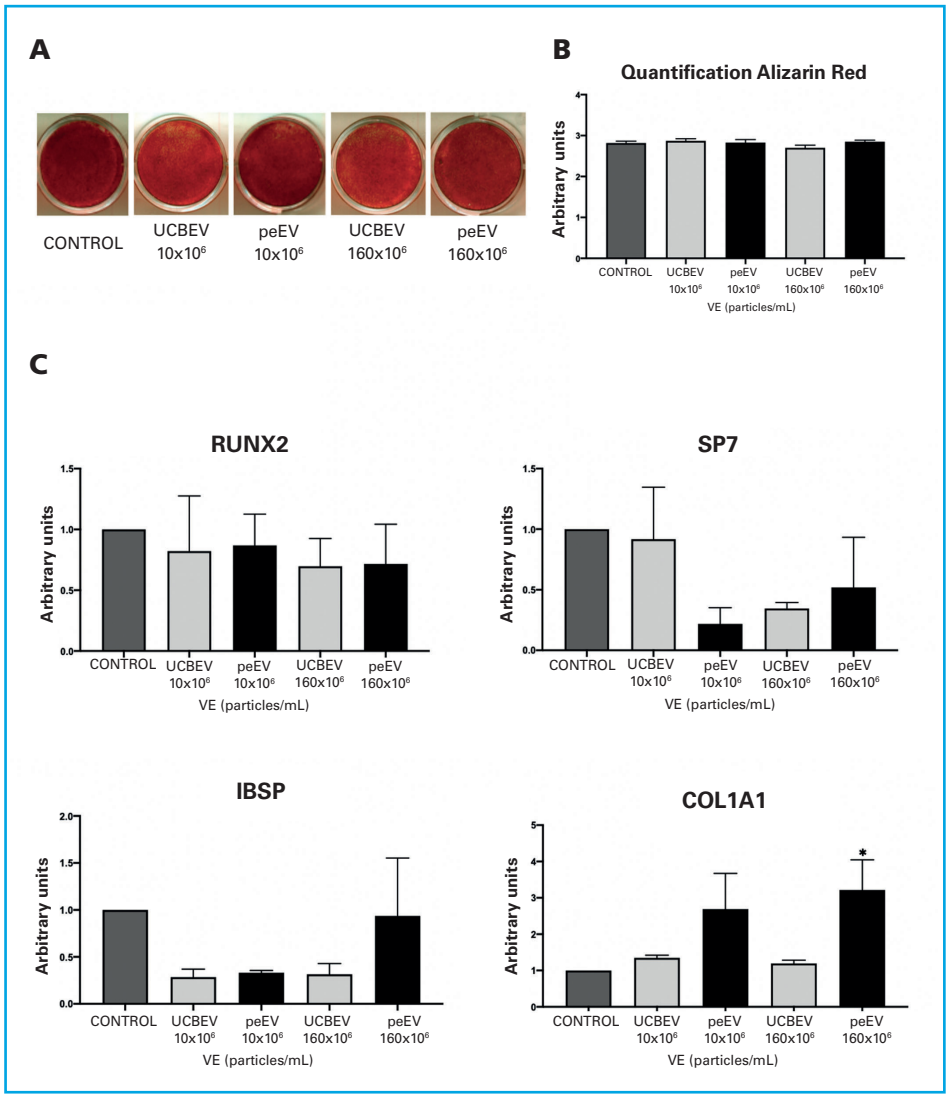

Figure 4. Study of the osteoblastic differentiation capacity of MSC treated with different concentrations of UCBEV and peEV. A. Representative images of Alizarin red staining for extracellular matrix mineralization in MSC cultures induced to osteoblasts and treated with different concentrations of UCBEV or peEV for 21 days. B. Quantification of Alizarin red staining. C. Gene expression of osteoblastic markers (RUNX2, SP7, IBSP, and COL1A1) in MSC cultures treated with UCBEV or peEV, 10 days after induction to differentiate into osteoblasts. *$p < 0.05$ compared to untreated cultures (control).

matic fractures. Although cell therapy has been widely evaluated in bone regeneration with promising results (23), it is currently considered that the use of EVs derived from MSCs as a cell-free therapeutic tool could avoid the drawbacks of producing and implanting progenitor cells for therapeutic purposes in bone (7). However, obtaining EVs derived from MSCs, even from immortalized cell lines, requires the establishment and maintenance of stable cell cultures, which increases the complexity of their isolation and requires suitable facilities for clinical use. Therefore, it is advisable to evaluate the potential therapeutic capacity of other sources of EVs that are easily accessible, abundant, and do not present significant ethical issues.

Thus, the aim of this work was to evaluate the effect of EVs derived from umbilical cord blood and from healthy adult individuals on processes related to bone regeneration, such as angiogenesis in endothelial cells and osteoblastic and adipogenic differentiation in bone marrow-derived MSCs.

Our results showed that both types of EVs did not significantly affect osteogenic differentiation but reduced adipogenesis in MSCs and increased angiogenesis in HUVECs. While UCBEV seem to favor angiogenesis more, and peEV may intervene more significantly in fat metabolism through inhibition of FABP4, we did not detect other major differences between both types of EVs. This could be partly because, in addition to nutrients and oxygen from the maternal blood, the umbilical vein blood also transports EVs from the mother (24). Therefore, besides fetal-origin exosomes, there would also be adult-origin exosomes.

The response to tissue damage requires a series of molecular and cellular events, including cell migration among others (25). According to the results obtained, migration of MSCs and HUVECs decreased, although with UCBEV, the reduction in cell migration was smaller than with peEV. These results suggest that the content of both types of EVs favored differentiation but reduced cell migration.

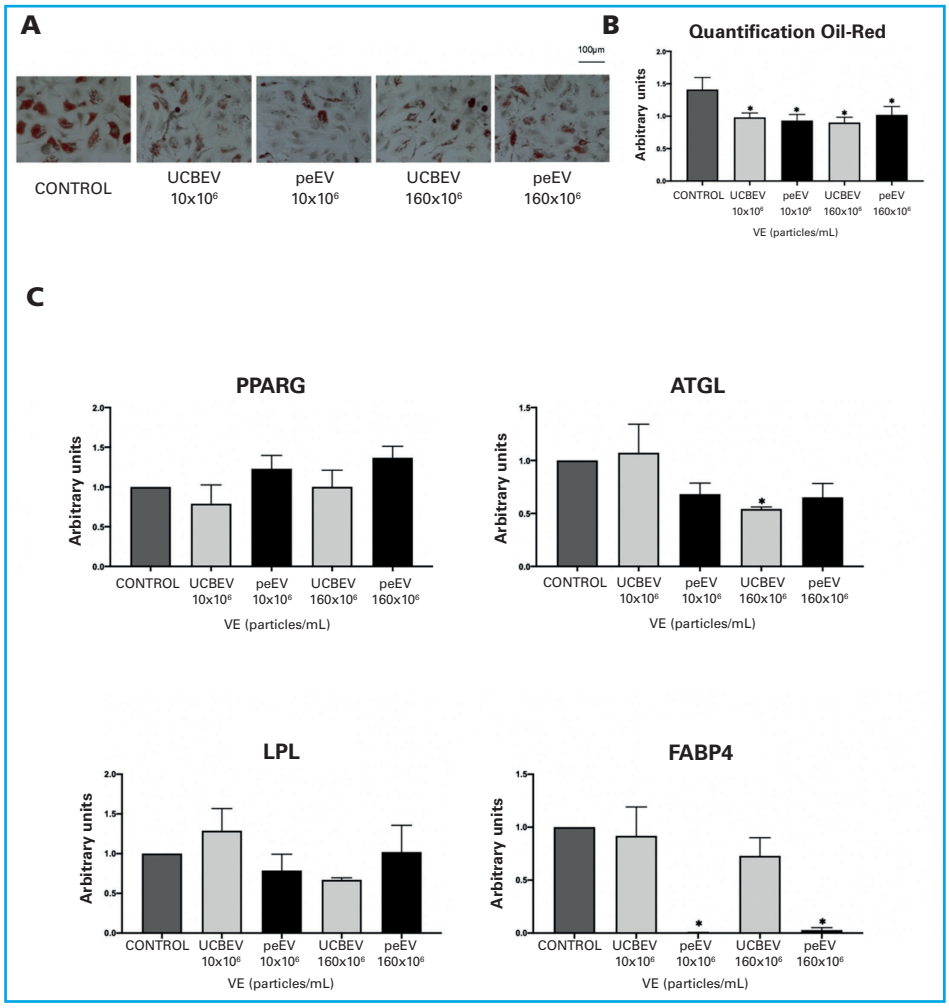

Figure 5. Study of the adipogenic differentiation capacity of MSC treated with different concentrations of UCBEV and peEV. A. Representative images of "Oil Red O" staining of fat vesicles in MSC cultures induced to adipocytes and treated with different concentrations of UCBEV or peEV for 14 days. B. Quantification of "Oil Red O" staining. C. Gene expression of adipogenic marker genes (PPARG, ATGL, LPL, and FABP4) in MSC cultures treated with UCBEV or peEV, 10 days after induction to differentiate into adipocytes. *$p < 0.05$ compared to untreated cultures (control).

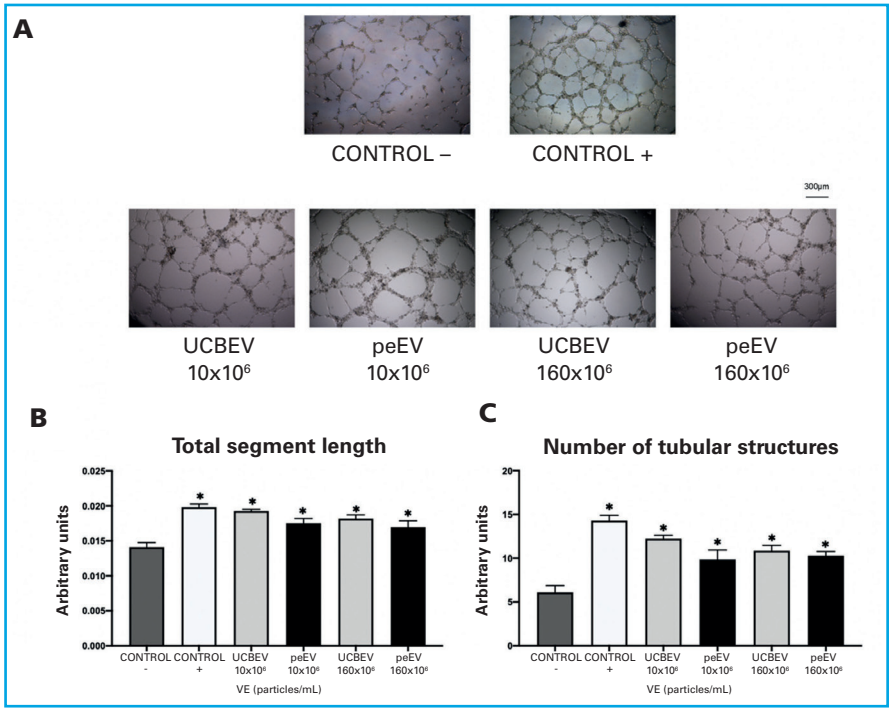

Figure 6. Study of angiogenesis in HUVEC cultures. A. Representative images of tubular structure formation in HUVEC cultures on matrigel and treated with different concentrations of UCBEV or peEV. The control (-) corresponds to untreated cultures, and the control (+) to cultures treated with bFGF (30 ng/mL) as an angiogenic factor. B and C. Graphical representation of the quantification in the angiogenesis assay of the total segment length and the number of tubular structures, respectively. *$p < 0.05$ compared to untreated cultures (control [-]).

