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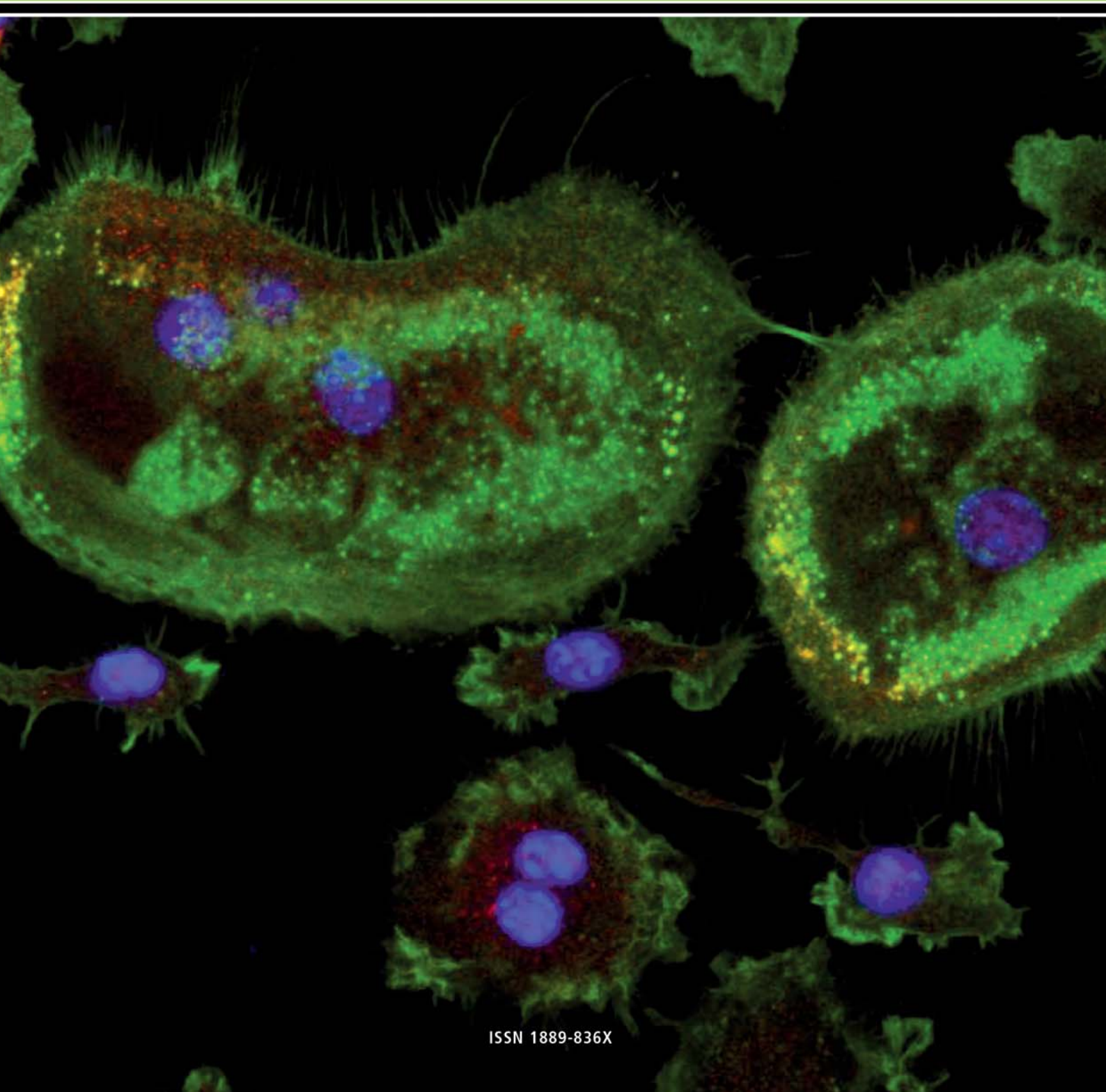

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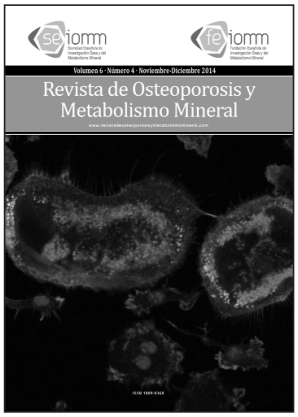

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SUMMARY

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Paget's disease of bone

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On 14th November 1877, the British doctor James Paget presented to the Medical and Surgical Society of London five cases of a condition which was called "Osteitis Deformans", a slowly developing bone disease characterised by the lengthening, softening and deformation of the bones, above all affecting the cranial bones and the long bones of the lower limbs. He published the first report in Medical-Surgical Transactions in 1877, in which he described in detail a man he had treated over a period of 20 years[1]. He subsequently published, more cases in 1882 as well as saying that he had not known that Czerney had used the term "Osteitis Deformans" in 1873.

Since this date many cases have been published and a large amount of information has been gathered relating to its etiology, prevalence, epidemiology, diagnosis and treatment, and "Osteitis Deformans" is now known as Paget's disease.

Today, Paget's disease of bone (PDB) is defined as a non-diffuse bone disease characterised by an increase in bone remodelling whose principle agent is the osteoclast. It is an entity of unknown etiology, sited segmentally in different areas of the skeleton. PDB may affect any bone and may be monostotic or polyostotic. The bones most affected are the pelvis (up to 70%), femur (30-55%), lumbar spine (25-50%) cranium (20-40%) and tibia (15-30%). The disease progresses along the affected bone and the appearance of a new location some years after the first diagnosis is very rare. This affectation leads to deformation of the bone with an increase in its size and deformity which may produce bone pain, arthralgia and nerve compression syndromes in the cranial nerve pairs, spinal stenosis or compression of the spinal cord. It also results in a greater risk of fracture in the affected long bones. It should also not be forgotten that pagetic tissue may suffer a neoplastic transformation with a higher incidence of sarcomas, especially in the polyostotic type which develop in 0.3-1% of cases[2,3].

PDB is asymptomatic in 50-75% of cases, and the doctor is alerted when the typical deformities appear (increased growth in the skull or bowing of the tibia), or when an increased level of alkaline phosphatase is detected in a routine analysis, or findings in an X-ray examination for another reason. In many cases the diagnosis of PDB is made after the complications have occurred, and if the Paget's is active, the markers for bone turnover are elevated. Among the markers for bone turnover the most useful appear to be amino-terminal telopeptide of collagen type 1, bone-specific alkaline phosphatase and amino-terminal propeptide of procollagen 1. However, taking into account its ease of use and low cost, the determination of concentrations of alkaline phosphatase is still a valid alternative.

The diagnosis of PDB is carried out primarily using X-rays with its characteristic images. Bone gammagraphy is not a specific method, but for us is useful to see the locations and spread of the disease. CAT and MRI scans are useful in evaluating neurological symptoms in the context of PDB and may also be of use to determine the extent and nature of neoplastic degeneration of the Pagetic tissue.

PDB has an interesting geographic distribution. The highest incidence is found in the United Kingdom (4.5% in those over 55 years of age) and within this country the highest incidence is in the northeast, with the best known concentration being Lancashire in which 7% of the population over 55 years of age is affected. It is quite common in the northeast of France, Spain and Italy. In Spain the prevalence of PPDB is at least 1% in people over 55 years of age, with notable variations according to geography and age. The best known predominant concentrations of PDB in our country are those of the province of Salamanca and the Sierra Norte de Madrid (Northern Sierra of Madrid) among others. It also occurs in the majority of other European countries, with the exception of the Scandinavian countries. In the rest of the world it is also common in countries which have seen high levels of immigration from Britain and other European countries during the 19th and 20th centuries such as: Australia, New Zealand, the United States and some regions of Canada. PDB is rare in the Indian sub-continent, Malaysia,

Indonesia, China and Japan. The disease affects both sexes, with a slight predominance in men in most series (the male/female ratio is approximately 1.4:1 in the United Kingdom), is rare before the age of 50, and its prevalence increases with age and affects up to 5-8% in the eighth decade of life in some countries[4-7]. Although there is no doubt that PDB has a genetic basis, the incidence and seriousness of this disease has diminished over recent decades[8-10].

Those patients with PDB often have a family history of the disease and it is estimated that the risk of PDB affectation in a first degree relative is increased seven-fold. In many families the disease is inherited in an autosomal dominant fashion with high incomplete penetrance, increasing with age. Great advances have been made in the last 15 years in the understanding of the genetics of PDB. Linkage analysis has identified some loci which are potential candidates in the chromosomes 2p36, 5q31, 5q35, 10p13 y 18q21[11].

The genes and loci which predispose for PDB have been identified through a correlation analysis of families. Among those genes and loci which have been associated with PDB or related syndromes are: CSF1 (located in 1p13), SQSTM1 (located in 5q35), in the 7q33 chromosome (the genes NUP205, SLC13A,4, and CNOT4), TM7SF4 (also known as DCSTAMP, located in 8q22) TNFRSF11B (located in 8q24), VCP (located in 9p13), OPTN (located in 10p13), TNFRSF11A (located in 18q21) and RIN3 (located in 14q33) and in the chromosome 15q24 (genes GOLGA6 and PML). In some of these the causal variant remains to be discovered. More studies are still required to determine the association of the different genes, as well as the importance of environmental factors which influence the development of PDB with these genetic alterations[11-16].

Some mutations of SQSTM1 may act as predisposing factors but are not sufficient to induce PDB, with additional factors (genetic or environmental) possibly being necessary[10-12,17,18]. Mutations of this gene are the most common cause of familial PDB. Transverse studies indicate that 80% of the carriers of SQSMT1 mutations develop PDB in the eighth decade of their lives. There are data which show that the age of onset of the disease in families with PDB in the current generation, in those with SQSMT1 mutation, is delayed in comparison with their parents' generation[10-12,15]. This emphasises the importance of environmental factors in triggering the disease.

To date, the precise molecular mechanisms which lead to the development of pagetic lesions in carriers of the SQSMT1 mutation have not yet been defined. On the other hand, recent experimental data suggest that one or a number of environmental factors may be required to induce the complete pagetic phenotype in the presence of the SQSMT1 mutation. Since environmental factors, some of them toxic, play a significant role in the development of PDB, and as the response to these factors is genetically conditioned, presented in this number is a work by Dr Usategui-Martín et al.[19], designed to determine whether the variability of some of the genes involved in the metabolism of exogenous toxins are related to the risk of developing PDB.

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Osteoporosis: a look into the future from Primary Care

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Primary health care (PHC) is the first point of contact for patients in the health care system and is key to the suspicion of osteoporosis in postmenopausal women (PMO), as well as in the approach to its diagnosis and treatment and the establishment of the risk of fracture.

Osteoporosis is the most common bone metabolic disease in our environment, representing a serious public health problem world-wide[1], and specifically in our country[2]. The prevalence of osteoporosis determined by bone densitometry in the lumbar spine is especially high after the menopause[3,4]. It is estimated that in Spain, one in three women over 50 years of age suffer from osteoporosis, increasing to one in two for those over 70 years of age. Most of these patients are located in the 55-80 years age range[3,4], and it is estimated that 4% of those patients over 50 years of age with a hip fracture will die during hospitalisation, and 24% in the first year after fracture[5]. Vertebral fracture is the most common, and that of the hip the most serious and with a greater cost to the health system, while there may also be other fragility fractures such as the distal radius, humerus, rib and tibia[6].

The different guides consider those patients in whom two of the following factors exist, combined with low bone mineral density (BMD)[7], to be at high risk: being over 65 years of age; having family history, especially maternal, of femoral fracture; prolonged consumption of corticoids: and lastly, having had falls.

The aim of treatment is the prevention of fragility fractures, in both the short- and long-term. To achieve this, the correct identification of the origin of the fracture – traumatic or due to fragility – will enable the correct diagnosis and the correct clinical decision regarding treatment. In our setting, we simply calculate the risk of the main osteoporotic fractures, so-called major fractures (vertebral, hip, humerus and forearm), and of the hip at 10 years, using the FRAX tool[8]. These do not indicate the decision as to who to treat, but are the clinical criteria which govern that decision, which may follow the decision thresholds proposed by the European Guide to Osteoporosis[9], and above all, the guides of the different scientific societies which better fit our work environment.

Thus, following the criteria of the Canadian Scientific Advisory Council on Osteoporosis[10], for whom the risk is defined on the basis of the FRAX scores as: low risk (risk of fracture after 10 years <10%), intermediate risk (10-19%) and high risk (≥20%) for the main osteoporotic fractures, and low risk (<3%) or high risk (≥3%) for hip fractures; or Qfracture®[11,12]. Both tools would both support clinical decisions by identifying patients at high/low risk of osteoporotic fracture, as well as decision to treat, thus improving the predictive parameters for Spanish women in a way which is more cost-effective than the traditional model based on a T-score of ≤-2.5 in the DXA[13].

In respect of the increase in bone mass measured through densitometry, frequently used the primary care system in patients over the age of 65, this is not a good variable with which to measure the efficacy of drugs[14], this being one of the tests identified as having little clinical value in a study in 2012[15]. In fact, a number of clinical trials have indicated that antiresorptive drugs continue to prevent the appearance of fractures, even though the BMD diminishes. Therefore, DXA should not be carried out in a generalised and indiscriminate way, not simply on the basis of the age of the patient or when a postmenopausal women presents herself, but rather it should be requested on the basis of the presence of risk factors[14].

Regarding the efficacy of drugs used in the treatment of osteoporosis we must consider this to be very limited in secondary prevention (previous

fragility fractures), and, practically-speaking, not demonstrated in primary prevention. There has recently been published a systematic review[16], comparing the effectiveness of drugs used for the treatment of osteoporosis, which updates the review carried out in 2012[15], whose objective was to review the evidence to determine the salient aspects of the efficacy and safety of drugs indicated for the prevention of fractures. A striking conclusion of the study was the existence of good quality evidence supporting the idea that some medicines reduce the risk of fractures in people with a BMD in the osteoporotic range and/or previous vertebral or hip fractures, with a great variation in in efficacy between bisphosphonates, denosumab and teriparatide, and with serious but very uncommon adverse effects, such as atypical subtrochanteric fracture or osteonecrosis of the jaw. Also highlighted is the lack of direct comparisons of the benefits, and the harm which results from indirect comparisons, which do not allow one to indicate which drug is more efficacious than another[17].

There is no evidence that early treatment in people below the age of 65 brings any benefits, nor is there sufficient evidence to recommend treatment from the age of 80[16,18]. So, in a study by Sanfélix-Genovés et al.[19], they draw attention to the contrast in the high levels of pharmacological treatment which exists in the region of Valencia and the low prevalence of risk factors in adults (50-65 years) which, coupled with an overuse of BMD, translates into a very significant impact on health spending. We should not forget that the treatment of first choice is still to consider changing harmful habits, taking physical exercise or avoiding falls, combined with a sufficient intake of calcium and vitamin D, which are as effective as the increase in BMD obtained through drug treatment, or even more so. Doctors in the Spanish health system are aware of the existence of the high comorbidity of osteoporosis with cardiovascular risk factors which could indicate a closer physiopathological relationship. For this reason, 9 out of 10 patients who attend a clinic for osteoporosis receive information on healthy life styles, balanced diets and on how to achieve a sufficient intake of calcium[19]. However, in spite of the low efficacy of these drugs their consumption has shot up, multiplying six-fold in the last ten years. A report by the national health service in the UK which examined the use of medicines for osteoporosis developed countries put Spain as the top country in the prescription of these treatments, which is seriously incongruous if we take into account the fact that the incidence of osteoporosis in Spain is the lowest, not only in Europe, but in the world[20]. This is why drug treatment should be limited to high risk patients who are going to be those who really benefit[16,18,20].

In all the guides to clinical practice consulted, the first choice pharmacological treatment, due to their efficacy, safety and efficiency, are the bisphosphonates, essentially alendronate and/or risedronate[2,9,10,17], which are those which have been shown to be the most efficient in the different types of fractures, with as a possible alternative the oral administration of zoledronate in patients at high risk, and/or with osteoporosis.

Given the controversy over safety in recent years, in addition to the studies of new drugs and their combinations, studies are also being conducted of efficacy[21], of efficacy compared with new molecules[22,23], of treatment in men[24] and of the optimum duration of treatments[25]. To date, the scientific evidence only justifies prolonged use over more than five years in highly selected patients.

In those patients with intolerance or contraindications for bisphosphonates, denosumab[26], a SERM or strontium ranelate may be recommended. All of these have safety problems notified by PRAC (the Pharmacovigilance Risk Assessment Committee of the European Medicines Agency), such as the temporary suspension of the sale in January 2014 of strontium ranelate due to an increase in serious cardiac events, thromboembolisms and/or skin reactions, or the risk of atypical femoral fractures with the use of denosumab, following the critical review which was carried out of the FREEDOM study[26].

In short, once a new medicine is authorised by the regulatory agencies and more in this type of pathology, the information regarding its efficacy and safety comes from the baseline studies and, therefore, the data are very limited. This obliges us to limit its prescription or, at least be "conservative" and, consequently, before prescribing a new drug for this pathology a careful risk-benefit assessment must be carried out, as well as assessing its suitability for each patient as against alternative more efficient therapies, with critical reviews carried out by professionals which allow comparison of the available information. This is an approach which addresses the patients and not the disease.

One of the issues which most concerns medical staff is adherence to treatment by the patient. In the work of Martínez et al. two out of three participating doctors considered that there was a level of non-adherence of 20%. Hence, one of the major challenges which doctors face is to successfully increase the adherence of patients to the recommendations and treatments they provide[27], involving all those concerned: patients, pharmacists and doctors. Adherence is understood to be as defined by the WHO in 2003, for whom it is: "the degree to which the conduct of a patient, in relation to the taking of medication, the following of a diet or the modifications of lifestyle correspond with the recommendations given by health professionals".

It can be deduced from this definition that, apart from the professionals and their communication with their patients, adherence also depends on the psychological connotations, experiences and knowledge of the patients themselves. Those people at risk of fragility fracture should take the opportunity to take informed decisions about their care

and treatment, in collaboration with the health professionals treating them. If the patient is in agreement, the families and carers should have the opportunity to participate in the decisions about the patient's treatment and care. The families and the carers should also have the information and support they need.

Good communication between health professionals and their patients is essential. This should be supported by written information based on the evidence, which suit the patients' needs and which should be culturally appropriate. It should also be accessible to those with additional needs such as physical, sensory or learning disabilities.

In short, in primary health care new paradigms are being opened up in relation to the management of a chronic disease, as is osteoporosis, in which the patients, in their environment, and the professionals involved should be jointly responsible for the development of their disease. It is known that a well-informed patient and a high degree of empathy with their family doctor enables an improvement in the doctor-patient relationship and, therefore, brings better health outcomes[27].

The health professionals face new challenges in improving therapeutic efficiency at this first level of care. For this reason they must carry out an effective selection of patients according to their risk (we ought not to forget that most patients with fractures are older, polymedicated and with multiple comorbidities and, up until now, not included in clinical trials), as well as the costs associated with their treatment and hospitalisation. In view of what lies ahead, this requires the establishment of protocols and guides to clinical practice agreed among the scientific societies in primary and specialised care (called "guides to guides", given the multidisciplinary nature of treatment in this pathology) and based on scientific evidence, which provide those aforementioned elements, creating integrated multidisciplinary educational programmes for the management of osteoporosis which allow the continuous updating and perfecting of the skills of doctors in primary care through active training programmes based on[28]:

1. Diagnostic and therapeutic decision algorithms.
2. Criteria for referral/monitoring.
3. Instructions for improving compliance
4. Criteria for the quality of care, of treatments, of life.
5. Cost criteria with evaluation of information on the economic impact of osteoporosis and associated fractures on their revenue.

In the primary care sector we should be more active in the search for new diagnostics, essentially promoting the use of the FRAX® tool, in women who attend clinics with diseases in which there is documented a high comorbidity with osteoporosis. It would seem to be essential to improve the quality of treatments of pluripathological and polymedicated patients, in whom new approaches, which incorporate the use of new drugs with demonstrable action on the risk of fracture and with widely-spaced doses, may help to resolve problems of adherence.

Throughout this process there will be key aspects of the management of osteoporosis, recommendations related to healthy lifestyles, diet, physical exercise, prior to the initiation of drug treatment, together with the need to emphasise that patients should take the recommended daily doses of vitamin D and calcium.

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Study of the deletions in the GSTM1 and GSTT1 genes and of the *Ile*105*Val* polymorphism of the GSTP1 gene in patients with Paget's disease of bone

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Summary

Background: Paget's disease of bone (PDB) is a disorder focussed on the bone with an increase in the number, size and activity of the osteoclasts. Some epidemiological data support the theory of its relationship with toxic or infectious environmental agents, whose interaction with some predisposing genetic alterations may lead to PDB. The glutathione S-transferases (GST) are involved in the metabolism of toxins, by catalysing the nucleophilic attack of the physiological substrate, reduced glutathione or GSH (g-Glu-Cys-Gly) on the electrophilic centre of a great number of toxic structures. We studied whether the variability of the GSTM1, GSTP1 and GSTT1 genes is related to the risk of developing PDB.

Patients and methods: We analysed 148 patients diagnosed with PDB, and 207 control individuals matched in sex and age with no history of bone alterations. Using genomic DNA obtained from peripheral blood the presence-absence of the GSTM1 and GSTT1 genes was studied by means of multiplex PCR. The study of the *Ile105Val* GSTP1 gene was carried out using PCR and subsequent digestion with the restriction enzyme BsmAI. The distribution of genotypes was analysed by means of the Pearson chi-square test. When statistically significant differences were found we carried out a multivariate logistical regression to determine the risk which the presence of a particular genotype could generate. We used the CSPSS 21.0 program. Differences were considered to be statistically significant when the value of $p<0.05$.

Results: We found differences in the distribution of the presence-absence of the deletion in the GSTM1 gene; not being a carrier for the deletion or being a heterozygous carrier in the GSTM1 gene confers a lower risk of developing PDB (OR=0.56, 95% CI: 0.36-0.87; p=0.011). In the study of the GSTT1 and GSTP1 genes there were no significant differences.

Conclusion: The detoxifying activity diminishes when two copies of the GSTM1 gene with deletions are inherited by reducing in enzyme activity, which has been associated with a greater susceptibility to some cancers, alcoholic hepatopathy and other inflammatory problems. We are not aware of any description of its association with PDB. PDB is observed more frequently in carriers of the homozygous deletion in the GSTM1 gene. This fact could explain the epidemiological findings which link PDB to exposure to certain environmental agents.

Key words: *Paget's disease of bone, glutathione S-transferase, genetics, polymorphism.*

Introduction

Paget's disease of bone is the most common bone metabolic disease after osteoporosis[1]. It is a bone disorder characterised by an increase in bone turnover in a disorganised way: a large increase in bone resorption, followed by bone formation of the same proportions. The result is bone with a structure which is irregular and anarchic, which alters its morphology and mechanical properties. Some patients are asymptomatic, while others have pain, degenerative arthropathy, fractures, bone deformities, deafness or other syndromes of nerve compression. The main change resides in the osteoclasts which increase in number size and activity[2,3].

There are currently two etiopathogenic hypotheses which attempt to explain the origin of PDB: the influence of environmental factors and the existence of genetic determinants[1].

There is evidence that genetic changes play a significant role in the development of the disease. There is a strong tendency to family aggregation (15-40%), with a seven-fold increase in relative risk of suffering the disease in families of patients with PDB[3-6]. In most of these families there is an autosomal dominant pattern with high penetrance in the sixth decade[3]. Recently, alterations in the genes SQSTM1, CSF1, OPTN, TNFRSF and TM7SF4[7,8], have been associated with a higher risk of developing PDB. Some epidemiological data, such as its heterogeneous distribution, or more recent changes in its incidence and seriousness, support the idea of the influence of environmental factors in the development of the disease. Its association with diets poor in calcium and vitamin D during infancy[9,10], exposure to environmental toxins[11], contact with animals during infancy[12-14], consumption of non-controlled meat[15], consumption of untreated water[16] and infectious agents such as viruses (paramixoviridae)[14,17,18], have been reported.

Neither environmental nor genetic factors alone explain the etiopathogeny of this disease. The most accepted model considers PDB to be the result of the synergetic action of both environmental and genetic factors. The genetic determination would explain the individual susceptibility to developing the disease following the exposure to the participating environmental factor[2].