This effect has been previously described by other authors, who showed that during osteogenic and chondrogenic differentiation of MSCs, migration decreased as differentiation progressed (26). Also, other authors have described that EVs derived from plasma of healthy individuals inhibit migration of microvascular endothelial cells (27).

Osteogenic differentiation of MSCs is a complex process regulated by various factors, such as the bone microenvironment, which significantly influences osteogenesis (28), and in which EVs participate, regulating different physiological aspects of stem cells (29). In this context, our results showed that the gene expression of COL1A1 increased in cultures treated with peEV, which could favor mineralization (30). However, in the expression of other osteoblastic genes, no significant changes were observed with treatments of peEV or UCBEV. This can be related to the fact that none of the EVs evaluated affected the mineralization of MSCs differentiated into osteoblasts. Induction of bone formation by EVs derived from MSCs has been observed in numerous previous studies (31). However, the application of exosomes derived from plasma of healthy adolescents on undifferentiated MSCs did not significantly affect osteogenic differentiation, although when treated with primary osteoblast cultures, an increase in alkaline phosphatase (ALPL) activity was observed (7). However, in that study, mineralization was not studied, so the possible effect of those exosomes on the final maturation of the treated osteoblasts cannot be concluded. In another study, it was shown that treatment of MSC cultures derived from bone marrow with EVs from umbilical cord blood plasma increased mineralization (32). The differences with the results obtained in the present study could be due to the concentration of exosomes used (which cannot be compared as it is expressed in µg/mL) and the MSCs being derived from mice and not humans.

Regarding adipogenic differentiation, treatment with the evaluated circulating EVs led to a decrease in fat vesicle formation. This was accompanied by a decrease in the expression of the ATGL gene in cultures treated with UCBEV and of FABP4 in peEV, involved in fat metabolism and fat vesicle formation (33,34). It is interesting to note that the expression of the PPARG gene was not affected by the treatments, suggesting that the EVs used may affect more the maturation than the early differentiation of adipocytes.

The fact that each of the evaluated circulating EV types affected the expression of different adipogenic genes suggests differences in their cargo, which could include miRNA content. It has been demonstrated that the expression of FABP4 can be inhibited by several miRNAs, such as miR-369-5p and miR-455 (35), while ATGL can be inhibited by hsa-miR-214-3p (36). Therefore, in subsequent studies, it would be interesting to determine the cargo differences between UCBEV and peEV to identify possible mechanisms of action on MSC differentiation. It should also be emphasized that, regarding the effect of circulating EVs on adipogenesis, it is important to consider the nature and health of the donors. Thus, circulating exosomes from obese adolescents favor adipogenic differentiation more than osteogenic differentiation compared to those derived from healthy adolescents with normal weight (31).

It has been reported that the increase in adiposity in bone marrow during aging is caused by changes in the marrow microenvironment, which favor differentiation of MSCs into adipocytes rather than osteoblasts (37). Therefore, although our results do not show an effect of UCBEV and peEV on osteoblastogenesis, they have shown their ability to decrease adipogenesis. Therefore, the application of these vesicles could prevent the increase in adiposity in bone marrow and consequently favor bone formation through a potential increase in osteoprogenitors. This is supported by studies showing that intravenous injection of EVs from umbilical cord blood in old mice for two months, once a week, reduced age-related bone loss, stimulating bone formation and inhibiting bone resorption (32).

Blood vessel formation is essential in regenerative processes to provide nutrients, oxygen, and facilitate the arrival of progenitor and immune cells. Our results indicate that the evaluated circulating EVs increased angiogenesis in HUVECs. Other authors have shown an angiogenic effect of UCBEV in pigs (38) and other animal models. *In vitro* studies have shown that EVs derived from umbilical cord blood and from the mother increase angiogenesis in human microvascular endothelial cells (HMEC) (39) and HUVECs (40). Exosomes derived from serum of healthy humans aged 20-30 years also have a proangiogenic effect through the inhibition of inflammation in macrophages. Thus, local application of these exosomes along with bone grafts favored bone regeneration in a model of mandibular bone defects in rats through reduced inflammation and increased angiogenesis (41). Therefore, our results are in line with these studies and support that both healthy adult and umbilical cord blood could represent an abundant source of EVs for therapeutic purposes. Potential therapeutic applications would include those related to bone formation due to their effects on MSC differentiation and the induction of angiogenesis.

CONCLUSIONS

In conclusion, both types of EVs did not affect mineralization in MSCs differentiated into osteoblasts but decreased adipogenesis in MSCs and increased angiogenesis in HUVECs. These findings suggest that both types of EVs, from abundant sources without significant ethical issues and easy to isolate, have high potential in regenerative medicine applied to bone, inhibiting bone marrow adiposity and favoring angiogenesis.

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Review

Biogenesis of circular RNAs, biological functions, and their role in the development of osteoarthritis

Sergio Rafael Carrillo-Patiño[1,2], Fiordaliso Carolina Román-Carraro[3], Brenda Anabel López-Ruiz[4], Juan Fernando Montes-García[2,5], Martha Alicia Ballinas-Verdugo[6], Emma López-Espinosa[7], Rogelio Frank Jiménez-Ortega[1,7]

[1]Laboratory of Bone Metabolism Genomics. Instituto Nacional de Medicina Genómica (INMEGEN). Mexico City, Mexico. [2]Academic Unit of Health Sciences. Universidad ETAC Coacalco Campus. Coacalco de Berrizobal, State of Mexico, Mexico. [3]Department of Processes and Technology. Universidad Autónoma Metropolitana. Cuajimalpa Unit. Mexico City, Mexico. [4]Botanical Garden. Instituto de Biología. Universidad Nacional Autónoma de México (UNAM). Mexico City, Mexico. [5]Genetics Laboratory. Unit of Morphology and Function. Faculty of Higher Studies Iztacala UNAM. Tlalnepantla de Baz, State of Mexico, Mexico. [6]Department of Immunology. Instituto Nacional de Cardiología Ignacio Chávez. Mexico City, Mexico. [7]Unit of Rehabilitative Human Acupuncture. Universidad Estatal del Valle de Ecatepec (UNEVE). Ecatepec de Morelos, State of Mexico, Mexico

Abstract

Osteoarthritis (OA) is the most common joint disease worldwide, and its progression is irreversible. Currently, the processes that lead to the development of this condition are not fully understood. However, evidence suggests that epigenetic mechanisms could play a key role in the development of this disease. Among these mechanisms are non-coding RNAs (ncRNA), which include circular RNAs (circRNAs), a class of RNA with a covalently closed loop structure that is highly stable and conserved. Most circRNAs present characteristics of abundance, stability, and conservation and often exhibit a tissue- or stage-specific manner of development with unique structures, and their deregulation has been associated with changes to various biological processes such as tumorigenesis, growth, invasion, metastasis, apoptosis, and vascularization, favoring the development of different diseases including OA. Recent studies suggest that circRNAs play key roles by acting as microRNA (miRNA) sponges or protein scaffolds, proposing them as promising biomarkers with potential for prevention, diagnosis, and therapeutic targets for the treatment of OA. Therefore, this review presents the concept and the main characteristics of circRNAs and describes the main biological functions and clinical relevance of this type of RNA, as well as their expressions and regulatory mechanisms, which provide evidence of the possible uses in the diagnosis and treatment of OA.

Keywords:
Osteoarthritis. circRNAs. ceRNAs. miRNAs. Splicing. Bone metabolism.

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Correspondence:
Rogelio Frank Jiménez-Ortega. Unit of Rehabilitative Human Acupuncture. Universidad Estatal del Valle de Ecatepec (UNEVE). Ecatepec de Morelos, State of Mexico. Mexico
e-mail: rogeliofrank.jimenez@uneve.edu.mx

INTRODUCTION

Bones are dynamic organs that undergo constant changes throughout the life of vertebrates. This process is known as bone remodeling and is responsible for maintaining the structural integrity of the skeletal system while also contributing metabolically by facilitating calcium and phosphorus absorption in the body. The bone remodeling mechanism under normal conditions involves the maintenance of homeostasis in phosphate-calcium metabolism. It can also be induced by tissue damage, activating the process of resorption of damaged or deteriorated bone, followed by the formation and deposition of new bone material (1). Both processes are functionally balanced in the creation and maintenance of an optimal functional structure of the skeletal system in accordance with functional demands. However, disturbances in the physiological balance of these processes can manifest as pathological osteogenesis, such as abnormal bone growth or bone protrusions on the articular surfaces, known as osteophytes, which can affect joints and lead to the development of OA (2). Physiological and pathological osteogenesis are similar processes based on the basic principles of bone tissue biology: osteoinduction and osteoconduction. The principle of osteoinduction is based on molecular factors acting on the proliferation and differentiation of the bone cell phenotype (3), while osteoconduction is based on the continuous internal reconstruction of bone tissue and the skeletal tissue throughout life. Osteoconduction aims to maintain optimal skeletal architecture in response to mechanical, static, and humoral circumstances during prenatal, neonatal, and childhood stages, which are periods of bone development and growth (4). To understand the onset of OA progression through bone remodeling, it is necessary to understand the physiological limits and differences between the compartments of the subchondral bone, which are described below. Subchondral bone refers to any bone located distal to the calcified cartilage, beneath which is a cortical bone plate 1 to 3 mm thick, identical to cortical bone in other skeletal locations but less rigid than diaphyseal cortical bone (5). Subchondral bone is located distally to the subchondral trabecular bone, which is more porous, metabolically active, and has lower volume, density, and rigidity than the cortical plate. Therefore, subchondral bone refers to both subchondral trabecular bone and the cortical plate without making an adequate distinction between their differences (6) (Fig. 1A). Therefore, it is important to distinguish between both bone regions, as in advanced OA, the changes occurring in the subchondral cortical plate differ from those occurring in the trabecular bone (7). In OA, it has been observed that the calcified cartilage separating the subchondral cortical bone from the non-mineralized articular cartilage may contribute to the development of sclerosis, which is seen in advanced stages of this disease and is referred to as the "tidemark" (8). With the progression of OA, the process of endochondral ossification establishes at the tidemark, which can be histologically detected through the presence of multiple marks. As a result of this renewed development process, cartilage becomes more calcified than bone, thickening, causing the underlying trabecular cartilage layer to be unable to produce enough new cartilage to maintain its volume, thus becoming thinner (9). In adult humans, approximately 25% of trabecular bone tissue and about 3% of compact bone tissue are replaced annually through bone remodeling, allowing bones to renew and respond in the medium and long term to the mechanical and metabolic needs of the body to optimize the architecture of the skeletal system and adapt it to biomechanical conditions. This process occurs throughout life but reaches its peak bone mass in the third decade, which is maintained with slight variations until the age of 50 (10,11). Currently, the absence of a specific molecular signature with prognostic importance in OA treatments motivates the scientific community to identify new biomarkers for the development of more effective therapeutic and diagnostic strategies. In the last decade, several studies have reported that osteoclastogenesis, osteoblastogenesis, and chondrogenesis may be regulated not only by genetic factors but also by epigenetic factors, where alterations in these mechanisms may underlie diseases associated with changes in bone remodeling (12). Epigenetics is the study of hereditary and reversible changes in gene expression that do not affect DNA sequences, which include mechanisms such as DNA methylation, histone remodeling, and non-coding RNAs, including circRNAs (13). circRNAs are a class of non-coding RNAs produced through a non-canonical splicing event known as "backsplicing," where a downstream splice donor site covalently binds to an upstream splice acceptor site, creating a circRNA (Fig. 1B). Some circRNAs have been identified through high-throughput technologies such as RNA sequencing (RNA-seq) and specific bioinformatics tools that relate expression patterns in specific tissues. Most circRNAs originate from protein-coding genes and consist of one or more exons. The RNA products resulting from alternative splicing types can be found within circRNAs, some of which contain exons not included in linear transcripts (14). circRNAs are molecules that lack polyadenylation (Poly A) and the 7-methylguanosine (7mG) cap, and like messenger RNAs (mRNAs), circRNAs are located in the cytoplasm (15). During splicing, intron retention caused by failure in the intronic loop disassembly during the canonical pathway may lead to the production of circRNAs that contain sequences derived from both exons and introns. Recently, circRNAs containing both types of sequences (exon-intron circRNA) have been described, as shown in fiugre 1C (16).