Involved in the metabolization of toxins are the glutathione S-transferases (GSTs). These are a family of enzymes which participate in cell detoxification. These enzymes catalyse the nucleophilic attack of the physiological substrate, reduced glutathione or GSH (g-Glu-Cys-Gly) on the electrophilic centres of a great number of toxic structures, allowing their degradation. They are classified in seven families (alpha, kappa, mu, pi, sigma, theta and zeta) which are differentiated both in their sequences, as well as in their immunological properties and physiological roles[19,20]. GSTM1, GSTP1 and GSTT are the most studied GSTs, and those which have been most commonly associated with human pathologies[21].

The role of environmental factors, some of which are toxic, appears to be significant in the development of the disease. Given that the individual response to the toxic factors is genetically determined, we have designed this study with the aim of trying to determine whether the variability of the GSTM1 GSTP1 and GSTT1 genes (involved in the metabolization of exogenous toxins) is related to the risk of developing PDB.

Materials and methods

Patients and controls

We studied 148 patients diagnosed with PDB. In the case of patients with family history of the disease, we only selected one patient per family to avoid familial genotype bias. The patients were diagnosed by the rheumatology service of the University Hospital of Salamanca. As a control group, we analysed 207 individuals, matched in sex and age with the group of patients, without history of bone alterations, and coming from the same geographic area. From each of the patients were gathered clinical characteristics such as sex, age at diagnosis, family history, number of bones affected, presence of fractures, affectation of skull and affectation of cranial nerve pairs. All the subjects studied, both in the group of patients and the control group, gave their informed consent to participate in the study, which was approved by the ethics committee of the hospital.

DNA extraction and analysis of polymorphisms

In both the patient and control groups the extraction of genomic DNA from peripheral blood was carried out using the standard phenol-chloroform procedure.

The study of the presence-absence of the deletions in the GSTM1 and GSST1 genes was carried out using multiplex PCR under conditions described in Table 1. The study of the *Ile*105*Val* polymorphism of the GSTP1 gene was conducted using PCR and subsequent digestion with the restriction enzyme BsmAl. The conditions used are set out in Table 1.

Statistical analysis

The distribution of genotypes among patients and controls was analysed using the Pearson chi-square test. In those polymorphisms in which statistically significant differences were found we carried out a multivariate logistical regression to determine the risk which the presence of a particular genotype could generate. The statistical analysis was carried out using the SPSS 21.0 program. Those differences whose p value was <0.05 were considered as statistically significant.

Results

We studied a total of 148 patients and 207 controls. The clinical characteristics of the patients are set out in Table 2. The distribution of the presence-absence of deletions in the GSTM1 and GSST1 genes and the distribution of the genotypes for the *Ile*105*Val* polymorphism in the GSTP1 gene, and

their relationship to the risk of developing PDB are shown in Table 3.

We found statistically significant differences in the distribution of the presence-absence of deletion in the GSTM1 gene: not being a carrier for the homozygous deletion in the GSTM1 gene confers a lower risk of developing PDB (OR=0.56, CI 95%: 0.36-0.87; p=0.011). In the study of the GSTTT1 and GSTP1 genes we found no statistically significant differences (Table 3).

No statistically significant differences were found in the analysis of the clinical characteristics of the patients in relation to the variability of the GSTM1, GSTT1 and GSTP1 genes.

Discussion

PDB lesions occur as the result of an increase in bone resorption followed by an increase in its formation. The main change is located in the osteoclasts which increase in number, size and activity. There is a range of evidence which indicates that the etiopathogeny of the disease is a synergy between a series of environmental factors and the existence of certain genetic determinants[2]. Through a study of the variability of the GSTM1, GSTT1 and GSTP1 genes (involved in the metabolism of endogenous toxins) we intended to evaluate the relationship between these variables and the risk of developing PDB. As far as we know, this is the first work which examines the influence of the changes in these genes on the development of this disease.

The GSTM gene is located in the 1p13 chromosome, and to date, five allelic variants are known: GSTM1, GSTM2, GSTM3, GSTM4 and GSTM5. A reduction in detoxification activity occurs when the deletion in gene GSTM1 is inherited, meaning that being a homozygous carrier of a deletion in the GSTM1 gene causes a reduction in enzyme activity. The theta class of GSTs comprises two genes which code for the two proteins GSTT1 and GSTT2. As with the GSTM1 gene, if a homozygous deletion in the GSTT1 gene is inherited, there is a reduction in detoxification activity. In terms of the sub-family of GSTP, it comprises a single gene GSTP1 in which have been described two allelic variants which differ in the base 313 of the cDNA, one adenine (A) being substitute by a guanine (G). This difference results in a change of a valine (Val) to an isoleucine (Ile) in the 105 codon of the amino-acid sequence, causing a defective bond between the enzyme and the substrate, and thus, a reduction detoxification activity[19,20,22,23].

Being a homozygous carrier for deletion in the GSTM1 and/or GSTT1 genes has been associated with a greater susceptibility to developing different types of cancer[21,22,24], alcohol-related liver disease[25] and other inflammatory diseases[25,26], because it causes poorer metabolization of toxic agents, with the synthesis of free radicals which damage DNA[20]. Our results show that not being a homozygous carrier for the deletion in the GSTM1 gene brings a lower risk of suffering PDB. In the study of the GSTT1 and GSTP1 genes we found no statistically significant difference between the patient and control groups. We found no statistically significant differences between the clinical expression, extent and activity of the disease in relation to the variability in the GSTM1, GSTT1 and GSTP1 genes in the group of patients with PDB.

One of the causes postulated as the origin of PDB is exposure to environmental toxins from the production of cotton, meat or drinking water without adequate control of sanitation, which may alter the maturation and activity of the osteoclasts, the increase in activity fostering the development of PDB[11,15,16]. Our hypothesis is that to have the homozygous deletion in the GSTM1 gene assumes poor metabolization of environmental toxins which, by a mechanism yet unknown, may increase the function of the osteoclasts and osteoclast precursors which, combined with other genetic changes not yet well described, could result in the development of PDB.

In conclusion, in those individuals who are carriers of the GSTM1 gene with homozygous deletions, PDB is more frequently observed. This fact could explain the epidemiological findings which associate PDB with exposure to certain environmental agents. Even so, functional studies of these polymorphisms are required in order to validate our hypothesis.

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Table 1. Amplification conditions and digestion for GSTM1 GSTM1, GSTT1 and GSTP1 genes

Amplification conditions GSTM1 and GSTT1 genes
Primers
C(+):
Sense: 5′-CGCCATCTTGTGCTACATTGCCCG-3
GSTM1:
Sense: 5`-ATCTTCTCCTCTTCTGTCTC-3′
Anti sense: 5′-TCACCGGATCATGGCCAGCA-3′
GSTT1:
Sense: 5′-TTCCCTTACTGGTCCTACATCTC-3
Anti sense: 5′-TCACCGGATCATGGCCAGCA-3
PCR program
95ºC 5 minutes
30 cycles (94ºC 30 seconds/58ºC 30 seconds/72ºC 45 seconds)
72ºC 8 minutes
Resulting PCR fragments and correspondence with the genotype
231, 450 y 158 pb: GSTM1(+)/GSTT1(+)
231 y 158 pb: GSTM1(+)/GSTT1(-)
450 y 158 pb: GSTM1(-)/GSTT1(+)
158 pb: GSTM1(-)/GSTT1(-)
Amplification conditions and digestion for GSTP1 gen
Primers
Sense: 5′-ACCCCAGGGCTCTATGGGAA-3
Anti sense: 5′-TGAGGGCACAAGAAGCCCCT-3′
PCR program
95ºC 5 minutes
30 cycles (94ºC 30 seconds/55ºC 30 seconds/72ºC 30 seconds)
72ºC 5 minutes
Amplicon: 176pb
Enzyme: BsmaI **Digestion:** 37ºC / 4 hours
Fragments resulting from digestion and correspondence with genotype
176 pb: AA
176, 91 y 85 pb: AG
91 y 85 pb: GG

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Table 2. Clinical characteristics of patients with PDB

		Patients (N)
Sex	Man Woman	79 69
Age at diagnosis	More than 60 years Less than 60 years	115 33
Family history	Sporadic Family	129 19
Number of affected bones	Less than three More than three	103 45
Presence of fractures	Yes No	9 139
Involvement of cranium	Yes No	61 87
Involvement of cranial nerve	Yes No	25 123

Table 3. Distribution of genotypes of the studied polymorphisms in genes GSTM1, GSTT1 and GSTP1 and its association with the risk of developing PDB

SNP	Genotype	Patients PDB N (%)	Controls N (%)	Value of p	OR (IC 95%)
GSTM1	-/-	98 (66.2%)	109 (52.7%)	0.011	1.00
	+/+ ; +/-	50 (33.8%)	98 (47.3%)		0.56 (0.36-0.87)
GSTT1	-/-	28 (18.9%)	49 (23.7%)	0.299	----
	+/+ ; +/-	120 (81.1%)	158 (76.3%)		
GSTP1	AA	70 (47.3%)	76 (39.0%)	0.280	----
	AG	65 (43.9)	97 (49.7%)		
	GG	13 (8.8%)	22 (11.3%)		
	AA+AG	135 (91.2%)	173 (88.7%)	0.477	----
	GG	13 (8.8%)	22 (11.3%)		
	AA	70 (47.3%)	76 (39.0%)	0.125	----
	AG+GG	78 (52.7%)	119 (61.0%)		

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Knowledge of osteoporosis, and the pharmaceutical expenditure it entails, in the primary health care system of the Canary Islands

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Summary

Background: Osteoporosis is a disease which can be managed by different specialisms, one of which is the family doctor. In this study we analyse the knowledge of osteoporosis, and the diagnostic and therapeutic approach taken to this disease, among primary care doctors in the Canarian archipelago, as well as making a first approximation of the expenditure on drugs used to treat this disease in 2013.

Material and method: Observational, descriptive, transverse study conducted between May 2013 and May 2014 with all primary care doctors in the Canarian health service. An anonymous survey covering 13 points was developed. The capture of the data about expenditure on drugs was facilitated by the service for the control of supply and rational use of medicines of the Canarian health service.

Results: 28.60% of primary care doctors in the Canarian archipelago responded to the survey. Of these, 75.30% reported using risk factors in evaluating the risk of fractures. Not a very high percentage, approximately half of the respondents, request densitometries, while 28.60% routinely use scales for the evaluation of risk of fracture and 32.80% use them occasionally. 90% of the professionals recommend non-pharmacological measures for the prevention of fractures in their patients, although 91% do not normally request a determination of blood levels of vitamin D.

In 2013 the expenditure on drugs for osteoporosis by the Canarian health service amounted to € 7,684,393.61, of which € 7,265,491.06 was in primary care.

Conclusions: The Canarian primary care doctors who responded to the survey had, in general, a good knowledge of osteoporosis, and of its risk factors, but focussed their professional activity more on prevention than treatment. The drug most commonly used in the treatment of osteoporosis in primary care is risedronate. Expenditure on drugs for osteoporosis in the Canarian archipelago in 2013 amounted to € 7,684,393.61, 94% of which was in primary care.

Key words: *osteoporosis, primary care, doctor, knowledge, drug expenditure.*

Introduction

The medical specialisms which manage osteoporosis, their approach, diagnosis and treatment, are highly heterogeneous[1]. There are patients who remain without treatment in spite of being at high risk of fracture, while others receive medication solely on the basis of a bone densitometry, and some with neither prior densitometry or risk evaluation. It is essential to differentiate between those patients with a higher risk of fracture who will benefit from pharmacological treatment, with the aim of optimising interventions to ensure a positive risk-benefit relationship. To achieve this, different instruments have been developed which estimate the risk of fractures based on risk factors, among which are software applications FRAX®[2,3] and Qfracture®[4], which allow the estimation of the risk over 10 years of a major fracture (any fragility fracture) or specifically fracture of the hip. Many patients with osteoporosis attend primary care clinics, which means that family doctors should have sufficient skills and diagnostic tools to deal with these patients. However, we found great variability in the application of the FRAX®[2,3] and Qfracture®[4] tools, as well as between the different national and international guides for the management of osteoporosis[5-7].