In recent years, it has been shown that circRNAs function as microRNA (miRNA) sponges, implying a higher level of regulation, since miRNAs are negative

regulators of various genes, such as genes encoding transcription factors. However, the practical use of circRNAs as regulators of specific miRNAs is still under development, as despite their high presence in nature, most mammals show low levels of circRNAs, representing 5-10% of total linear RNA, which implies relatively fewer miRNA binding sites (17).

Recently, numerous OA-related circRNAs have been identified, suggesting that these molecules may play an important role in the onset and progression of this disease and could have clinical applications as potential markers for OA progression. Therefore, this review describes the most recent advances in the biogenesis and biology of circRNAs, as well as the biological processes in which they are involved.

PROPERTIES OF circRNAs

According to their formation type, circRNAs can be categorized into 3 main groups: exonic circRNA (EciRNA), exon-intron circRNA (EIciRNA), and circular intronic circRNA (ciRNA) (18,19). circRNAs are derived from canonical splicing sites, and this has been demonstrated through mutational analysis in circRNA expression vectors, where inhibition of spliceosome assembly has shown that circRNA biogenesis is dependent on the canonical splicing machinery (20). Most circRNAs are of the EciRNA type, which are non-collinear single-chain molecules made up of one or more exons (22). circRNAs are expressed at lower levels than linear RNAs, so the biological relevance of circRNAs was underestimated until the advent of next-generation sequencing (NGS),

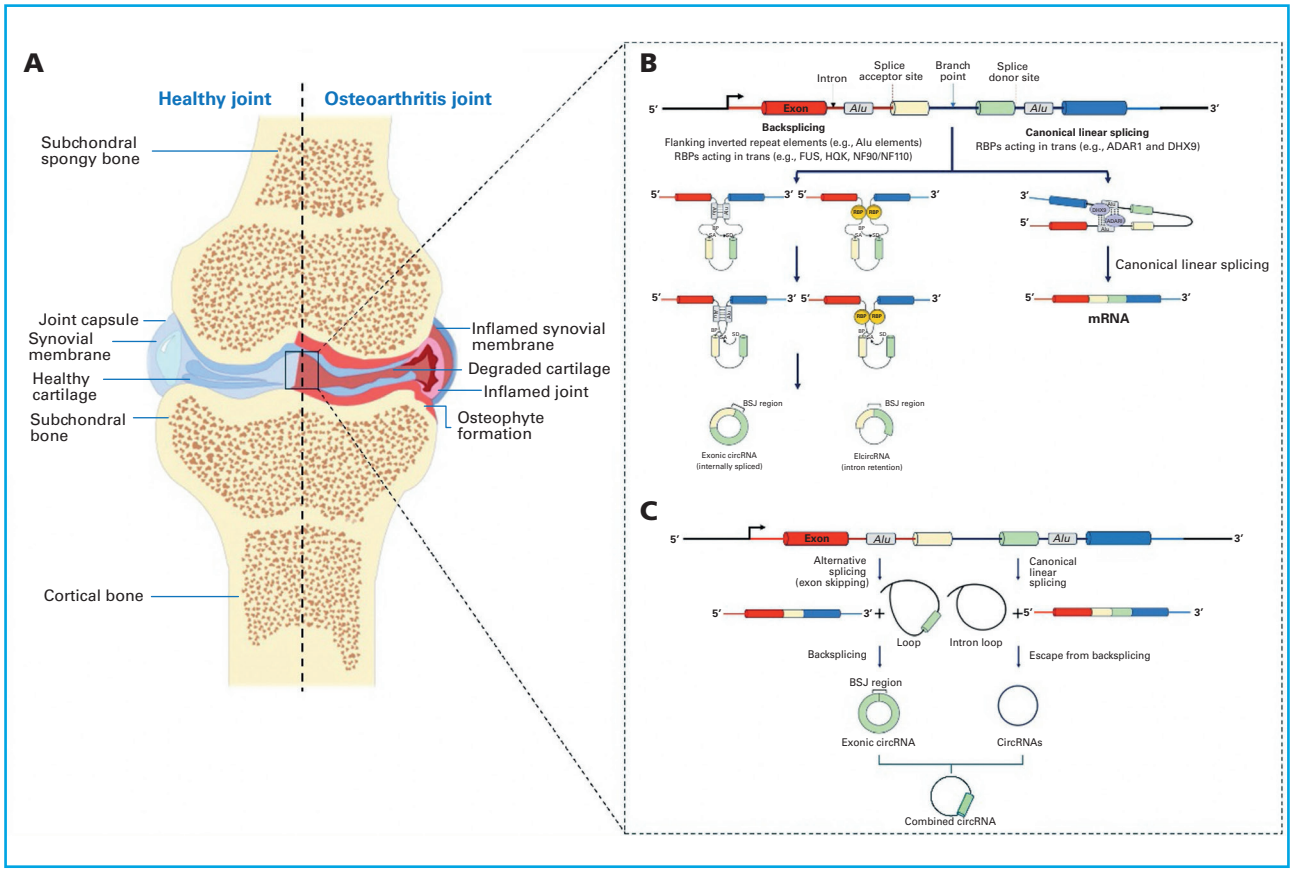

Figure 1. Structure of cartilage and biogenesis of circRNA involved in OA. A. Different stress stimuli that can activate chondrocytes, leading to the loss of phenotypic stability and extracellular matrix degradation in cartilage. On the left, the structures that make up a healthy joint are shown, while on the right, structures affected by OA are shown, such as inflammation and cartilage degradation. B. The biogenesis of circRNA is shown, where the backsplicing mechanism is on the left and linear splicing is on the right. Backsplicing occurs during the transcription of most human genes and is favored by long flanking introns, inverted repeat elements (Alu elements), and RNA-binding proteins (RBPs) that act in trans. RNA-binding proteins such as FUS, quaking protein (HQK), NF90, and NF10 are protein products of the interleukin enhancer-binding factor gene that promote backsplicing. On the other hand, canonical linear splicing (right) is favored by exons surrounded by short flanking introns and by introns bound by RBPs that act in trans, adenosine deaminase acting on double-stranded RNA (ADAR1), and ATP-dependent RNA helicase A (DHX9). RBP proteins interrupt base pairing between inverted repeat elements, allowing the splicing machinery to generate a linear mRNA. C. circRNAs can be generated from splice intermediates known as loop precursors, which are created by an exon skipping event during linear splicing (left) or from intronic loop precursors that escape the backsplicing step of canonical linear splicing (right).

which allowed their efficient detection. Some characteristics of circRNAs are their high stability, conservation, and tissue specificity (22), making it plausible to consider these molecules as potential biomarkers for the early detection of diseases such as OA and for their potential as targets in clinical research.

BIOGENESIS OF circRNAs

circRNAs are molecules derived from canonical splicing sites that depend on the splicing machinery, and it has been shown that inhibition of the spliceosome by reducing small nuclear ribonucleoprotein U2 (snRNP U2) elements, a component of the spliceosome, significantly increases the ratio of circRNA to linear RNA (23). Therefore, when pre-mRNA processing events slow down, the nascent RNA can be directed to alternative pathways that facilitate backsplicing. This mechanism was supported by a study in *Drosophila melanogaster* where it was demonstrated that the loss of splicing factors increases circRNAs formation (24). Backsplicing involves the formation of loops from the intron sequences flanking the upstream splice donor site. These formations can be regulated by base pairing between inverted repeat elements (Alu elements) found in the upstream and downstream introns or by the dimerization of RNA-binding proteins (RBPs) such as quaking protein (HQK) or FUS45, which bind to specific sites in the flanking introns (25,26). However, work in *D. melanogaster* suggests that the biogenesis of many circRNAs results from a combination of cis-elements and trans-acting splicing factors, including heterogeneous nuclear ribonucleoproteins (hnRNPs) and proteins containing long serine and arginine residues (SR) (24). On the other hand, adenosine deaminase enzymes (ADAR) prevent the activation of the innate immune system by editing adenosine to inosine in endogenous double-stranded RNA (dsRNA) (27), while RNA helicase DHX9 suppresses circRNA biogenesis that depends on base pairing between inverted repeats, specifically the editing of adenosine to inosine and the development of helicoidal dsRNA structures that prevent loop formation in the intronic sequences (Fig. 1B) (28). Furthermore, the products of the ILF3 enhancer-binding factor, NF90 and NF110, play a role in host antiviral mechanisms and can promote circRNA production through stabilization of intronic RNA pairs (29). An event where alternative exons are removed from the final mRNA product and end up contained within the spliced loop is referred to as "exon skipping," where the loop undergoes internal backsplicing, leading to the formation of circular RNA (30). Finally, intronic loops released from disassembly can lead to the formation of circRNAs.

The most abundant circRNAs usually have long introns flanking the exons involved in backsplicing and are often derived from genes with constitutive promoters (31) (Fig. 1C). Additionally, changes in epigenetic mechanisms within histones and gene bodies can affect alternative splicing and have a direct impact on circRNA biogenesis (32). It has been reported that the deletion of DNA methyltransferase 3B (DNMT3B) causes changes in the expression of host genes in a linear form (33), meaning that methylation can modify gene expression during circRNA biogenesis according to the genetic context. In this regard, circRNAs can directly influence the epigenetic regulation mechanisms of the promoter regions of their host genes. It has been shown that through the transcription factor Friend leukemia integration 1 (FLI1), circRNA FLI1 is produced, an exonic circular RNA (FECR1) related to the development of breast cancer, which induces the demethylation of CpG sites in cis by recruiting methylcytosine dioxygenase (TET1), an Fe(II)/2-oxoglutarate-dependent dioxygenase that induces active DNA demethylation (34). Finally, it has been reported that the mean transcription elongation rate is much higher in circRNA-producing genes than in genes that do not produce them (35).

FUNCTION OF circRNAs AS SPONGES

The localization of circRNAs within the cytoplasm and cellular stability suggests that these molecules may act as competitive endogenous RNAs (ceRNAs). A recent study reported that the gene for the 1 antisense RNA protein related to cerebellar degeneration (CDR1as) is involved in the degeneration of cerebellar neurons and produces around 70 highly conserved miRNA target sites with the ability to inhibit mRNA activity (Fig. 2A), demonstrating that circRNAs can function as miRNA sponges, as well as proteins dependent on RBPs (36) (Fig. 2B).

It has been reported that the CDR1as gene contains miR-7 binding sites, whose interaction results in the positive regulation of the expression of the downstream target gene of miR-7, while suppression of circCDR1as results in the negative regulation of downstream target genes of miR-7, including phosphatidylinositol 3-kinase (PI3K) (37), which plays a key role in the subchondral tissue of mice with OA, promoting osteogenic differentiation and osteoblastic proliferation, resulting in the formation of aberrant bone tissue (38,39).

circRNAs AS ENHANCERS OF PROTEIN FUNCTION

Most circRNAs can be localized in the cytoplasm in the form of exons. However, a different type of circRNAs with distinct properties, such as EIciRNA, tends to enrich in the nucleus to promote transcription through RNA Pol II. For example, ci-ankrd52

promotes transcription through its binding with RNA Pol II, while its suppression results in reduced expression of its parental gene (40). On the other hand, EIciRNA such as CircEIF3J and CircPAIP2, located in the nucleus, tend to bind to the U-small ribonucleoprotein and subsequently to RNA Pol II to regulate gene expression (41) (Fig. 2C). Furthermore, various reports have shown that circRNAs can recruit proteins to specific locations and have cis-regulatory effects on the transcription of coding genes (42) (Fig. 2D). In recent years, advances in circRNAs research have demonstrated that these molecules play an important role in the pathogenesis of OA, especially in the endogenous competitive mechanisms regulated by circRNAs.