Few studies exist in Spain as to the degree of knowledge primary care doctors have about osteoporosis, although they are one of the fundamental pillars of care for patients with osteoporosis. Furthermore, there are no current studies in the autonomous community of the Canary Islands to enable the evaluation of the actions of its professionals and the degree to which the aforementioned risk scales are utilised.

The main objective of this study was to obtain a first approximation of the extent of the knowledge of osteoporosis, and of the approach, diagnosis and treatment of this disease in doctors working in primary care in the Canarian health service, as well as estimating the expenditure incurred in primary care on drugs used for the treatment of this disease during the year 2013.

Material and method

Observational, descriptive, transverse study carried out between May 2013 and May 2014 of all primary care doctors in the Canarian health service.

An anonymous self-completed survey covering 13 items was developed (Annex 1), which asked questions about the knowledge and attitudes of professionals in dealing with osteoporosis. The survey was designed by the authors of this article. Using data provided by the primary care administration of the Canarian health service in Tenerife, the survey was sent by email to the directors of all the health centres in the Canarian archipelago, enabling it to reach anonymously the 1,168 family doctors who were working at that time in primary care. The completed surveys were returned by email to the authors of this article, indicating which to which health centre they belonged.

The study was evaluated and authorised by the research section of the primary care administration in Tenerife. All the data obtained were treated confidentially.

To estimate the expenditure on drugs used for the treatment of osteoporosis we requested the data regarding this expenditure during 2013 from the service for the control of supply and rational use of medicines, in the general directorate for assistance programmes of the Canarian health service. This service sent us three spreadsheets in Excel 2007: the first, entitled "Indicator", contained two tables, one which specified the percentage use of drugs of first choice for osteoporosis in each of the hospitals in the Canaries, and the other in which are indicated their use in each of the seven primary care administrations. The second spreadsheet, which is entitled "Consumption", contains three tables which specify the drugs by active ingredient, by number of packets and expenditure in euros (the first table in which was not known where expenditure originated, the second table with the expenditure incurred by specialist care, both in number of packets and euros, and a third with the expenditure incurred by primary care). Lastly, a third spreadsheet called "Total Consumption" in which we include a table which specifies the names of the medicines and the total consumption in both specialised and primary care, both in packets and in euros.

The codification of the data was carried out with the SPSS (Statistical Package for the Social Sciences) program version 21 for which we held the appropriate licences.

Results

Of the 1,168 doctors in primary care invited to participate in the study, a total of 332 responded to the questionnaire, which is 28.6% of all such doctors in the Canarian archipelago. The distribution of doctors who responded to the questionnaire by island is shown in Table 1. The islands with the highest percentage of respondents were El Hierro and Tenerife.

The percentages for each response to each item are shown in Table 2. To the question as to whether the professional considers risk factors in the appearance of fractures (item 1), 75.30% said they usually considered them and 21.10% occasionally. Only 3% of respondents did not consider these factors in their consultation.

In relation to the use of densitometry for screening during the menopause (item 2), 72.30% did not use it for screening and 16.60% used it only sporadically. Densitometry to monitor osteoporosis in treatment was only requested by half the professionals who responded (item 3). On the other hand, we found that 14.80% confirmed their use of conventional radiography as a method for diagnosing osteoporosis, 30.70% used it occasionally and 51.50% did not use it at all for diagnosis (item 13).

91% of the professionals who responded recommend non-pharmacological measures for the prevention of fractures in their patients, as opposed to 1.20% who did not recommend any measures and 7.80% who said that they sometimes did so (item 4).

44% of the doctors do not routinely monitor the height of their patients, and only 24.40% usually do so (item 5). In spite of this, 51% of respondents said that they had requested X-rays of the lumbar spine in cases of a reduction in height (item 6).

In relation to the use of scales for the evaluation of risk of fracture, 28.60% said that they did so routinely and 32.80% occasionally. 38% do not use scales for this pathology in their clinic (item 7).

In cases in which fragility fractures are detected, 70.20% of the doctors responding requested a complementary test (although the question did not specify which), 13.60% requested no such test and 15.10% only requested one occasionally (item 8).

91% of respondents did not routinely request a test for vitamin D levels in the monitoring of patients with osteoporosis or at risk of fractures (item 9). 83.70% said they ensured a good supply of calcium and vitamin D as a function of age, sex and other related factors (item 10).

To find out which is the drug most used in the first instance for the treatment of osteoporosis , an open question was posed in which the professionals could explain which treatment they considered to be the first choice for their patients (item 11). The responses were grouped in 10 categories whose distribution can be seen in Table 3.

The duration of treatment with bisphosphonates was checked in 85.20% of cases, while 12% of those responding do not do so (item 12). Lastly, in relation to the data obtained from the service for the rational use of medicines[8], in the year 2013 the expenditure on medicines for osteoporosis in the Canarian health service[8] totalled € 7,684,393.61.

In primary care the expenditure was € 7,265,491.06, the top drug prescribed being risedronic acid, followed by ibandronic acid (Figures 1 and 2). It should be clarified that included within the figure for expenditure in primary care is the expenditure originating both in prescriptions from primary care, as well as those from specialised care. With reference to drugs which should be used as first choice for the treatment with osteoporosis, in primary care these represented 13.71%.

Discussion

The family doctor is an essential pillar of support for the care of osteoporosis in all its aspects: preventative, educative and therapeutic. For this reason they need to be capable of identifying the population at highest risk of osteoporotic fracture in the early silent phase, before the first fracture appears[9].

Osteoporosis is an asymptomatic disease which is difficult to diagnose in the absence of a fracture[10,11]. And even if there are fractures, these often do not produce symptoms. A number of authors have indicated that is it important that the doctor has adequate diagnostic methods at their disposal, but it is also necessary that they have the correct information regarding the treatment of this disease[12-15]. In Spain, until the publication of the first guide to osteoporosis by the semFYC[16], the study of this disease in primary care was not well documented, neither was it included in the programme of preventative and health promotion activities. In the ABOPAP 2000 study carried out in Spain[17], the approach to osteoporosis in primary care was studied. Notable among the results of this study was the fact that around a quarter of doctors had access to bone densitometry, whereas around 50% said that they continued to study patients suspected of having osteoporosis. It is also interesting that screening for risk factors is lower than expected in certain risk situations, such as family history of osteoporosis or hip fracture, chronic treatment with glucocorticoids, etc. As was expected, those doctors who had available the best diagnostic tools also carried out greater screening for risk factors[18].

Table 1. Distribution of primary care physicians (in percentage) who answered surveys on each island of the Canary Islands

Tenerife	53.54%
Fuerteventura	40.74%
Gran Canaria	3.74%
Hierro	87.5%
Lanzarote	22.78%
La Gomera	35.29%
La Palma	8.16%

Another study published in Spain[19], which analysed approaches to osteoporosis in a primary health care centre concluded that the family doctors rarely complied with directives emanating from guides to diagnosis and treatment.

In this study, according to the results from the doctors surveyed, we are able to say that, in general, what primary care doctors in the Canaries carry out best is prevention, which is shown in the high percentage of those who responded who took into account risk factors (96.40%), ensured a good intake of calcium and vitamin D (83.70%) and routinely applied non-pharmacological measures to the general population (90%).

Even so, it is notable that only 61.40% said that they used risk factor scales to a greater or lesser extent. Given the simplicity of the tests, they could be of general use in primary care. It is possible that these staff are not yet convinced by the assessment of risk factors, perhaps due to the risk scales not being completely accepted consensually among researchers.

On the other hand, 71.60% of the doctors who responded did not measure patients' heights in their clinic. This is contradictory since this data is necessary when using the scales. We raise the question as to whether doctors really take into account height or not, since this parameter is measured by the nurse, and in the survey we do not ask who measured it. This may also reflect a lack of knowledge on the part of many staff of the significance of loss of height as an indicator of vertebral fracture.

Table 2. Percentage of responses on each item

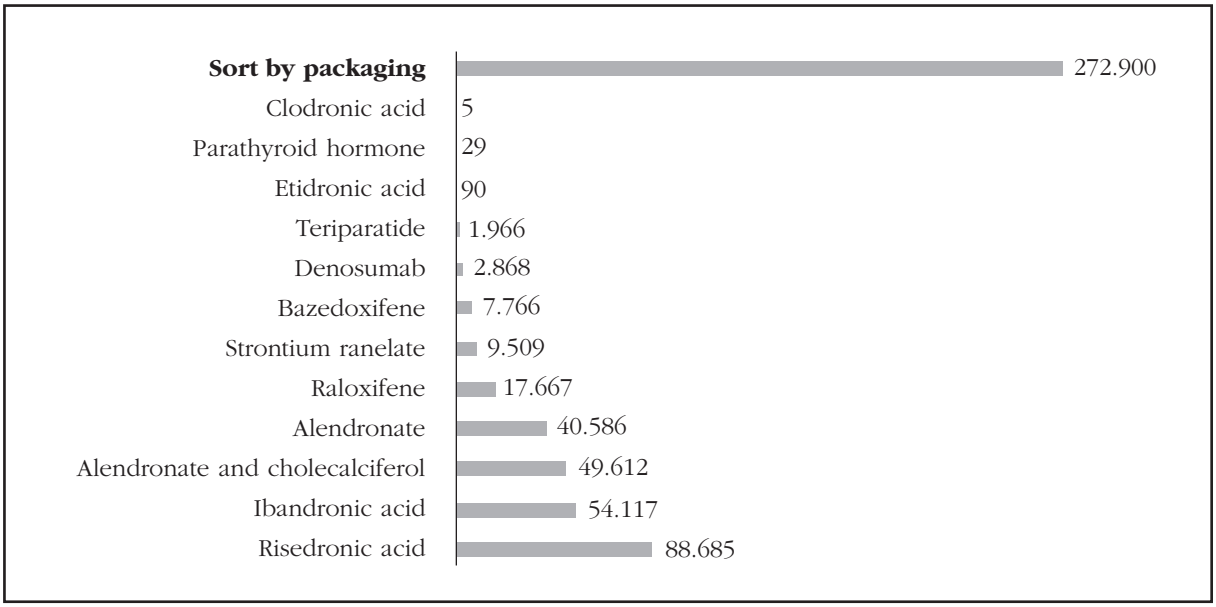
Question	Yes	No	Sometimes	No answer
1. Do you consider risk factors?	75.30%	3%	21.10%	%
2. Are you applying densitometry screening?	11.10%	72.30%	16.60%	0%
3. Are you applying densitometry in osteoporosis treatment every two years?	50.90%	46.10%	NE	3%
4. Do you apply nonpharmacological measures?	91%	1.20%	7.80%	0%
5. Do you measure the size in question?	24.40%	44%	31.60%	0%
6. If decrease in size, do you ask radiograph?	51.20%	44.90%	NE	3.90%
7. Do you use scales to assess the risk of fragility fracture?	28.60%	38%	32.80%	0.60%
8. If fragility fracture, other proof do you ask?	70.20%	13.60%	15.10%	1.20%
9. Are you applying vitamin D?	8.40%	91%	NE	0.60%
10. Do you ensure the intake of calcium and vitamin D?	83.70%	13.90%	0.30%	2.10%
11. What is the drug most commonly used primarily for the treatment of osteoporosis?	The responses were grouped in 10 categories, distributed as shown in Table 3			
12. Do you review the years taking bisphosphonates?	85.20%	12%	0.30%	2.40%
13. Do you use X-rays to diagnose osteoporosis?	14.80%	51.50%	30.70%	3%

Table 3. Drugs used by physicians who responded to the survey

Therapeutic group	Nº	%
Bisphosphonates	127	38.25
Calcium/vitamin D + bisphosphonates	69	20.78
Calcium/vitamin D	43	12.95
Non-pharmacological measures	10	3.01
Non-pharmacological measures, calcium/vitamin D + bisphosphonates	7	2.11
Non-pharmacological measures, calcium/vitamin D	6	1.81
Other treatments for osteoporosis	5	1.51
Otros tratamientos para osteoporosis	3	0.90
No specific treatment	14	4.22
Unanswered	48	14.46
Total	332	100

Another point of note is that 91% of doctors do not request a vitamin D test in patients with osteoporosis or at risk of fracture, so we believe either that there is a lack of knowledge of the usefulness of such a test, or that there is an administrative difficulty in requesting one, although the fact that 83.70% said that they ensured a sufficient supply of vitamin D to their patients inclines us to the second explanation. In terms of the diagnosis, it is notable that nearly 50% used X-rays, occasionally or routinely, to diagnose osteoporosis. And, if a reduction in height is detected, only 50% request a spinal X-ray. This leads us to suspect that there is an under-diagnosis of possible vertebral fractures, and in some way corroborates the possibility of a lack of knowledge about the loss of height as being indicative of vertebral fracture as has been pointed out before. Furthermore, according to the results, if a doctor were to detect a fragility fracture 28.70% would not, or would only occasionally,

Figure 1. Pharmaceutical expenditure on medicines for osteoporosis, in 2013, ordered by packaging made by Primary Care in the Canarian Health Service



request a complementary test. In this respect, the low use of bone densitometry may reveal the difficulty primary care doctors have in accessing this diagnostic test.