MECHANISM OF TRANSLATION INDEPENDENCE

Some circRNAs are translatable independently through two main mechanisms, even without the presence of the 7mG-5′ cap (43). The first mechanism involves the internal ribosomal entry site (IRES), which is a relatively short RNA sequence segment that regulates ribosome binding to RNA without relying on the 7mG-5′. It has been reported that circFBXW7 contains an open reading frame (ORF) initiated by IRES, allowing translation initiation independently of the presence of 7mG-5′, and this translation increases the expression of the tumor suppressor gene known as F-box and WD-7 repeat protein (FBXW7), inducing the ubiquitination degradation of c-Myc in breast cancer (44) (Fig. 2E). The second mechanism involves an N6-methyladenosine (m6A)-dependent form of circRNA that can be translated even without IRES sequences. Methylation at the N6 position of adenosine in RNA is a dynamic and reversible modification, with 499 associations related to circRNA identified, of which 25 were validated by seq-RNA (45). It was shown that RNA translation is promoted by circRNAs through the demethylation of the gene for Fat-Mass-and-Obesity-Associated-Gene. Therefore, it has been suggested that the translational function of circRNA may be common in the human transcriptome. However, depending on the specific circular structure of the circRNA, these IRES- and m6A-induced translation mechanisms need to be addressed (Fig. 2F).

MECHANISMS INVOLVED IN circRNAs-REGULATED OA

Initially, OA was thought to be the result of anatomical and functional joint injuries derived from cartilage degradation. Recently, it has been reported that inflammatory mediators produced by the synovial membrane, cartilage, and subchondral bone are responsible for the pathogenesis of OA (46). It has also been reported that synovial joints are filled with inflammatory cells, including T and B cells, which interact with other joint cells, constituting a vicious cycle where, during the early stages of OA, chondrocytes are activated in a compensatory manner to enhance extracellular matrix synthesis. Additionally, they produce and release proinflammatory cytokines, such as tumor necrosis factor-α (TNF-α) and interleukin 6 (IL-6), which damage cells and the extracellular matrix, while degradation products stimulate inflammation. At this point, circRNA can regulate inflammatory reactions through ceRNA-induced mechanisms involved in OA, combining apoptosis, oxidative stress, autophagy, mechanical stress, and cell proliferation (47).

OVERREGULATED circRNAs INVOLVED IN OA

circ-NFKB1

This circRNA is derived from reverse splicing of exons 2, 3, 4, and 5 of the gene encoding the nuclear factor NF-kappa B (NFKB) on chromosome 4, lacks a poly-A tail, and has recently been reported to be upregulated and involved in the regulation of the NFKB signaling pathway in inflamed chondrocytes and cartilage with OA. Inhibition of circ-NFKB1 prevents extracellular matrix (ECM) catabolism and restores ECM anabolism damaged by IL-1β activity, while ectopic expression of circ-NFKB1 promotes chondrocyte degradation *in vitro.* In murine models, intra-articular injections of circ-NFKB1 adenovirus in mice trigger spontaneous cartilage loss, promoting OA development. Therefore, it is suggested that circ-NFKB1 interacts with α-enolase (ENO1), regulating the expression of its parental gene, NFKB1, maintaining NF-κB signaling pathway activation in chondrocytes (48).

circMELK

This circRNA exhibits abnormal upregulation and has been reported to promote autophagy and apoptosis in human chondrocytes, leading to OA development. While inhibition of circMELK inhibits apoptosis and enhances chondrocyte autophagy, preventing OA progression in articular cartilage, this could be a promising therapeutic strategy for OA treatment. In work by Zhang et al. (2022), it was reported that circMELK is a sponge for miR-497-5p, which in turn regulates MYD88 expression in IL-1β-stimulated chondrocytes. Upregulation of MYD88 expression triggers NF-κB pathway activation, promoting apoptosis and inhibiting autophagy in chondrocytes, leading to OA development. Therefore, circMELK expression promotes chondrocyte apoptosis and inhibits autophagy in OA by activating the MYD88/NF-κB signaling axis through miR-497-5p (49).

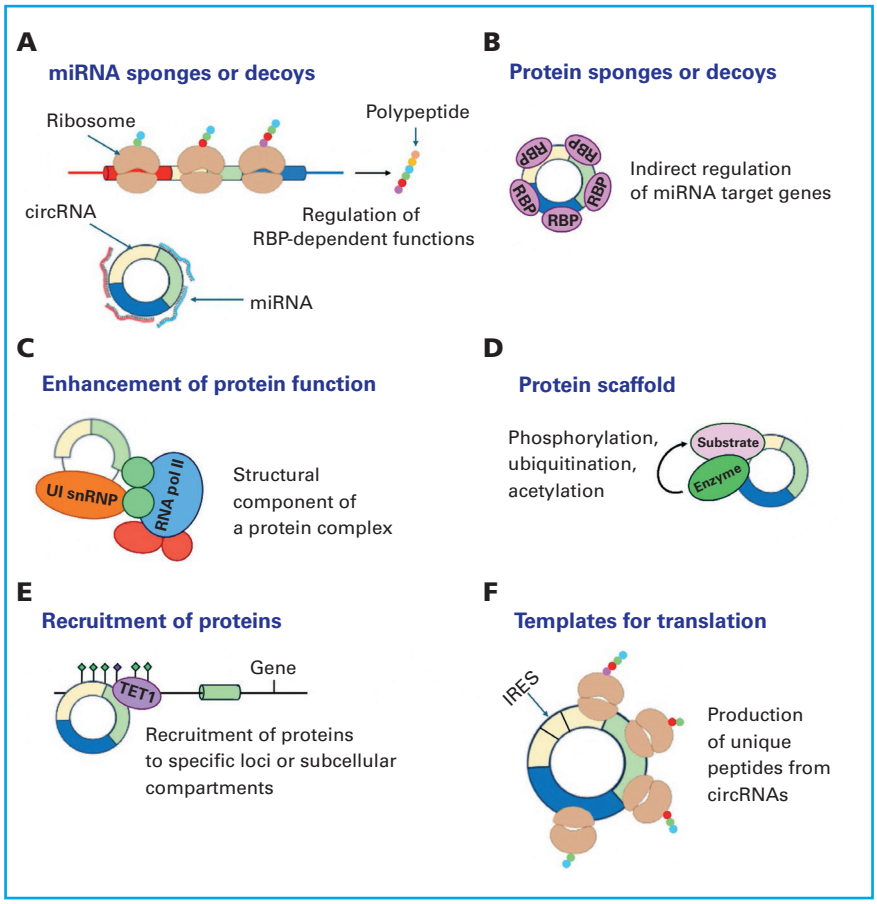

Figure 2. Mechanisms of circRNAs functions. A. circRNAs can function as sponges or decoys for miRNA, protecting target mRNAs from degradation induced by these molecules. B. circRNAs contain RBP-binding regions that can function as sponges or decoys for these proteins and directly regulate their functions. C. circRNAs can interact with specific proteins and enhance their function. The RNA polymerase II contains small nuclear ribonucleoprotein U1 (snRNP). D. Some circRNAs function as protein scaffolds, facilitating the localization of enzymes (phosphatases, acetylases, and ubiquitin ligases) and their substrates to influence the reaction kinetics. E. circRNAs can recruit specific proteins to certain loci or subcellular compartments. For example, the circRNA FLI1 (FECR1) recruits the methylcytosine dioxygenase TET1 to the promoter region of its own host gene. F. circRNAs with internal ribosome entry site (IRES) elements and AUG sites can be translated under certain circumstances, resulting in unique peptides.

circ_0136474

This circRNA has been implicated in OA development. Cheng et al. (2023) reported that the upregulation of this circRNA inhibits cell proliferation, restricts apoptosis, ECM degradation, and the inflammatory response by regulating the miR-140-3p/MECP2 axis in IL-1β-stimulated CHON-001 cells. The authors observed an increase in circ_0136474 expression levels in cartilage tissue samples with OA and suggest that IL-1β may modulate inflammation in OA development. On the other hand, inhibition of circ_0136474 expression promoted cell proliferation and reduced apoptosis, ECM degradation, and the inflammatory response in chondrocytes with OA. Thus, this may be a new mechanism to help understand OA pathogenesis and provide a potential therapeutic target or biomarker (50).

circ_0022383

This circRNA is involved in OA development. It is predominantly in the cytoplasm of chondrocytes and functions as a sponge for miR-3619-5p, forming a feedback loop, circ_0022383/miR-3619-5p/SIRT1. In a study by Qian et al. (2022), it was observed that inhibition of miR-3619-5p protects chondrocytes from IL-1β-induced damage, while positive regulation of miR-3619-5p inhibits the protective action of circ_0022383 on chondrocyte function. SIRT1 is a NAD^+-dependent deacetylase involved in regulating many physiological activities, including cellular senescence, inflammation, apoptosis, and metabolism, promoting longevity and counteracting age-related diseases (51,52). Of note, many studies have shown a chondroprotective role for SIRT1. However, its haploinsufficiency could cause OA development by inducing excessive apoptosis and catabolic responses. The authors explain that a decrease in SIRT1 in OA patients and primary chondrocytes stimulated with IL-1β counteracts the inhibitory action of miR-3619-5p deletion on chondrocyte ECM degradation, inflammation, and apoptosis, suggesting the possible involvement of the miR-3619-5p/SIRT1 axis in OA development (53). Other overregulated circRNA related to OA development are shown in table I (48-58).