In terms of the prescribed treatment, most responded saying that they use bisphosphonates, and in second place, the same drugs, associated with calcium and vitamin D. Fewer than 10 doctors responded that the treatment depended on the age of the patient and associated risk factors.

Various studies in primary care have been conducted in our country regarding the applicability of the FRAX© tool[2,3] to determine the absolute risk of fracture in postmenopausal women and, as a function of the results, to consider recommendations in relation to the convenience of requesting of bone densitometry and/or of initiating treatment with antiresorptives[20,21].

Patient care overload, combined with the large amount of knowledge which the family doctor needs to carry out their daily tasks, means that osteoporosis is seen as a priority, or not, according to the preferences of each doctor. To this can be added the lack of unanimity in the guides available at the time of requesting a bone densitometry and, above all, when defining which patient to treat[17].

The main objective of this work is to obtain a first approximation for the understanding and management of osteoporosis by primary care doctors in the Canary Islands, so that once the reality is known, attempts can be made to increase the use of tools for the evaluation of risk of fracture, to reduce the use of tests and treatments in low risk patients and to increase the use of these resources in those at high risk, hence seeking the most efficient use of resources. It is certain that a significant limitation of the study was that the percentage of doctors who answered the survey was less than was hoped for, but we may consider them a representative sample of primary care doctors in the Canary Islands. It is possible that those who completed the questionnaire were those most involved in the disease, for one reason or another, and if we take this into account (as well as the chronic lack of time which staff in primary care possess) the percentage of responses is satisfactory. Other limitations of the study are: firstly, that the survey used had not been validated by other researchers, given that we could not find any survey which could be adjusted to the objectives of this study; and secondly, in some of the smaller islands it is not possible to request densitometry, which means that the response to whether densitometry was requested every two years for screening, and if densitometry for monitoring is requested, could be biased.

Conclusions

Our results show that primary care doctors in the Canary Islands who responded to the questionnaire consider prevention of osteoporosis as part of their work, whereas they show less knowledge and determination, as well as having fewer means available, in relation to the diagnosis and treatment of the disease.

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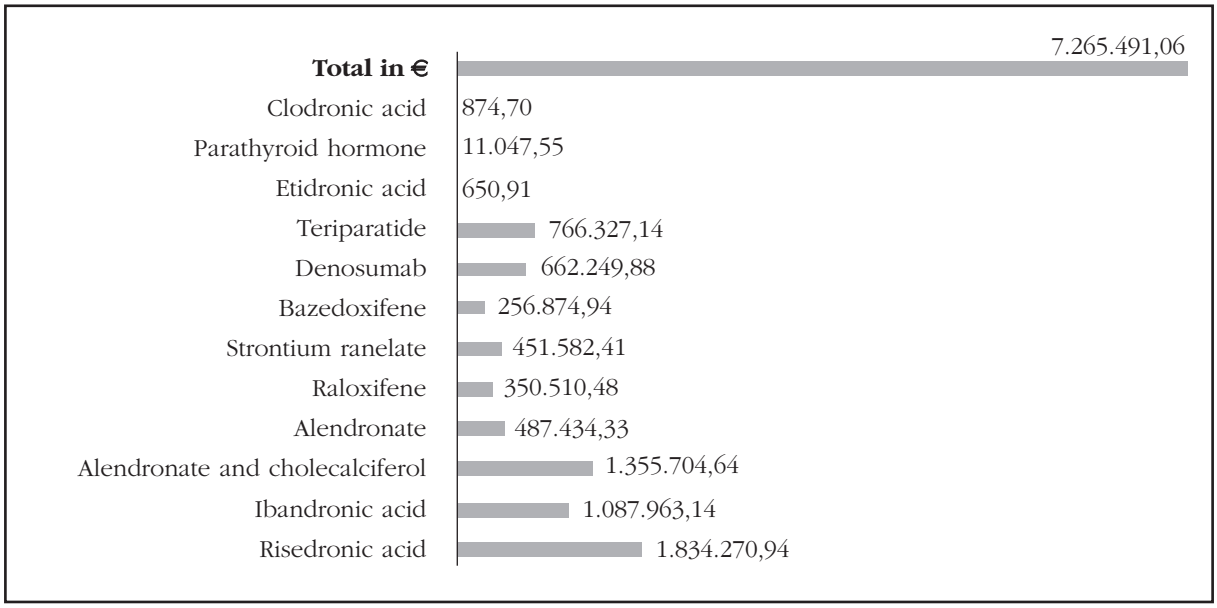
Figure 2. Pharmaceutical expenditure on medicines for osteoporosis in 2013, in euros (€) by Primary Care Health Service Canario



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Annex I. Survey data collection

Knowledge about osteoporotic fractures in primary care professionals
1) Do you consider all risk factors for fracture: age, personal history of fracture, risk of falls and use of corticosteroids, as usual in the query? ☐ YES ☐ No ☐ SOMETIMES
2) Are you applying routinely densitometry, screening for all postmenopausal women? ☐ YES ☐ No ☐ SOMETIMES
3) Are you applying control densitometry every two years in patients with osteoporosis treated? ☐ YES ☐ No
4) Do you apply nonpharmacological measures the general population routinely: no smoking, no drinking, physical exercise regularly, diet rich in calcium? ☐ YES ☐ No ☐ SOMETIMES
5) Do you measure regularly in consultation up to their patients? ☐ YES ☐ No ☐ SOMETIMES
6) In case of decreasing size do you ask x-thoracic and lumbar spine? ☐ YES ☐ No
7) Do you use any scale (FRAX, QFracture) to assess the risk of fragility fracture? ☐ YES ☐ No ☐ SOMETIMES
8) In case you are facing a fragility fracture patient with some complementary test do you ask? ☐ YES ☐ No ☐ SOMETIMES
9) Are you applying vitamin D levels routinely? ☐ YES ☐ No
10) Do you ensure good calcium intake as needed (depending on age, sex, ...) and vitamin D? ☐ YES ☐ No
11) What treatment for osteoporosis using home?
12) In patients taking bisphosphonates Do you regularly check how many years have you been taking it? ☐ YES ☐ No
13) Do you use radiography as a method to diagnose osteoporosis? ☐ YES ☐ No ☐ SOMETIMES

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Effect of spinal cord injury recently in bone turnover and in bone mass evolution of complete motor. Preliminary findings

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Summary

Background and aim: Spinal cord injury (SCI) has been associated with a marked increase in bone loss and a higher incidence of skeletal fractures, however the pathogenesis and clinical management of this condition remains unclear. The aim of this study was to analyze the bone mineral density (BMD) evolution in patients with complete SCI and its relationship with parameters of bone metabolism and bone turnover markers.

Methods: Patients with a recent complete motor SCI (ASIA A)(<6 months) were prospectively included. Bone metabolism parameters (calcium, phosphate, PTH and 25-OHD), bone turnover markers (bone formation: procollagen type 1 aminoterminal propeptide -P1NP-, bone alkaline phosphatase -bone AP-, osteocalcin -OC-; bone resorption: C-telopeptides of type I collagen -CTx-) and BMD were assessed in all patients at baseline and at 6 months. The results were compared with a control group.

Results: 23 men with complete SCI (ASIA A) and a mean age of 38±15 years were included at 102±33 days of SCI onset. 52% had paraplegia. 12 patients were assessed at 6 months of follow-up. Patients with SCI showed a significant increase in bone turnover markers, especially P1NP and CTx, compared to controls (P1NP: 191±90 vs 51±19 ng/ml, p<0.001; CTx: 1.37±0.49 vs 0.51±0.23 ng/ml, p<0.001). At 6 months, bone turnover markers decreased (P1NP: -34%, p=0.005 and CTx: -26%, p=0.002) and BMD had a mean decrease of 12% at total femur (p=0.002) compared to baseline, with osteoporosis development in 50% of patients. Bone markers (bone AP, P1NP and OC) were negatively correlated with total femur BMD values.

Conclusions: Patients with complete SCI show a marked increase in bone turnover and bone loss, especially at the proximal femur, with the development of osteoporosis being observed in 50% of these patients at 6 months of follow-up. These findings indicate the need to implement preventive measures within the therapeutic approach in these patients.

Key words: *osteoporosis, spinal cord injury, bone metabolism, bone turnover markers.*

Introduction

An absence of mechanical load on the skeleton is associated with a marked loss of bone mass which may result in the development of osteoporosis and fractures. Spinal cord injury (SCI), especially when they are complete, are a common cause and an archetypal example of an absence of load on the skeleton. So, a marked loss of bone mineral density (BMD) after an SCI has been reported, of the order of 35% after two years from its occurrence, and the development of osteoporosis and fractures in more than 50% of patients[1-5]. Although the physiopathology of this process is not well understood, after an SCI there has also been observed a marked increase in bone turnover, especially during the first year after the SCI[6-10]. While the absence of load is the main factor related to this finding, the regulatory mechanism for this process is not clear. This fact, together with the absence of guidelines aimed at the prevention and treatment of osteoporosis after an SCI, could be the cause of defective treatment for these patients. Indeed, a study recently carried out in our unit found evidence that after a complete SCI fewer than 10% of patients had obtained anti-osteoporotic treatment, even after having had fragility fractures[2], a fact which has also been observed in other studies[11]. It is important to remember that the individuals who have a complete SCI are usually young people, which means that the risk of developing fractures over their life time is very high, clearly increasing at 3-5 years after the SCI[2], which indicates the necessity of adopting preventative measures in these patients.

The objective of this study is to analyse the development of BMD and bone turnover in patients with recent SCI, and the factors relating to the loss of bone mass in this process. This preliminary analysis shows the development of the BMD and the markers for bone turnover in the short-term, in the first 6 months of monitoring.

Patients and methods

Population of the study

Prospective study in which were included patients with recently occurring (<6 months) SCI of traumatic origin and severe in character (complete motor SCI [ASIA scale: A or B]). The patients were recruited consecutively (from August 2010 to January 2012) at the Guttman Institute for Neurorehabilitation and then referred to the bone metabolism pathology unit of the rheumatology service of the Clinical Hospital of Barcelona.

Those included were patients over 18 years of age, while those having diseases or processes which affected bone metabolism (Paget's disease of bone, rheumatoid arthritis, hyperparathyroidism, hypercortisolism, malabsorption syndrome, malignant tumours, transplants, recent pregnancy or breastfeeding) and/or who were following treatment with drugs which would interfere with bone metabolism (bisphosphonates, strontium ranelate, selective estrogen receptor modulators, calcitonin, hormone therapy, denosumab and teriparatide, amongst others) were excluded.

In all the patients the risk factors for osteoporosis were evaluated, including: family history of femoral fractures, personal history of fractures, tobacco and alcohol consumption, dietary intake of calcium (mg/day) and history of renal lithiasis. In addition the cause, level (tetraplegia/paraplegia), severity and type (spastic/flaccid) of SCI and associated complications were analysed.

The severity of the SCI was evaluated using the ASIA (American Spinal Injury Association) scale which, classifies MLs into 5 categories according to motor function and residual sensitivity: A: complete motor and sensory loss; B: complete motor and partial sensory loss; C and D: partial motor and sensory loss; E: without motor or sensory lesion[12].

The results were compared with a healthy control group of the similar age and sex.

The study was carried out with the approval of ethics committee of the hospital and adjusted in accordance with directives pertaining to research in humans. All the patients signed their informed consent for inclusion.