UNDERREGULATED circRNAs INVOLVED IN OA

circ_0114876

It is a circRNA derived from the inverse splicing of the transcription of the protein tyrosine phosphatase re-

Table I. Overregulated circular RNAs involved in the development of OA in humans

circRNA	Mechanism	Effect	Model	Reference
circ_0008012	NFKB1	Chondrocyte degradation	Human chondrocytes	(48)
circ_0009127	miR-497- 5p/MYD88/NF-κB	Induction of apoptosis, inflammation, and autophagy in chondrocytes	Human chondrocytes	(49)
circ_0136474	miR-766-3p/DNMT3A miR-140-3p/MECP2	Induction of apoptosis and oxidative stress in chondrocytes	CHON-001	(50)
circ_0022383	miR-3619-5p/SIRT1	Inhibition of apoptosis and extracellular matrix degradation	Human chondrocytes	(51,52)
circ_0092516	miR-337-3p/PTEN	Chondrocyte differentiation and inhibition of apoptosis	Human chondrocytes	(53)
circ_0000205	miR-766-3p/ADAMS5	Reduction of proliferation and inhibition of apoptosis	Human chondrocytes	(54)
circ_0032131	miR-502-5p/ADAMTS5 miR-145/HGF/c-MET miR-140-3p/ADAM10	Inhibition of proliferation and migration	Human chondrocytes	(55)
circ_0043947	miR-671-5p/RTN3	Induction of apoptosis and inflammatory response	Human chondrocytes with IL-1β induced damage	(56)
circ_0005526	miR-142-5p/TCF4	Induction of apoptosis and inflammatory response	Human chondrocytes with OA	(57)
circ_SPG11	miR-337-3p/ADAMTS5 miR-665-3pGREM1	Induction of apoptosis and extracellular matrix degradation	Human chondrocytes with OA0	(58)

ceptor type A (PTPRA). Its negative regulation is associated with cell proliferation, inhibition of apoptosis, and promotion of the inflammatory response. In a study by Ou et al. (2023), the action mechanism of circ_0114876 was explored, and it was observed that this circRNA shares binding sites with miR-1227-3p, which targets ADAM10, a gene involved in regulating chondrocyte injury and extracellular matrix loss, partially alleviating the effects of miR-1227-3p. Therefore, the downregulation of circ_0114876 could promote the development of OA (59).

circ_0004662

This circRNA is involved in the progression of OA, as it has been observed in human chondrocytes that circ_0004662 regulates the expression of miR-424-5p, which targets vascular endothelial growth factor A (VEGFA), crucial for chondrocyte survival. Negative regulation of this circRNA would allow the expression of miR-424-5p and, consequently, the negative regulation of VEGFA, promoting the development and progression of OA (60).

circCDK14

Negative regulation of circCDK14 has been involved in the development of OA since it contains binding sites for miR-1183, which regulates the expression of Krüppel-like factor 5 (KLF5), a gene involved in various cellular functions, including proliferation, apoptosis, autophagy, pluripotency, invasion, and migration. Its dysregulation has been linked to pathological processes in bones and joints. Thus, the negative regulation of circCDK14 would allow the expression of miR-1183, inducing the negative regulation of KLF5, favoring the development of OA (61).

circ_0020093

Negative regulation of circ_0020093 in chondrocytes has been associated with the development of OA. In a C28/I2 cell model, it was observed that circ_0020093 regulates the expression of miR-181a-5p, which targets the gene related to erythroblast transformation (ERG). This gene has been associated with joint formation as it drives chondrocytes to a permanent developmental

Table II. Underregulated circular RNAs involved in the development of OA in humans

circRNA	Mechanism	Effect	Model	Reference
circ_0114876	ADAM10 and miR-1227p	Regulation of proliferation and apoptosis	Chondrocytes from OA patients	(59)
circ_0004662	miR-424-5p/VGFA	Regulation of proliferation and inhibition of apoptosis	Human chondrocytes with IL-1β induced damage	(60)
circ_0001721 (circCDK14)	miR-1183/KLF5	Regulation of proliferation and inhibition of apoptosis	Human chondrocytes with IL-1β induced damage	(61)
circ_0020093	181a-5p/ERG	Regulation of apoptosis and extracellular matrix degradation	Human chondrocytes with IL-1β induced damage	(62)
circPDE4D	miR-4306/SOX9	Regulation of apoptosis and extracellular matrix degradation	Human chondrocytes	(63)
circ_0072688 (circADAMTS6)	miR-324-5p/PI3K/AKT/mTOR	Regulation of proliferation and extracellular matrix degradation	Chondrocytes from OA patients	(64)

pathway, turning them into joint-forming cells. Therefore, the negative regulation of circ_0020093 could allow the upregulation of miR-181a-5p, negatively regulating ERG and decreasing cartilage formation (62). Other subregulated circRNAs related to the development of OA are shown in table II (59-64).

CONCLUSIONS

A large number of studies have been reported on the functional role of circRNAs in the development of OA, aiming to demonstrate their performance as regulatory molecules of this pathology. However, many deficiencies remain in current research. For example, the functions of circRNAs have been limited to acting as miRNA sponges, without considering the characteristics of various diseases. Despite these limitations, the potential of circRNAs has been confirmed in various conditions, indicating the direction for studying the role of circRNAs in bone metabolism-related diseases. circRNAs are essential competitive inhibitors of miRNAs, and their high level of conservation and stability could allow more effective treatment of OA. However, the mechanism of circRNAs should not be limited to ceRNA, as they can play other roles such as protein interactions and regulation of transcription/translation. Therefore, circRNAs may regulate biological processes such as proliferation, apoptosis, differentiation, chondrocyte autophagy, extracellular matrix degradation, oxidative stress processes, and inflammatory processes, all of which are related to OA. On the other hand, circRNAs can also modulate the intra-articular environment, such as the synovial membrane, meniscus, and subchondral bone, and therefore may be considered as potential biomarkers in procedures such as liquid biopsy for OA detection. Finally, despite the availability of multiple studies, many deficiencies remain concerning the mechanisms, construction in animal models, and disease heterogeneity.

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Special Article

Delphi consensus on the management of osteoporotic patients in primary care

Cristina Carbonell-Abella[1], Rafael Manuel Micó-Pérez[2], Francisco Vargas Negrín[3], José Carlos Bastida-Calvo[4], Pilar Aguado Acín[5]

[1]Family and Community Medicine. Vía Roma Health Center. Universitat de Barcelona. Barcelona, Spain. [2]Family and Community Medicine. Fontanars dels Alforins Center. Xátiva-Ontinyent Health Department. Valencia. Spanish Society of Primary Care Physicians (SEMERGEN). [3]Family Medicine. Dr. Guigou Health Center. Tenerife, Spain. Working Group on Rheumatic and Musculoskeletal Diseases. Spanish Society of Family and Community Medicine (semFYC). [4]Family and Community Medicine. Marín Health Center. Pontevedra, Spain. National Coordinator, Osteoarticular Pathology/ Osteoporosis Group, Spanish Society of General and Family Physicians (SEMG). [5]Rheumatology Department. Hospital Universitario La Paz. Madrid, Spain. Spanish Society for Bone and Mineral Metabolism Research (SEIOMM)

Abstract

Introduction: numerous clinical guidelines exist on the identification, treatment, and follow-up of patients with osteoporosis, but they show significant differences in their recommendations, often leading to confusion and uncertainty among healthcare professionals.

Objective: to reach a consensus and unify criteria regarding the identification, evaluation, treatment, follow-up, and role of patients with osteoporosis.

Methods: after reviewing major guidelines on osteoporosis management, an expert committee identified aspects with the most controversy or the least evidence and developed a Delphi questionnaire with 92 statements grouped into the following sections: 1. Identification and evaluation; 2. Treatment; 3. Monitoring and follow-up; 4. Referral criteria; and 5. Patient perspective.

Results: consensus was reached on 77 statements (83.7 %). Panelists agreed on the importance of properly identifying this condition by stratifying patients according to their fracture risk, considering factors such as bone mineral density, age, sex, fall risk, family and personal history of fractures, and other clinical factors. Emphasis was also placed on the importance of exercise and nutrition, as well as the timing, duration, and potential treatment holidays of pharmacological therapy, with individualization as needed.

Conclusions: family physicians recognize the importance of identifying, evaluating, treating, and monitoring patients with osteoporosis to reduce the risk of fragility fractures. However, some aspects still cause confusion and require further scientific evidence.

Keywords:
Primary care.
Consensus. Delphi.
Osteoporosis.
Patient.

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Correspondence:
Cristina Carbonell-Abella. Family and Community Medicine. Vía Roma Health Center. Barcelona, Spain
e-mail: ccarbonell@ub.edu

INTRODUCTION

Osteoporosis affects millions of people worldwide, and its prevalence is expected to increase as the population ages. In Spain, it represents a significant challenge, with a considerable number of individuals at risk of fractures and related complications, leading to substantial health, economic, and social costs.

Despite its severity, clinical practice guidelines issued by scientific societies worldwide regarding the identification, treatment, and follow-up of osteoporosis patients exhibit significant differences in their recommendations. This variability can create confusion and uncertainty among healthcare professionals and, ultimately, impact patient management and the quality of care they receive (1-5).

Therefore, this Delphi consensus was developed to discuss, agree upon, and unify criteria for the identification, evaluation, treatment, and follow-up of osteoporosis patients, as well as the relationship between primary and hospital or secondary-level care.

METHODS

STUDY DESIGN

We designed this study using the Delphi method, a structured communication technique that gathers expert opinions on complex or controversial topics with insufficient or uncertain evidence (6,7). Additionally, this method allows the exploration and unification of expert opinions while avoiding the biases and logistical challenges of in-person consensus meetings, such as influence bias or lack of confidentiality. The study was conducted in several phases: 1) Formation of a scientific committee of experts representing the Spanish Society of Primary Care Physicians (SEMERGEN), the Spanish Society of Family and Community Medicine (semFYC), the Spanish Society of General and Family Physicians (SEMG), and the Spanish Society for Bone and Mineral Metabolism Research (SEIOMM); 2) Review of major national and international reference guidelines on osteoporosis diagnosis and treatment to identify controversial or low-evidence aspects; 3) Creation of a Delphi questionnaire with statements addressing the issues identified in the previous step; 4) Two successive rounds where a panel of experts rated their level of agreement with the proposed statements; and 5) Compilation, analysis, and discussion of the results.

PARTICIPANTS

The study involved a scientific committee, an expert panel, and a technical team. The scientific committee consisted of one coordinator and four experts in osteoporosis treatment. The expert panel was selected by the scientific committee from members of medical societies, ensuring adequate territorial representation across Spain. The participating medical societies were SEMERGEN, semFYC, SEMG, and SEIOMM, with the latter leading the project. The panel mainly included primary care physicians and other specialists involved in osteoporosis and fragility fracture care.

THE DELPHI QUESTIONNAIRE

After reviewing and discussing controversial or low-evidence aspects of major clinical practice guidelines, the scientific committee developed a Delphi questionnaire comprising 92 statements grouped into the following sections: 1. Identification and evaluation (20 statements); 2. Treatment (47 statements), further divided into subcategories: exercise (3 statements), nutrition (8 statements), pharmacotherapy (12 statements), treatment initiation (15 statements), treatment duration (2 statements), and treatment holidays (7 statements); 3. Monitoring and follow-up (8 statements); 4. Referral criteria (11 statements); 5. Patient perspective (6 statements).

A 9-point Likert scale (7) was used for assessment, structured into three groups based on agreement level: 1-3: Disagreement; 4-6: Neutral (neither agreement nor disagreement); 7-9: Agreement.

DELPHI CONSENSUS PHASES

Following the Delphi methodology (8), the questionnaire was sent to the expert panel for response. In the 1st round (May-June 2024), panelists completed the online questionnaire and had the option to add open-text comments. The technical team analyzed and presented first-round results using bar charts to facilitate individual feedback and clarifications. Non-consensus statements were re-sent to panelists for evaluation in a 2nd round (June-July 2024). Results were then tabulated and descriptively analyzed, concluding with a final meeting of the scientific committee to discuss findings.

ANALYSIS AND INTERPRETATION OF RESULTS

To analyze the opinions of the expert panel and the type of consensus reached for each statement, the median and interquartile range of the scores obtained for each statement were used. Consensus was considered to have been reached for any statement when two-thirds or more of the respondents (≥ 66.7 %) scored within a 3-point range (1-3 or 7-9) containing the me-

dian. The type of consensus for each statement was determined by the median score value. Consensus in agreement was indicated when the median score was ≥ 7, and consensus in disagreement was indicated when the median score was ≤ 3. No consensus was considered to have occurred when one-third or more of the panelists (≥ 33.3 %) scored in the 1-3 range and another third or more in the 7-9 range. When the median score fell within the 4-6 range, the statements were considered uncertain for a representative majority of the group.

RESULTS

DELPHI CONSENSUS

Of the 76 experts consulted, 72 completed the 1st round and 66 the 2nd round of the Delphi consensus without proposing new statements. In the 1st round, consensus was reached on 61 of the 92 statements, all in agreement. The remaining 31 statements that were not agreed upon were sent back to the panelists for reconsideration in a 2nd round, in which 16 were agreed upon: 15 in agreement and one in disagreement. After 2 rounds, consensus was reached on 77 statements (83.7 %): 76 in agreement (82.6 %) and 1 in disagreement (1.1 %). The remaining 15 statements (16.3 %) remained without consensus.

Figure 1 shows the results of the 2 rounds, and Tables I to V present the overall results of all the analyzed statements.

BLOCK 1. IDENTIFICATION AND EVALUATION

A total of 18 out the 20 statements proposed on the identification and evaluation of osteoporosis were agreed upon after 2 rounds, all in agreement (Table I).

Panelists widely agreed on aspects such as the lack of awareness or interest in osteoporosis in primary care (82.9 % agreement), the need to stratify patients based on fracture risk (98.7 %), and the way to assess this risk, considering bone mineral density (BMD) and other factors such as age, fall risk, and other clinical factors (> 93 %).

Additionally, they considered that the FRAX (Fracture Risk Assessment Tool) is useful for classifying patients according to fracture risk (68.7 %) but should not replace the clinician's judgment, which evaluates all risk factors collectively (92.1 %). They also agreed that FRAX underestimates the risk of major osteoporotic fractures in Spain (72.4 %). However, there was no agreement on whether FRAX's usefulness is the same for evaluating risk in men and women.

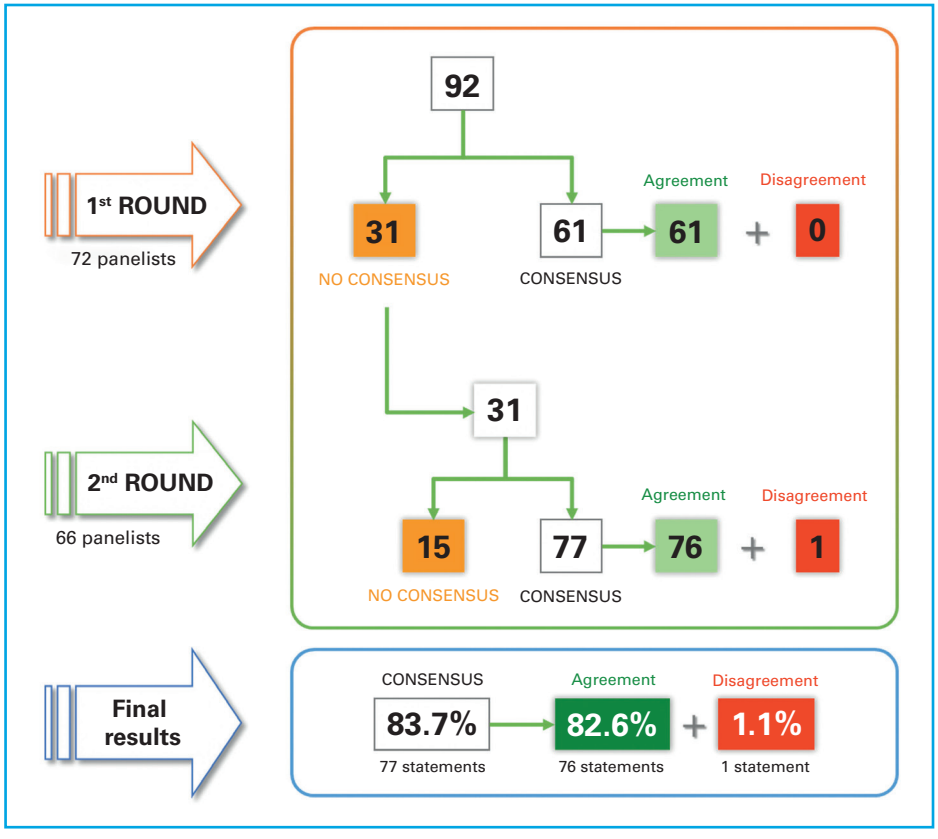

Figure 1. Main results of the Delphi consensus.

Table I. Results obtained by the expert panel after two rounds of consultations for the "Identification and Evaluation" block

Statements	Me	IQR	Agreement %
1. There is a lack of awareness of osteoporosis, lack of interest, or even ignorance of the existing protocols for its management in primary care	8	2	82.9 %
2. It is essential to stratify patients according to fracture risk as very high, high, and moderate	9	1	98.7 %
3. The evaluation of fracture risk due to fragility takes into account both bone mineral density and clinical risk factors	9	1	94.7 %
4. Fracture risk should be assessed by age and risk factors	8	2	93.4 %
5. The assessment of fall risk is relevant for evaluating fracture risk	8	2	96.1 %
6. The FRAX tool, with or without bone mineral density assessment, is useful for classifying patients according to fracture risk	7	3	68.7 %
7. The FRAX tool is useful for assessing hip fracture risk in both men and women	7	3	61.2 %
8. The FRAX tool underestimates the risk of major osteoporotic fractures in Spain	7	3	72.4 %
9. The FRAX tool should not replace the judgment and clinical discretion of the physician who considers all the patient's risk factors as a whole	8	2	92.1 %
10. A FRAX value for major fracture ≥ 10 is considered high fracture risk	7	5	69.7 %
11. A FRAX value for hip fracture ≥ 3 is considered high fracture risk	8	4	83.6 %
12. A high fracture risk is considered when there are at least two clinical risk factors strongly associated with fractures	8	2	85.5 %
13. If there are clinical risk factors, the appropriate age to perform a bone densitometry via DXA (dual-energy X-ray absorptiometry) to measure fracture risk is from 50 years old	7	6	67.2 %
14. Bone densitometry via DXA should be performed from 65 years old	6	6	55.2 %
15. In the evaluation of a patient with osteoporosis, an analytical study should be performed to rule out secondary causes	9	2	94.7 %
16. Bone turnover markers (e.g., CTX and P1NP) are not necessary in the initial evaluation of an osteoporosis patient, but they are useful if they can be measured	7	3	76.1 %
17. A lateral spine X-ray is always a test to consider in the evaluation of fracture risk	8	6	71.1 %
18. The presence of a fragility fracture should be evaluated and considered as an osteoporotic fracture and taken into account for possible treatment	9	1	98.7 %
19. The presence of a fracture in the last 2 years acts as a multiplying factor for more fractures	9	2	94.7 %
20. Fracture Liaison Services (FLS) intervene in both the identification and evaluation of patients with fragility fractures	8	3	88.2 %

IQR: interquartile range; Me: median. Green: consensus on agreement; orange: no consensus.

They agreed that DXA (dual-energy X-ray absorptiometry) should be performed to assess fracture risk starting at age 50 only if there are clinical risk factors (67.2 %), but no consensus was reached on performing it routinely or systematically from age 65 onwards.

Regarding patient evaluation, there was consensus on conducting laboratory tests to rule out secondary causes (94.7 %), the usefulness of lateral spine X-rays (71.1 %), and that bone turnover markers are not necessary but convenient if they can be measured (76.1 %).

There was a wide consensus that Fracture Liaison Services (FLS) play a role in both identifying and evaluating patients with fragility fractures (88.2 %). Other key agreements included that a fragility fracture should be

assessed and considered an osteoporotic fracture and taken into account for potential treatment (98.7 %), and that a fracture within the past 2 years significantly increases the risk of future fractures (94.7 %).

BLOCK 2. TREATMENT

A total of 37 out of the 47 statements proposed regarding osteoporosis treatment reached consensus in agreement. The remaining 10 statements were not agreed upon. This block was divided into 6 sub-sections (Table II).

Most of the proposed statements received strong support from the panelists, especially those related to exercise and treatment duration (all were agreed upon) and those regarding treatment initiation and therapeutic holidays (only one statement from each of these sub-sections was not agreed upon). The panelists expressed more uncertainty regarding statements related to pharmacotherapy.

The role of exercise in improving balance and strength, reducing falls, and thus lowering fracture risk was emphasized. There was also consensus on the role of supplementation with calcium, vitamin D, and proteins unless the patient follows a balanced diet. However, there was no agreement on the usefulness of supplementation with vitamin K and magnesium or the role of protein intake in fracture healing and faster recovery.

Regarding pharmacotherapy, consensus was reached on supplementing pharmacological treatments with calcium and vitamin D (80.3 % agreement), that not all treatments indicated for women are also indicated for men (85.1 %), considering contraindications of anabolic drugs in cancer patients (90.8 %), using anabolic drugs before antiresorptives in patients older than 75 years with severe vertebral or multiple vertebral fractures (69.7 %), and that the risk of jaw osteonecrosis with antiresorptives is low (80.3 %) but increases with exposure and bisphosphonate dosage, especially in patients receiving intravenous bisphosphonates and cancer patients (82.9 %).

Table II. Results obtained by the expert panel after 2 rounds of consultations for the "Treatment" block

Statements	Me	IIQR	Agreement %
Exercise			
21. Low-intensity physical activity (such as yoga, Pilates, or walking) can improve balance and strength, reduce falls, and consequently decrease fractures	8.5	2	96.1 %
22. The effect of protein intake alone is lower than the effect of protein intake combined with an exercise plan on physical performance	8	2	92.5 %
23. The effect of protein intake alone is lower than the effect of protein intake combined with an exercise plan on muscle strength in older adults	8	2	94.7 %
Nutrition			
24. The necessary calcium intake should be between 800-1200 mg per day	8	3	88.2 %
25. A daily calcium intake of less than 2000 mg does not increase cardiovascular risk	7	4	74.6 %
26. Supplementation with calcium, vitamin D, or protein is likely to have little effect on fracture risk in individuals with a balanced diet and no deficiency in any of the three elements	8	3	80.6 %
27. At least 800 IU of vitamin D should be supplemented daily for individuals over 65 with fracture risk and those with vitamin D deficiency, limited sun exposure, or inadequate calcium intake (< 700-800 mg daily)	8	1	90.8 %
28. Calcium should primarily be obtained through the diet, mainly from dairy products	8	3	85.5 %
29. Supplementation with vitamin K and magnesium is not useful for preventing fragility fractures	7	5	61.2 %
30. The necessary protein intake should be 1-1.5 g/kg body weight per day	8	3	81.6 %
31. Protein supplementation helps consolidate fractures and enables faster recovery	6	4	47.8 %
Pharmacotherapy			
32. All pharmacological treatments should be accompanied by calcium and vitamin D supplementation	8	3	80.3 %

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Table II (cont.). Results obtained by the expert panel after 2 rounds of consultations for the "Treatment" block