Analytical tests

Blood was taken from all patients at between 8 and 10 in the morning after overnight fasting. A biochemistry profile was performed which included calcium, phosphate and creatinine, determined by standard techniques, and levels of 25-hydroxyvitamin D (25-OHD) and parathyroid hormone (PTH) were assessed using automated chemoluminescence (Liaison, Diasorin and Advia Centaur XP, Siemens, respectively). In addition, the following biochemical markers for bone formation were determined: bone alkaline phosphatase (Bone AP, IDS, Vitro); osteocalcin (OC, radioimmunoassay, Elsa-Osteo-Cis, Gif-sur-Yvette, France) and amino-terminal propeptide of collagen type 1 P1NP, Cobas e411 automated method, Roche), and for bone resorption: carboxyl-terminal telopeptide of collagen type 1 (CTx, Cobas e411 automated method, Roche).

Bone mineral density

The BMD in the lumbar spine, proximal femur (femoral neck and total femur) and in the lower limbs (EI) were determined in all patients by means of double X-ray absorptiometry (DXA; Lunar Prodigy, Radiation Corporation Madison, WI, US). The densitometric risk categories (normal BMD, osteopenia and osteoporosis were defined according to the criteria of the WHO[13].

Statistical analysis

The results are expressed as the mean ± standard deviation of the mean (SD). The differences between the means of the continuous variables were analysed using the Mann-Whitney nonparametric U test, and the differences between proportions, by the Fisher test. For the comparison of paired variables the Wilcoxon test was used. To evaluate the association between variables the Pearson correlation coefficient was used. A value $p<0.05$ was considered statistically significant. The statistical analysis of the data was carried out using the SPSS program (version 18.0, Chicago, US).

Results

The clinical characteristics of the patients included in the study are shown in Table 1.

The study included 23 males with an average age of 38±15 years (range: 18-64) at an average of 102±33 days from suffering the SCI. All the patients had a severe SCI (ASIA A); 48% had tetraplegia and 52% paraplegia. The majority of patients (83%) had an SCI of the spastic type. All the patients had a severe residual functional affectation: 3 patients (13%) remained totally immobilized in bed, the rest (87%) required a wheelchair for mobility. The main cause of the SCI was a traffic accident (57%). The rest of the patients had an SCI due to falling (17%), diving into shallow water (13%), a sporting accident (9%) or domestic accident (4%). 12 of the 23 patients (7 with tetraplegia and 5 with paraplegia) were newly assessed after 6 months of follow up.

After the SCI a marked increase in markers for bone turnover (OC, P1NP and CTx) were observed compared with the control group (Table 2). No significant differences were observed in the value of bone markers as a function of the degree of lesion (patients with tetraplegia vs those with paraplegia). Also, those patients with SCI had a significant increase in levels of phosphate in the blood, and a reduction in values of PTH compared with the control group (Table 2). 39% of the patients had vitamin D deficit (<20 ng/ml), however, no differences were observed in levels of 25-OHD or in values of calcium in the blood compared with the control group.

At 6 months of follow up a significant reduction in markers for bone turnover (P1NP: -34%, p=0.005 and CTx: -26%, p=0.002) were observed, although they remained higher with respect to the control group, and there was a normalisation of the parameters for phosphorous-calcium metabolism (baseline phosphate: 4.4±0.4 mg/dl vs 3.9±0.4 mg/dl in follow up, p=0.011; baseline PTH: 32±20 pg/ml vs 40.8±22.9 pg/ml in follow up, p=0.09).

The BMD in the proximal femur and in the EI reduced significantly at 6 months follow up (total femur: -12.3+4.9%, p=0.002; femoral neck: -12.8+6.7%, p=0.002; EI: -7.7+3.7%, p=0.003) compared with baseline values (Figure 1). No significant changes were observed in the development of the lumbar BMD (1.214±0.2 g/cm^2 baseline vs 1.224±.2 g/cm^2 at 6 months, p=n.s). 50% of the patients had criteria for densitometric osteoporosis after 6 months follow up. No significant differences were observed in the development of BMD as a function of the level of SCI (tetraplegia vs paraplegia) or the type of lesion (spastic vs flaccid). None of the patients had skeletal fractures during the first 6 months of monitoring.

A negative correlation was observed between the values of BMD in the total femur and markers for bone turnover (Bone AP: r=-0.63, p=0.001; P1NP: r=-0.459, p=0.028; OC: r=-0.454, p=0.051). The values of CTx were not related to the values of BMD. The change in BMD at 6 months was not related to the change in markers for bone turnover.

Discussion

The results of this study show that after a complete motor SCI a marked increase in bone turnover and in loss of bone mass occurs, especially in the proximal femur, which leads to the development of osteoporosis in half of the patients, a complication which is already observed after 6 months of follow up, and which indicates the need to adopt preventative measures in the therapeutic approach with these patients.

Hence, over a period of only 6 months the patients included in this study had a loss of BMD in the proximal femur of 12% after an SCI and 50% developed densitometric osteoporosis. The BMD in the lumbar spine, however, remained stable during the follow up. These results coincide with those of earlier studies which indicate that the loss of bone mass after an SCI occurs early, already being evident at 6 weeks from the lesion[1,14] and of greatest magnitude during the first two years after the SCI, with loss of BMD which varies between 8 and 35%, depending on it location and the time over which it had developed[1,4,6,15,16], which leads to the development of osteoporosis and fractures in more than 50% of patients[4]. One of the main characteristics of the loss of bone mass associated with an SCI is its location, since, as seen in our study, it occurs below the level of the lesion, affecting, above all, the lower limbs[1,17,18]. This fact appears to be associated with the absence of mechanical load in the said location and which explains, furthermore, the high incidence of fractures in lower limbs which is observed in these patients, especially in the femur and tibia[2,19,20]. Also, even though it has been suggested that there is a greater loss of trabecular bone following an SCI[15], other studies show that the bone loss occurring in this process takes place in various sections. Hence, a study which analysed the development of bone mass using peripheral quantitative computerised tomography (pQCT) in the proximal femur of patients with a recent SCI, describes cortical bone loss and an alteration in bone microarchitecture and strength after SCI, which would be measured by an increase in trabecular and endosteal bone resorption[14]. The results of our study also suggest an early affectation in both bone sections; thus, the magnitude of the bone loss in the different sections of the proximal femur, femoral neck and total femur, was similar after 6 months of follow up, of the order of 12% in both type of location. However, no significant changes were seen in the lumbar BMD after 6 months of follow up, a finding which has also been reported in other studies[1,4], and which confirms the determining effect which the absence of mechanical load has in the lower limbs on bone loss associated with this process.

The majority of studies, both in experimental and human studies, report a marked increase in bone turnover after an SCI[1,6,8-10], especially during the first year of the lesion, a finding which we also observed in our patients. In fact, the patients included in this study had an increase in markers for

Table 1. Clinical characteristics of the patients include in the study

	Patients with SCI (n=23)
Age (years)	38±15
Males (n)	23
Risk factors for osteoporosis:	
BMI (kg/m^2)	24±5
History of renal lithiasis (%)	13
Active smoker (%)	30
Daily alcohol consumption (%)	13
Family history:	
Femoral fracture (%)	13
SCI characteristics:	
Development period of SCI (days)	102±33
Paraplegia/tetraplegia (%)	52/48
Spasticity (%)	83
Causes of SCI:	
-Traffic accident	57
-Fall from a height	17
-Diving into shallow water	13
-Sporting accident	9
-Direct axial trauma	4

SCI: spinal cord injury; BMI: body mass index.

bone turnover, especially P1NP and CTx in the blood, after an SCI of the order of 2 to 3 times higher than the control group, which, in addition –as has been observed in the study of Sabour et al.[21]– are negatively correlated with values of BMD in the proximal femur. This increase in the markers for bone turnover diminishes progressively, such that at 6 months, although they remain slightly higher, their values have reduced significantly. Furthermore, in those patients it was also observed that there was a reduction in the values of PTH and a secondary increase in phosphate, two findings previously described in patients with recent SCI and which had been attributed to the marked increase in in bone turnover which occurred after the lesion[6,8,9,22]. Both parameters, PTH and phosphate, were normalised after 6 months follow up. Even though a high prevalence of hypovitaminosis D has been seen in patients affected by SCI[23], this has been observed mostly in patients with a longstanding SCI. Our patients had recent MLs, less than 6 months earlier, a fact which may explain the absence of difference in values of vitamin D compared to the control group. Furthermore, although the increase in bone turnover which is observed after a recent SCI is a finding reported in most of the studies, the magnitude of this increase, and above all, its long-term development are aspects which are less well-documented. Hence, although the increase in turnover is particularly evident during the first few months after the SCI, some authors indicate the persistence of this increase some years after the SCI. Thus, Zehnder et al.[6] observed that around 30% of patients had an increase in bone resorption, evaluated by determining levels of free deoxypyridinoline ten years on from the SCI. This is a fact which coincides with the persistence in BMD loss, although at a lower level, which patients have after several years with an SCI[6,24], and which confirms the necessity of adopting preventative measures for the monitoring and treatment of this process.

The physiopathology of the alterations in bone turnover and loss of bone mass associated with an SCI is unclear, nevertheless, the absence of mechanical charge appears to be the determining factor for the loss of bone mass associated with this process[1]. Other factors such as the denervation which occurs after an SCI could also contribute as an additional factor in this loss. In this vein, experimental studies indicate a higher loss of bone in mice with an SCI than in mice subject to load on their extremities[16,25].

One of the main limitations of this study is the loss of subjects during the follow up. However, this is an initial analysis which includes a highly homogeneous sample of patients, all having had a complete motor SCI in the past 6 months, which therefore allows the application of the results to other patients with similar characteristics. Furthermore, it is remarkable in featuring a prospective cohort which includes one of the highest numbers of patients with SCI.

In summary, the results of this preliminary study show that after a complete motor SCI there is a marked increase in bone turnover and loss of bone mass below the level of the lesion, which leads to the development of osteoporosis in half the patients during the first year of follow up. These results confirm the necessity of establishing preventative measures against the development of osteoporosis as part of the therapeutic approach taken with these patients.

Conflict of interest: There are no conflicts of interest on the part of the authors.

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Figure 1. Variation in BMD in the lumbar spine (white bar), total femur (light grey bar), femoral neck (dark grey bar) and lower limbs (black bar) 6 months after SCI

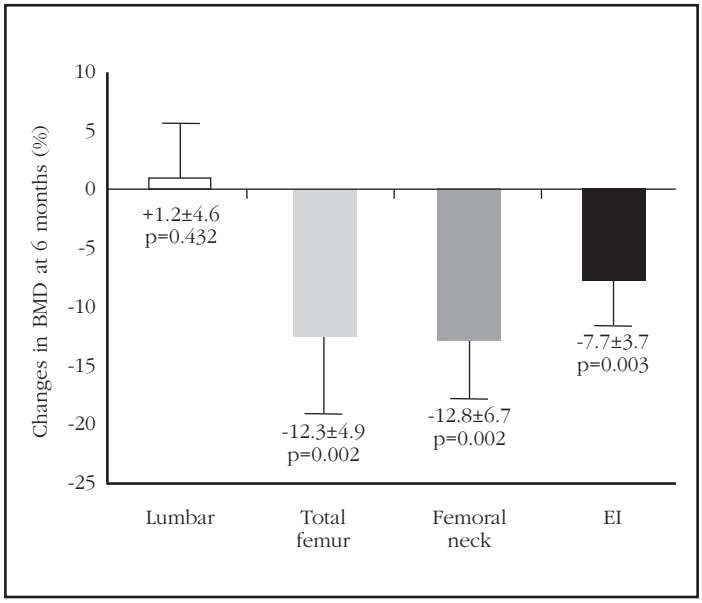

Table 2. Comparison of the parameters for phosphocalcium metabolism and markers for bone turnover between patients with SCI and the control group

	SCI n=23	**Controls** n=36	**p**
Age (years)	38±15	38±15	0.953
Males (%)	100	100	
BMI (kg/m^2)	24±5	24±3	0.843
Metabolism parameters phosphocalcic:			
Calcium (mg/dl)	9.5±0.5	9.5±0.4	0.612
Phosphate (mg/dl)	4.3±0.4	3.5±0.5	<0.001
PTH (pg/ml)	27±16	53±18	<0.001
25-OHD (ng/ml)	24±13	21±8	0.289
Bone turnover markers:			
Bone AP (ng/ml)	13.3±3.8	12.7±4.6	0.632
OC (ng/ml)	25.8±10.1	20.5±7.7	0.05
P1NP (ng/ml)	191±90	51±19	<0.001
CTx (ng/ml)	1.37±0.48	0.51±0.23	<0.001

SCI: spinal cord injury; BMI: body mass index; PTH: parathyroid hormone; 25-OHD: 25-hydroxyvitamin D; Bone AP: Bone alkaline phosphatase; OC: osteocalcin; P1NP: procollagen type 1 aminoterminal propeptide; CTx: carboxy-terminal telopeptide of type 1 collagen.

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Advances in the study of the mechanisms involved in the modulation of the expression of sclerostin in human cells

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Summary

Sclerostin plays an important role in the regulation of bone metabolism, as is shown in the dramatic changes in bone mass which occur when its activity is inhibited by means of monoclonal antibodies. However, the mechanisms which regulate its expression are still not well-understood. Various studies have shown an association between polymorphisms of the SOST gene promoter (which codes for sclerostin) and bone mineral density. Also, the degree of methylation of a CpG island near the start of the transcription is associated with marked changes in the expression of the gene. Therefore, it appears that the production of sclerostin is influenced by both genetic and epigenetic mechanisms, in addition to other hormonal and mechanical factors. A greater knowledge of these mechanisms would not only contribute to a better understanding of bone biology, but could open up new therapeutic opportunities.

Key words: *sclerostin, SOST, methylation, epigenetic.*

Introduction

Sclerostin is a protein coded by the SOST gene. This protein is secreted specifically by the osteocytes and has a negative effect on bone formation, through the inhibition of the Wnt canonical pathway[1]. The inhibition of this pathway has profound consequences for the activity of the osteoblasts; specifically, their differentiation is inhibited and their apoptosis induced[2,3]. The importance of sclerostin in bone biology has been seen in the description of cases of mutations of the SOST gene in humans which provoke an altered bone phenotype, with an increased bone mass[4,5]. On the other hand, the inhibition of sclerostin through the use of neutralising antibodies has been demonstrated to have a powerful anabolic effect in bone, both in animals and in humans[6,7].

Although the significance of sclerostin in bone homeostasis appears indubitable, there are various aspects of its biology which still remain unknown. Some of the least-known aspects are the factors which regulate the expression of sclerostin and the mechanisms involved. For example, it is not known why solely the osteocytes, and not other osteoblast line cells, are capable of expressing sclerostin. Possibly even more intriguing is the fact that there are osteocytes within the bone producing sclerostin, while others located within a few microns of them do not[8].

In some experimental models various effectors have been identified which are capable of regulating levels of sclerostin. On the one hand, among the positive effectors are the bone morphogenetic proteins (BMPs)[9] or the combined action of the tumor necrosis factor (TNF) and the tumour necrosis factor-like weak inducer of apoptosis (TWEAK)[10]. On the other, notable among the negative regulators are parathyroid hormone (PTH)[11,12], prostaglandin E2 (PGE2)[12] and mechanical load, this last factor being of special significance due to the role which this type of stimulus has on bone homeostasis[13]. Unfortunately, many of these experiments have been carried out in murine models and it remains to be seen to what extent they are transferable to human bone. Furthermore, although the effects of these factors have been described, the molecular mechanisms which underlie their effects on the expression of SOST have hardly been identified.

One of the obstacles which researchers are encountering when studying the regulation of sclerostin production is the absence of systems in which this gene is actively expressed. Currently, there are no human osteocyte lines available. The generation of some murine lines has been reported, but in spite of the fact that these show some of the characteristic phenotypes of osteocytes, their production of sclerostin is barely detectable. It would therefore of great interest to find a good system in which the factors involved in the regulation of the expression of this gene may be identified.

Curiously, not the entire promoter sequence for the SOST gene is preserved between species, which suggests that the regulation may differ as a function of the species. This is still more evidence of the necessity of developing human models. Some works suggest that region 5' of the gene would have two sections: one, close to the start of the transcription, which shows marked transcriptional activity, and the other, situated at some 1,000 base pairs' distance from the start of the transcription, which could have an inhibitory effect[14]. On the other hand, it is interesting to note that various groups, including ours, have shown an association between some polymorphisms located in the 5' region of the gene and bone mineral density (BMD)[15,16]. In the same vein, in genome-wide association studies (GWAS) some polymorphisms of a nucleotide (SNPs) have been found near this gene associated with BMD[17]. This suggests that these polymorphisms may have a functional impact and modulate the expression of the gene, but actually, it is not known if this really is the case or what would be the molecular mechanisms involved.

Given the importance attributed to sclerostin in bone formation, the identification of the molecular mechanisms which regulate its levels could open new areas of investigation in bone biology, and perhaps help to identify new therapeutic targets related to the inhibition of its production, which would have an anabolic effect on bone. Furthermore, the validation of new models of cells of human origin in which it would be possible to study these mechanisms could be crucial for the advancement of the understanding of the regulation of sclerostin. In this article we briefly review some recent results from our laboratory and those of other researchers to shed some light on these questions.

DNA methylation and the regulation of gene expression

A good number of the cytosines of mammalian DNA are methylated, especially when they are followed by a guanine, which is to say, when forming CG dinucleotides (often also known as CpGs, the "p" indicating the phosphate group which links the two bases). It is supposed that the methylation brings stability to the DNA and avoids "transcriptional noise" in the background. There are zones of DNA, called "CpG islands", which have a particular behaviour. These islands consist of regions of a few hundred nucleotides which are especially rich in CpG and which are found frequently in the promoter regions of many genes. In recent years it has been shown that the level of methylation in these CpG islands (and in the adjacent regions, called "CpG island shores") plays an important role in the regulation of the expression of many genes. In general, when the CpG of the promoter regions is highly methylated, the transcription of DNA to RNA is repressed, and, as a consequence, the levels of the protein for which the gene codes are reduced. Inversely, the demethylation of the promoter tends to be associated with the active transcription of the gene.

There are many molecular mechanisms involved in the regulation of gene expression through changes in methylation, of which only some are known. Thus, for example, the methylation of DNA may impede its bonding with some activating transcription factors. On the other hand, the methylated regions attract some proteins which bond specifically to these methylated regions. This is the case with MeCP2, the binding protein for methylated CpG[18]. However, it should also be taken into account that the methylation of DNA acts in combination with other epigenetic mechanisms, specifically with the postranslational modifications of the histones. In fact, when MeCP2 bonds to DNA, it recruits other proteins, such as HDACs (histone deacetylases) which modify the tails of the histones near this region. Together, these modifications contribute to the modulation of gene expression. For example, the highest levels of histone acetylation are usually associated with an activation of transcription; while, to the contrary, the methylation of certain lysines present in the histones is associated with gene repression[19].

The patterns of DNA methylation are transmitted through mitosis, which means that they are inherited from the cell which divides into two daughter cells. In this process an essential role is played by a family of enzymes called DNA-methyltransferases (DNMTs), in particular type 1[20,21].

DNA methylation can be a passive phenomenon, which is to say, it may appear during some cell divisions if the DNMTs do not perform their function of the remethylation the DNA daughter chains. But demethylation may also be an active process. This is to suggest that it is possible that some regions of DNA are demethylated without the necessity of cell division and the consequent replication of DNA having taken place. The process by which active demethylation occurs is not well understood, but the enzyme GADD45 and the conversion of the methylcytosines to hydromethylcytosines appears to play a special role in it[19,22,23]. In addition, its true significance in tissue homeostasis is also not well known. Nevertheless, it has been suggested that this process could be involved in osteoblast differentiation[24].

Our group has demonstrated that methylation and demethylation of some genes plays an essential role in variations in the patterns of gene expression which occur during the different stages of the differentiation of the osteoblast-lineage cells. For example, using the technique of laser assisted microdissection and subsequent DNA analysis of the cells thus captured, we have confirmed that during the step from osteoblasts to osteocytes there occurs a marked reduction in the methylation of the SOST gene promoter. Differently from that which occurs in the case of osteoblasts, this is a necessary requisite for osteoclasts to be able to synthesise sclerostin[25]. Other genes involved in the biology of the skeleton are also regulated, in part, through the level of methylation of their promoters. This is the case, for example, with osteoprotogerin, the ligand of RANK (RANKL), alkaline phosphatase, osterix or estrogen receptor[26-28].

DNA demethylation as experimental tool

The changes in the methylation of the CpG islands are very powerful regulation mechanisms. They are possibly not involved in the fine regulation of gene expression, but act as a type of molecular "interrupter" which starts and stops gene transcription. Once the demethylation allows transcription, other mechanisms, (humoral, physical, etc.) will be responsible for adjusting precisely the gene expression, in response to what is required at that moment[29].

The regulatory power of the mechanisms linked to methylation are shown in certain experiments in which DNA methylation is pharmacologically induced. For this, nucleotide analogs are often used, such as azacytidine and deoxy-azacytidine (or decytabine) which inhibit the activity of the DNMTs. Thus we have been able to demonstrate that the incubation of different types of cell with decytabine strongly induces the expression of sclerostin even when in normal conditions these cells do not express the gene[25].

This phenomenon has, furthermore, an interesting practical repercussion, in that it facilitates the study of the mechanisms which modulate the expression of sclerostin. Given that there are no human osteocyte lineages, or techniques to isolate viable osteocytes from human bone, it is complicated to explore the regulatory mechanisms for this gene in humans. Although there are different immortalised osteoblast lineages, and it is relatively easy to obtain osteoblasts from bone biopsies, these cells do not express sclerostin. However, the demethylation of its promoter with decytabine induces the expression of this gene, thus functioning, at least in theory, as an experimental model to analyse the physical and chemical factors involved in its regulation. But for this model to be really useful the osteoblasts should have a pattern of response to different stimuli similar to that of the osteocytes in *in vivo* animal experimental models[30,31].

In fact, this appears to be the case. The results which we have obtained with this model of osteoblasts treated with decytabine have confirmed the inhibitory effect of PTH and the stimulatory effect of the BMPs (bone morphogenetic proteins) on the expression of SOST[32]. We have also been able to confirm that the osteoblasts treated with decytabine maintain their response not only to humoral factors but also to mechanical stimuli. When these cells are subject to a pulsating flow of the culture medium (which simulates the stimulus of the osteocyte membranes by the liquid present in the lacunae and canaliculi of the bone) a series of biochemical responses is induced, notable among which is the induction of nitric oxide synthase (NOS), with its consequent accumulation in the medium. This response is maintained in the osteoblasts pre-

treated with decytabine. Furthermore, in these cultures it is possible to confirm that mechanical stimulus induces a reduction in the expression of sclerostin, in line with that demonstrated in *in vivo* experimental models[13,33]. The later experiments with nitric oxide inhibitors and donors have enabled the confirmation that nitric oxide synthesis really is involved in the inhibitor effect of SOST induced by mechanical stimulation[34].

SOST gene promoter, sclerostin and bone mass

Various studies of candidate genes and also those of genome association (GWAS) have found polymorphisms of the SOST gene associated with bone mineral density[15,35]. Hence, we have proved that women who are homozygous for the minor allele (G) of SNP rs851054, situated in promoter region 5' of the gene, have a BMD significantly lower than women with other genotypes. This suggest that this polymorphism may provoke differences in transcriptional activity as a function of the allele which is present. To explore further the mechanisms involved, we cloned the entire SOST promoter region (positions -1440/+30 in relation to the transcription start site or TSS) and confirmed its transcriptional activity in a luciferase reporter vector, after its transfection in different types of cell. The cloning of various regions of this fragment allowed us to confirm that the most active region appears to be in the first 500 nucleotides. In fact, the transcriptional activity of the vectors with insertion in the region -580/+30 is somewhat greater than that of the complete regions (-1440/+30). Contrarily, the most distal region (-1440/1030) is not active, while the intermediate region (-1032/-571) has a certain degree of activity, although clearly lower than that of the complete region or the region nearest to the TSS. Also, we found that BMP2 increases the transcriptional activity of these constructions, while PTH has no effect, which is in accordance with those studies which show that the effect of this hormone is mediated by an enhancer region located in several thousand base pairs[32].