Statements	Me	IIQR	Agreement %
Pharmacotherapy			
33. The benefits of pharmacological treatment in terms of anti-fracture efficacy or bone mineral density increase are the same in both women and men with primary osteoporosis	6	5	53.7 %
34. All osteoporosis treatments indicated for men are also indicated for women, but not all treatments indicated for women are approved for men	8	4	85.1 %
35. All osteoporosis treatments are indicated for the treatment of glucocorticoid-induced osteoporosis	2	3	19.4 %
36. Most postmenopausal < 65 years old with low bone mass, no fractures, and no other fracture risk factors do not require pharmacological treatment	6	5	55.2 %
37. The efficacy of all osteoformers is similar when fracture risk is very high	6	6	41.8 %
38. The efficacy of all antiresorptives is similar in terms of improving bone mineral density and reducing fracture risk when fracture risk is very high	4	5	28.4 %
39. In cancer patients, the contraindications of anabolic drugs should be considered	8	2	90.8 %
40. The most appropriate option for patients > 75 years old with hip fractures is a parenteral (IV or subcutaneous) antiresorptive rather than an anabolic	5	6	41.8 %
41. The most appropriate option for patients > 75 years old with severe vertebral fractures or multiple vertebral fractures is an anabolic rather than an antiresorptive	7	4	69.7 %
42. The risk of osteonecrosis of the jaw is low with antiresorptives used for osteoporosis treatment	8	4	80.3 %
43. The risk of osteonecrosis of the jaw in patients treated with intravenous bisphosphonates and cancer patients increases with the duration of bisphosphonate exposure and the dose	8	4	82.9 %
Start of treatment			
44. The choice of drug to start osteoporosis treatment should be based on fracture risk stratification	8	3	88.2 %
45. After a fragility fracture, the diagnosis of osteoporosis and initiation of treatment should be established as soon as the acute episode has been resolved	8	5	82.9 %
46. The initiation of osteoporosis treatment should be agreed upon between the doctor and the patient in postmenopausal women under 65 with T-scores < -3.0	8	2	88.1 %
47. The initiation of osteoporosis treatment should be agreed upon between the doctor and the patient in postmenopausal women under 65 with distal radius fractures, especially if there are doubts about the trauma intensity	8	4	80.6 %
48. The initiation of osteoporosis treatment should be agreed upon between the doctor and the patient in patients with grade 1 vertebral deformities, which are not always easy to interpret as fractures	8	4	79.1 %
49. Patients with a fragility fracture should be treated regardless of bone mineral density	8	3	85.5 %
50. Patients with a T-score ≤ -2.5 in the spine, femoral neck, or total hip and age ≥ 70 years should be treated	7	3	73.7 %
51. Patients with a FRAX major fracture value ≥ 10 should be treated	7	3	69.7 %
52. Patients with a FRAX hip fracture value ≥ 3 should be treated	7	4	68.4 %
53. Patients with osteopenia (particularly if the T-score is ≤ –2.0) who also have a high clinical fracture risk (2 strong clinical risk factors for fractures, FRAX major fracture ≥ 10, and FRAX hip fracture ≥ 3) should be treated	8	3	84.2 %

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Table II (cont.). Results obtained by the expert panel after 2 rounds of consultations for the "Treatment" block

Statements	Me	IIQR	Agreement %
Start of treatment			
54. Patients receiving chronic glucocorticoids (the equivalent of ≥ 5 mg/day of prednisone for more than 3 months) should be treated with anti-osteoporotic drugs	7.5	5	67.1 %
55. Women receiving aromatase inhibitors for breast cancer should always be treated with anti-osteoporotic drugs if they have fracture risk factors	8	6	77.6 %
56. Men undergoing hormonal deprivation (antiandrogens and GnRH analogs) for prostate cancer should always be treated with anti-osteoporotic drugs if they have fracture risk factors	7	3	65.7 %
57. In the absence of contraindication or intolerance, the treatment of choice for patients with moderate or high fracture risk (but not very high risk) starting anti-osteoporotic therapy is oral bisphosphonates	8	4	79.1 %
58. For patients at very high fracture risk, the preferred treatment option would be sequential therapy, starting with an anabolic followed by an antiresorptive	8	4	80.3 %
Duration of treatment			
59. The initial duration of treatment will depend on the patient's risk and the drug used	8	2	93.4 %
60. The duration of treatment should consider the chronicity of osteoporosis	8	2	92.1 %
Therapeutic holidays			
61. The risk of atypical femur fractures increases as the duration of exposure to antiresorptives increases, but decreases rapidly (within one year) when treatment is interrupted	7	5	67.2 %
62. During therapeutic holidays (around 2 years) from bisphosphonates, there is no increase in fracture incidence	5	5	43.3 %
63. Therapeutic holidays are not mandatory but an option for patients who have received bisphosphonate treatment for 5 years and still have moderate fracture risk	8	2	78.9 %
64. Therapeutic holidays are recommended only for patients treated with oral or IV bisphosphonates, not with osteoformers or other antiresorptives	8	4	73.7 %
65. Therapeutic holidays are not recommended for patients who have received bisphosphonates for 5 years and still have a high fracture risk	8	3	82.9 %
66. Temporary interruption of bisphosphonate treatment should be considered for patients with no incident fractures and a T-score > -2.5 in the femoral neck	8	3	75.0 %
67. If treatment needs to be interrupted or during a therapeutic holiday, it is recommended to repeat DXA (dual-energy X-ray absorptiometry) and clinical evaluation	8	4	80.3 %

IQR: interquartile range; Me: median. Green: consensus on agreement; orange: no consensus.

However, no consensus was reached on several aspects, such as whether the benefits of pharmacological treatment (in terms of fracture prevention or BMD increase) are the same for men and women with primary osteoporosis; whether all osteoporosis treatments are indicated for glucocorticoid-induced osteoporosis; whether most postmenopausal women younger than 65 years with low bone mass but no fractures or other fracture risk factors require pharmacological treatment; whether the efficacy of all bone-forming agents is similar in very high-risk fracture patients; whether all antiresorptives provide similar improvements in BMD and fracture risk reduction in very high-risk patients; and whether the best option for patients over 75 with hip fractures is a parenteral antiresorptive (intravenous or subcutaneous) rather than an anabolic drug.

Regarding the initiation of treatment, for most of the agreed-upon statements, there was consensus on the need to stratify fracture risk and start treatment as soon as possible in cases of fragility fractures. Additionally, it was agreed to make this decision collaboratively between the physician and the patients in cases where the evidence for indication is more uncertain, such as in postmenopausal women under 65 years

with distal radius fractures, grade 1 vertebral deformities, or women under 65 with a T-score < -3.0 (79.1-88.2 %). It was also agreed that treatment should be initiated in all patients with fragility fractures, regardless of bone mineral density (BMD); with two risk factors strongly associated with fracture; with a T-score ≤ -2.5 in the spine, femoral neck, or total hip and aged ≥ 70; with a FRAX score for major fracture ≥ 10 and for hip ≥ 3; those on chronic glucocorticoids; and women receiving aromatase inhibitors for breast cancer with fracture risk factors (67.1-85.5 %). Furthermore, there was consensus on the use of oral bisphosphonates in patients with moderate or high fracture risk (79.1 %) and the preference for sequential therapy (anabolic followed by an antiresorptive) in very high-risk patients (80.3 %). There was a near-consensus on the administration of antiosteoporotic medications to prostate cancer patients on hormone deprivation therapy if they have fracture risk factors.

Regarding the duration of treatment, there was consensus to consider the chronicity of osteoporosis (92.1 %) and that it should depend on the patient's risk and the medication used (93.4 %).

Finally, regarding therapeutic holidays, panelists agreed that they are only optional in cases where bisphosphonates have been administered for 5 years and the patient has moderate fracture risk (78.9 %). They were not considered appropriate in cases where osteoformers or other antiresorptives are used (73.7 %) or if the patient has received bisphosphonates for less than 5 years and has high fracture risk (82.9 %). Additionally, it was agreed to repeat the DXA and clinical evaluation if treatment is interrupted (80.3 %). There was no consensus on whether there is no increase in fracture incidence during the 2 years of bisphosphonate therapeutic holidays.

BLOCK 3. MONITORING AND FOLLOW-UP

DSix out of the 8 statements on osteoporosis monitoring and follow-up reached consensus in agreement (Table III).

Panelists agreed that there is insufficient evidence to support the use of bone turnover markers for monitoring fracture risk in patients on anti-osteoporotic treatment (67.1 % agreement), but there was no consensus on their use for predicting fractures during bisphosphonate treatment holidays. However, there was agreement on the usefulness of these markers in assessing treatment adherence (68.4 %).

They also agreed that DXA and blood tests are sufficient for follow-up, with DXA being performed at long intervals of 3 up to 5 years (67.1 %). Additionally, they agreed that osteoporosis patients receive little follow-up after hospital discharge (76.3 %). There was no consensus on whether early monitoring with DXA

Table III. Results obtained by the expert panel after two rounds of consultations for the "Monitoring and Follow-up" block

Statments	Me	IQR	Agreement %
68. There is insufficient evidence to support the clinical use of bone turnover markers to monitor fracture risk in patients receiving antiosteoporotic treatment	7	5	67.1 %
69. There is insufficient evidence to support the clinical use of bone turnover markers to predict fractures in patients starting therapeutic holidays with bisphosphonates	7	6	62.7 %
70. The use of bone turnover markers can help assess the patient's treatment adherence	7	3	68.4 %
71. A bone densitometry test via DXA (dual-energy X-ray absorptiometry) and a blood test are sufficient to monitor patients with osteoporosis	7	6	67.1 %
72. After a hip fracture, there is less antiosteoporotic treatment than needed due to insufficient follow-up of patients upon discharge	7.5	3	76.3 %
73. After a fracture, early monitoring of bone mineral density (before 3 years of treatment) has limited value in predicting responses to antiresorptive treatments, although it has more value with osteoformers	6	4	56.7 %
74. To detect changes in bone mineral density with antiresorptives, tests should be run at sufficiently long intervals (approximately every 3-5 years)	7	5	67.1 %
75. Evaluation, treatment, and follow-up should be shared between Fracture Liaison Services (FLS) (or in their absence, the bone metabolism specialist) and primary care	8	3	88.2 %

IQR: interquartile range; Me: median. Green: consensus on agreement; orange: no consensus.

(before 3 years) has limited value in predicting the response to treatment with antiresorptives, but more value with osteoformers.

There was broad agreement that the evaluation, treatment, and follow-up should be done collaboratively between the FLS (or in their absence, the bone metabolism specialist) and primary care (88.2 %).

BLOCK 4. REFERRAL CRITERIA

Nine out of the 11 statements on patient referral were agreed upon, except for one (Table IV).

Panelists disagreed that patient referral from hospital care to a non-face-to-face primary care consultation is sufficient (67.2 % disagreement, 10.4 % agreement). There was no consensus either on considering the patient as a candidate/eligible for secondary fracture prevention as a referral criterion between primary care and hospital care.

Panelists agreed on the other proposed referral criteria, such as the suspicion of secondary osteoporosis and inadequate treatment response (93.4 %), and the consideration of a specific e-consultation for osteoporosis in addition to the in-person consultation (93.4 %). They also agreed on the need for coordination between primary and hospital care (97.4 %) and that the first consultation after hospital discharge should always be in person in primary care (83.6 %).

BLOCK 5. PATIENT PERSPECTIVE

All 6 statements regarding the osteoporosis patient's perspective reached consensus in agreement (Table V).

Nearly all panelists agreed on the importance of understanding the patient's knowledge about their disease, ensuring the information provided is clear, confirming their understanding, and involving them in shared decision-making (> 94 % agreement). Additionally, they emphasized the need for regular assessment of treatment adherence and persistence (98.7 %) and highlighted the significant role of patient support programs in osteoporosis treatment (96.1 %).

Table IV. Results obtained by the expert panel after 2 rounds of consultations for the "Referral Criteria" block

Statements	Me	IQR	Agreement %
76. It is necessary to establish agreed-upon coordination and referral criteria between primary care and hospital care	9	1	97.4 %
77. For coordination between hospital and primary care, it should be considered appropriate to have a specific e-consultation for osteoporosis, in addition to the possibility of in-person referral	9	1	93.4 %
78. Referring patients from hospital care to a non-in-person primary care consultation is sufficient	2	2	10.4 %
79. Patients should always be referred from hospital care to an in-person primary care consultation, at least for the index visit after hospital discharge	8	5	83.6 %
80. The suspicion of secondary osteoporosis is a referral criterion between primary and hospital care	9	2	93.4 %
81. Juvenile osteoporosis is a referral criterion between primary and hospital care	9	1	98.7 %
82. Inadequate treatment response (not due to non-compliance), with significant progression of bone mineral density loss or new fractures, is a referral criterion between primary and hospital care	9	2	93.4 %
83. The presence of side effects/contraindications to treatment that make therapeutic management difficult is a referral criterion between primary and hospital care	8	4	86.8 %
84. The presence of comorbidities that make patients especially complex for therapeutic management is a referral criterion between primary and hospital care	8	3	84.2 %
85. A patient being a candidate/eligible to secondary fracture prevention is a referral criterion between primary and hospital care	7	6	64.2 %
86. An acute symptomatic vertebral fracture that is difficult to control with standard analgesic treatment is a referral criterion between primary and hospital care	8	2	89.5 %

IQR; interquartile range; Me: median. Green: consensus on agreement; red: consensus on disagreement; orange: no consensus.

Table V. Results obtained by the expert panel after 2 rounds of consultations for the "Patient's Perspective" block

Statements	Me	IQR	Agreement %
87. It is necessary to know the patient's understanding of their condition to improve treatment outcomes	9	2	94.7 %
88. It is important to ensure the information is given to the patient and in what format	9	1	98.7 %
89. It is necessary to ensure that the patient has understood everything explained about their treatment and self-care	9	1	98.7 %
90. The patient must always be involved in decision-making to ensure shared decisions	9	1	97.4 %
91. The patient's treatment adherence and persistence should always be periodically assessed	9	1	98.7 %
92. Patient care programs in osteoporosis treatment are very important	9	1	96.1 %

IQR: interquartile range; Me: median. Green: consensus on agreement.

DISCUSSION

The results of this Delphi consensus show a high degree of agreement among primary care specialists and other specialties with a particular interest in osteoporosis concerning its identification and treatment. Consensus was reached on 77 out of the 92 proposed statements (83.7 %). There was considerable difficulty in identifying patients, especially in detecting vertebral fractures within the context of primary care. However, the level of agreement achieved among the experts is notable, considering the existence of numerous clinical guidelines that, although providing practical recommendations, often differ from one another. This variability, along with the low evidence or controversy surrounding some recommendations, can generate confusion among healthcare professionals, particularly those with less specialization in the area of osteoporosis.

Osteoporosis, despite its high prevalence and serious consequences, remains an underdiagnosed and undertreated condition. In Spain, studies show that most patients with hip fractures do not receive specific bone treatment: in 2021, only 23.7 % were on any pharmacological treatment for osteoporosis (9). Since 2010, the treatment gap in patients with osteoporosis has grown by 25 % (10), and in 2022, the PREFRAOS study, conducted within the primary care setting, confirmed low diagnosis and treatment, especially in men (11). Therefore, it is essential to establish general lines of action that facilitate the proper management of these patients, unifying criteria so that all primary care physicians work under the same approach and protocol and promoting coordination between primary care specialists and second-level care physicians. This expert consensus seeks to establish common recommendations.

In diagnosis, all experts agree with national and international guidelines (1,2,12) that it is essential to stratify patients based on fracture risk, classifying them as very high, high, and moderate risk. To this end, using the FRAX tool is helpful, regardless of whether a BMD assessment has been performed. According to the SCOOP study, screening patients using FRAX for primary prevention was effective in preventing hip fractures and was also cost-effective (13,14). However, in the context of secondary prevention, when a fracture is present, initiating treatment is justified without requiring a BMD assessment or the application of FRAX. It should be noted that FRAX may underestimate the risk of major osteoporotic fractures in Spain (but not hip fractures) (1), so it should never replace the clinical judgment of the physician, who must consider all the patient's risk factors as a whole and individualize diagnosis. Experts did not reach an agreement on whether the usefulness of FRAX is the same in men and women. Scientific literature indicates that although FRAX considers clinical risk factors and BMD in a similar manner in both sexes, most studies evaluating the tool primarily included women (2,15), and its use is more common in postmenopausal women (3). FRAX predicts fracture risk comparably in older adults but may underestimate it in younger women over 40 with risk factors, in men with high BMD (16), and in patients treated with glucocorticoids or with a history of fractures (17). To date, FRAX underestimates high doses of glucocorticoids or scores the same for having more or fewer previous osteoporotic fractures. The development of FRAXPLUS introduces some nuances that help address these limitations (16).

For the treatment of patients with osteoporosis, guidelines recommend a combination of non-pharmacological measures and pharmacological treatments (1,2,12). Adopting a healthy lifestyle (avoiding smoking and moderate alcohol consumption) (2), engaging in regular physical exercise, and maintaining proper nutrition contribute to acquiring a higher peak bone mass during development and maintaining it afterward (3,15). Experts recommend performing low-intensity physical activity, as it improves balance and strength,

thereby reducing the risk of falls and potential fractures. Additionally, when combined with adequate protein intake, it helps improve physical performance and increase muscle strength in older adults. Regarding nutrition, experts recommend an adequate intake of calcium (800-1200 mg/day), protein (1-1.5 g/kg body weight per day), and vitamin D (at least 800 IU/day) through diet, but if these amounts are not met, supplementation should be considered. However, experts did not agree on the usefulness of vitamin K and magnesium supplementation for fracture prevention. Evidence regarding the impact of vitamin K on bone health is limited and contradictory (3). Some studies suggest that a dose of 1 mg/day could reduce bone turnover in postmenopausal women (12). However, systematic reviews have not found any significant effects on vertebral fractures or BMD in this population (18). Regarding magnesium, no clinical studies have evaluated its effect on fracture risk or BMD, and most people consume adequate amounts of this nutrient through diet (12).

Regarding pharmacological treatment, all experts align with European guidelines (1,2) and agree that the therapeutic approach for osteoporosis is based on fracture risk stratification. In patients at very high risk of fractures, the use of osteoformers or osteoanabolics is recommended, such as teriparatide, romosozumab, and abaloparatide, which was recently available in Europe and Spain (11,12,19). For patients at high risk of fractures, the recommended treatment includes antiresorptives, such as bisphosphonates and denosumab (20). Due to the chronic nature of osteoporosis, its treatment should be considered for the medium and long term. Therefore, the proposal for patients at very high fracture risk, or imminent fracture risk, is sequential therapy, starting with an osteoformer, such as teriparatide, abaloparatide, or romosozumab, and then continuing with an antiresorptive, such as bisphosphonates or denosumab (1,2,12). If any treatment cannot be prescribed in primary care, the patient should be referred to hospital care for evaluation.

Clinical practice guidelines suggest, and experts agree, that after completing a regimen of osteoformers, treatment should continue with an antiresorptive to maintain or increase BMD and, thus, reduce the risk of fractures. For high-risk patients attending the consultation, guidelines and therapeutic algorithms recommend bisphosphonates as the first-line therapy, sparing denosumab for patients for whom bisphosphonates are contraindicated or not tolerated (4). However, experts did not reach a consensus on the efficacy of antiresorptive treatments in very high-risk patients or whether more potent parenteral antiresorptives should be selected. According to the literature, denosumab was more effective than bisphosphonates in improving BMD in the femoral neck, hip, and lumbar spine (21,22), but the results are less conclusive or contradictory regarding fracture risk (21,22). On one hand, it has been observed that the reduction in fracture risk is comparable between zoledronic acid and denosumab (23). On the other hand, a recent study conducted in real clinical practice situations demonstrated that denosumab achieved a greater reduction in fracture risk than alendronate (20). In any case, in clinical practice, guidelines' recommendations are followed, prescribing bisphosphonates as the first option for most patients with moderate or high risk and for primary prevention.

Similarly, osteoformers are not the same in their efficacy and mechanism of action. It has been observed that postmenopausal women treated with abaloparatide experienced greater increases in BMD than those treated with teriparatide or placebo (24,25). It has also been confirmed that romosozumab is superior to other osteoformers, like teriparatide, in improving BMD, but there were no significant differences in terms of fracture risk reduction (26). This advantage of romosozumab in terms of BMD may be explained by its dual mechanism of action: it acts both in bone formation and in reducing bone resorption (27,28).

Consensus results also showed that experts did not agree on the efficacy of pharmacological treatment depending on sex. Scientific literature shows that bisphosphonates, denosumab, and teriparatide are effective in both sexes, although most studies have been conducted in postmenopausal women (2,15), and their results have been extrapolated to men under the assumption that they are comparable (3). In the case of alendronate, risedronate, zoledronic acid, and teriparatide, specific benefits in vertebral fractures have been found in men, and denosumab is effective in men undergoing androgen deprivation therapy (1,29).

Experts agree that treatment with osteoformers has a specific duration: teriparatide is administered for 24 months (30), abaloparatide for 18 months (31), and romosozumab for 12 months (32). Once this period is completed, and with the current available evidence, no more cycles can be given, as neither teriparatide nor abaloparatide can be administered again, at least in Spain (30, 31). Although the possibility of using more cycles with romosozumab was discussed, currently, only a phase II study addresses this option (33). These treatments are indicated for patients with very high fracture risk who should then be treated with an antiresorptive: bisphosphonates or denosumab. The main controversy arose about when to start using antiresorptives and for how long. In this regard, a common recommendation needs to be reached. Where there is no doubt is about "treatment holidays," which can only be considered in patients with moderate risk who have been treated with bisphosphonates (4).

Another aspect to consider in therapeutic management, and which represents a challenge for the primary care physician, is maintaining good treatment

adherence in osteoporosis patients. To achieve the expected benefit from treatment, adherence should be above 50 %, and ideally > 70 % (19,34). The degree of adherence or persistence to pharmacological treatments in osteoporosis can vary between 10-80 % (35-37). However, several studies have highlighted that adherence to osteoporosis treatment is generally low, and in the first year, the abandonment rate is between 30-50 % in most cases (38). Therefore, it is vital to put all available means to improve the therapeutic adherence of the patient. Physicians perceive that poor therapeutic adherence is primarily associated with lack of communication with patients or between professionals, side effects, and the drugs route of administration (39). In this regard, experts agree that treatment outcomes improve by ensuring that the patient understands information about their disease and making them part of therapeutic decisions. Furthermore, for positive treatment results, communication between professionals (FLS, first- or second-level care specialists) and having access to a specific osteoporosis e-consultation are beneficial. Experts recommend regularly assessing the patient's adherence level. On the other hand, scientific evidence shows that treatments administered less frequently, such as weekly or monthly bisphosphonates and semiannual denosumab, tend to improve adherence vs daily doses. This is due to the perceived convenience for patients and less interruption of treatment, thus increasing long-term effectiveness (40). Various studies have shown that administering drugs via parenteral routes improves adherence vs oral routes (41,42). These aspects should be considered by clinicians to ensure good treatment adherence (43).

Experts agree on the importance of patient follow-up by the primary care physician due to the chronic nature of the condition. After hospital discharge, a face-to-face consultation with the primary care physician should always be scheduled. Furthermore, most of the criteria for patient referral from primary care to hospital care are in line with national and international guidelines (12).

This Delphi consensus presents several limitations that must be considered when interpreting the results. The qualitative nature of the method may introduce subjective biases, and although the goal is to represent various specialties, the selection of experts may not encompass all clinical experience in managing osteoporosis, so the results should be interpreted with caution.

Lastly, it is essential to establish unified recommendations for the management of patients with osteoporosis that are accessible to all medical societies and to the entire primary care community to prevent underdiagnosis and undertreatment. These recommendations should include fracture risk assessment, implementation of preventive measures, proper treatment selection, and appropriate patient follow-up. Furthermore, it is important to provide specific training to primary care physicians to enhance their knowledge about the disease, as well as more diagnostic and therapeutic resources, as this will allow them to manage osteoporosis and its complications more effectively.

This Delphi consensus highlights the urgent need to unify criteria for the clinical approach to osteoporosis in Spain, where the gap in diagnosis and treatment continues to widen. The high concordance in recommendations among specialists establishes a solid foundation to align all healthcare professionals toward more consistent and effective care for this chronic disease. The consensus proposes clear guidelines for prevention, therapeutic selection, and patient follow-up, promoting better communication and adherence to treatment. These results represent an opportunity to transform osteoporosis management and optimize clinical outcomes through a coordinated strategy based on scientific evidence.

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