On the other hand, from the genomic DNA of individuals for various polymorphisms frequent in the SOST promoter (rs801054 and rs801056), we cloned the promoter regions with each of the possible alleles for these polymorphisms in luciferase reporter vectors. We then analyzed its transcriptional activity after transfection into various osteoblast-type lineages. However, the differences in activity of the difference alleles were small (data not published). This suggests that the demonstrated association of these alleles with bone mineral density should be measured by indirect mechanisms which are not reproduced in these experimental models. Among these should be considered: certain factors, physical or humoral, present *in vivo* but not *in vitro*; the interaction of other cell elements in the bone microenvironment; or complex actions which involve the three dimensional structure of chromatin and the involvement of other distant regions of the DNA. An obvious candidate is called the Van Buchem region, situated several thousand gene bases away, and in which have been described regulatory regions (such as that called ECR5), which appear to mediate the response to some factors, PTH in particular[36,37].

Conclusion

Sclerostin plays a significant role in the regulation of bone metabolism, as is demonstrated in the dramatic changes in bone mass which occur when its activity is inhibited by means of monoclonal antibodies[38,39]. However, knowledge of the mechanisms which regulate their expression is still incomplete. Nevertheless, in recent years new data have been generated which allow one to sketch out, albeit schematically, some of the factors and pathways involved (Figure 1).

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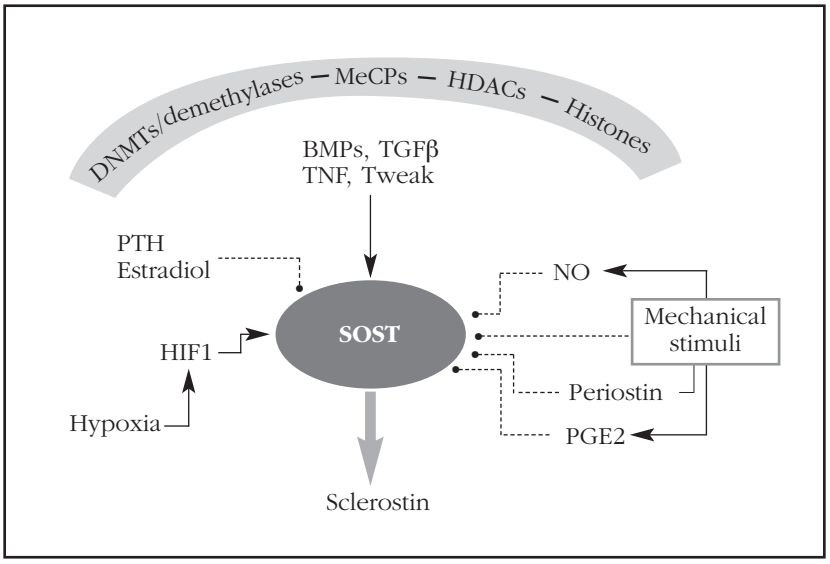
Figure 1. Schema of the regulation of the expression of sclerostin. The solid arrows indicate stimulatory effects; the dotted lines ending in a button, inhibitory effects



NO: nitric oxide; HIF1: inducible factor type 1 hypoxia.

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Osteoclasts: much more than bone remodelling cells

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Summary
The osteoclast has been considered classically as a cell with the exclusive function of bone remodelling, with a gregarious behaviour.
However, advances which have been made in recent years have changed this concept drastically, and we now know that this multinuclear cell is subject to complex biological regulation, necessary for it to exert a multifunctional role of unknown dimensions.
In addition to its participation as the only cell capable of reabsorbing the calcified bone matrix, the osteoclast is one of the cellular elements effective in the immune system, a function still little-known but expected, given its belonging to the monocyte-macrophage lineage. Its role in other processes, both local, such as as a collaborative element in osteoformation and hematopoietic stem cell niche maintenance, and systemic, is also beginning to be understood.
In this review the most significant findings contributing to our understanding of the biology of the osteoclast are analysed, with an eminently practical content and an approach aimed at understanding the possible molecular targets which will allow a better therapeutic treatment of such important diseases as osteoporosis, arthritis or cancer.

Key words: *osteoclasts, osteoporosis, arthritis, RANKL.*

Introduction

Osteoclasts (OCs), as the only cells capable of extracting the calcified bone matrix, are the protagonists in the delicate task of dissolving the crystals of calcium phosphate and digesting the collagen, by means of highly specialised structures[1]. Their pathogenic role in the induction of excessive bone resorption observed in pathological processes such as osteoporosis[2], arthritis[3], or cancer[4], is fundamental. The notable advances which have occurred since the start of this new century have allowed us to understand the intimate mechanisms which regulate the formation, activity and survival of OC, opening new possibilities for the design of drugs with more specific actions than those that already exist.

In recent years, the scientific effort dedicated to understanding the complex resorptive mechanisms has grown exponentially, with great advances being made through three main lines of research: 1) the study of a series of genetic diseases, relating the phenotypes observed to the dysfunction detected; 2) experimental studies based on the creation of animal models in which a determined gene is annulled or overexpressed; and 3) by obtaining precursors and mature cells in culture and analysing their responses to various stimuli. Taking into account the fundamental importance of OCs in the pathogeny of such significant diseases as arthritis, osteoporosis and cancer, along with the enormous quantity of information which has emerged in the last five years, we consider it necessary to carry out a review to update our knowledge in this important area of research.

General characteristics of osteoclasts

OCs are located on the internal surfaces of the Haversian canals of the cortical bone, in the trabeculae large than 200 microns and in the external walls of the bone, beneath the periosteum. Although potential precursors may be found in the peripheral blood, spleen and bone marrow, the mature cells are very rarely found away from the bone surfaces, except in pathological situations, such as in giant cell tumours. In the absence of the specific situation of high levels of remodelling, such as occurs at the metaphysis of the long bones during growth or in diseases such as primary hyperparathyroidism, OCs are scarce in the skeleton since they only comprise 1-2% of bone cells. They have a half-life of two weeks, and in normal conditions, after this period, undergo apoptosis[5].

In spite of their rarity in samples of non-decalcified tissue, their morphology is characteristic when activated, which enables them to be easily recognised as strongly polarised multinucleated structures, with a basal region for the interchange of external signals and a zone joined to the calcified matrix by a structure called the brush border. The OCs move, by means of podosomes, over the calcified surfaces, on which a single cell can form consecutively a number of Howship's lacunae. They have a number of immunohistochemical characteristics which facilitate their identification, among which are the expression of tartrate-resistant acid phosphatase (TRAP). Although TRAP mRNA has been identified in other tissues, such as the kidney, intestine and lung, as well as in activated macrophages, this enzyme continues to be an essential osteoclast marker whose expression appears very early, immediately before the mononuclear OC initiates the fusion mechanisms, increasing progressively through the different post-fusion stages until maturity is reached.

The OCs belong to the monocyte-dendritic-macrophage lineage, although, differently from other members of its progeny, it has the capacity to bond to bone by means of the $\alpha v\beta 3$ integrins, which are expressed in the surface of the podosomes and which have the property of interacting with the proteins of the matrix, such as osteopontin and vitronectin. Following the primary activation signal, the multinuclear OC is polarised and is stuck to the bone surface by means of specialised structure known as the brush border, at the ends of which are found the integrins which become bonded to the matrix producing a hermetic seal with the lacuna, an essential step for the interchange of ions and proteases necessary for proper bone resorption.

The basolateral zone of the membrane does not undergo significant morphological changes, but will play a role, which is poorly-understood, in cell communication and in the transport of ions. In the osteoblast cytoplasm there is a high level of carbonic anhydrase II activity which causes a dissociation of the cytosolic carbonic acid into protons (H^+) and bicarbonate ($HCO3^-$), the latter interchanged with chloride (Cl^-) by means of a specific channel, which allows the conservation of the intra-cellular isoelectric state. The proton is directed to the brush border, where a proton pump dependent on a specific ATPase (H^+-ATPase) transports it to the lacuna. In the vicinity of this pump is situated an ion channel (chloride 7 channel, ClC7) which is a simple ion interchanger which uses voltage gradient to obtain the energy necessary to transport them through the membrane. Specifically, this channel interchanges 2 Cl^- for 1H^+, and its function is highly important in the processes of lysosome acidification in general[6] and in bone resorption in particular.

The loss of function of the ClC7 is one of the most common causes of osteopetrosis[7] and is, together with the proton pump, an interesting therapeutic target[8], but limited, at the moment, due to the consequences of its extra-skeletal actions, above all, the risk of production of lysosomal diseases[9]. In the lacunae, through the union of these two ions, hydrochloric acid is formed, which acidifies the environment causing the hydroxyapatite to dissolve, liberating calcium and phosphate, while at the same time maintaining the cytoplasmic ionic charge in equilibrium. Lastly, through the lysosomes, a cysteine protease, cathepsin K, and a series of metalloproteases are secreted which, finally, cause the dissolution of the organic matrix. The resulting degradation products enter the OC by endocytosis and are transported to the basolateral region in vesicles rich in TRAP and released to the exterior by exocytosis.

Formation and activation of osteoclasts

The osteoblasts (OB) of mesenchymal origin reside, essentially, in the bone tissue and the adjacent bone marrow. However, the OCs and their precursors are a highly dynamic population, and the mechanisms which control their migration and arrival at the bone surfaces have recently emerged as essential elements of the homeostasis of the skeleton. OCs derive from hematopoietic stem cells, which will lead, through myeloid progenitors, to circulating monocytes and tissue macrophages[10]. The target organ will define the final characteristics of these cell populations, emitting different signals which will determine their different morphological and functional qualities: Küpffer cells in the liver, alveolar macrophages in the lungs, microglia in the central nervous system, histiocytes in the connective tissue, dendritic cells and macrophages in the lymphoid organs, and OCs in the bone. In spite of the fact that many of the properties of these differentiated myeloid cells, essentially their structure and function in the tissues, are known, there is still very little known of the intimate mechanisms which govern their differentiation and dynamics.

Migration of the precursors

Mononuclear lineage cells with the capability of differentiating into osteoclasts have been found in the bone marrow and in the bloodstream[11,12]. Although it is not known if there is a mononuclear precursor population specific to OCs, it is known that certain sub-classes of circulating monocytes and dendritic cells, as well as progenitor cells of monocyte-macrophage lineage resident in the bone marrow, have the capability of being transformed into OCs if they are subject to certain specific signals[13]. Using innovative fluorescence techniques which allow the visualisation of the behaviour of cells *in vivo*, Kotani et al. have recently shown that the mature OCs situated in the resorption surfaces come from the circulating monocytes which migrate to these regions of the bone where they undergo fusion, polarisation and development of the elements of the cytoskeleton which characterise active OCs[14].

The signals which attract the circulating precursor population towards the bone surfaces are starting to become understood, constituting an interesting group of molecules of potential therapeutic interest. These cells, which should express RANK in their membranes, become attracted to the bone marrow or the quiescent surfaces where, after receiving the RANKL signal, they are transformed into mature, polarised OCs with the characteristic cytoskeleton. This main signal comes from the mesenchymal cells of the bone marrow, from lining cells or from the osteocytes situated in the depths of the calcified matrix.

The RANKL signal is essential for the final activation of the OCs, although it is probably only executed in the target organ, there being signals which we could consider to be "anterior" which provoke the migration of the precursors from the circulation system. To date, various recruitment signals have been identified, notable among which is chemokine CXCL12, strongly expressed in stromal cells located in the perivascular regions of the bone marrow. The osteoclast precursors express the receptor of chemokine CXCR4, whose union with CXCL12 promotes the recruitment and survival of the OCs[15]. The CXCL12/CXCR4 axis has become a target of great interest in oncology[16,17] due to its key role in the migratory behaviour of tumour cells, although, taking into account the above, it is highly probable that it also participates in functions such as accelerated bone remodelling which occurs in postmenopausal osteoporosis, or in the different forms of bone destruction which characterise rheumatoid arthritis.

Another chemokine axis of interest is that featuring CXCL1 (fractalkine), expressed in osteoblasts, and its receptor, CX3CR1, expressed in OCs whose action could also be important in the recruitment of precursors[18]. Nevertheless, the design of small molecules with activity inhibitory to chemokines[19] is encountering a number of difficulties due to the toxicity caused by their poor specificity.

Another group of molecules with recruiting action are the bioactive sphingolipids. Known for their structural role in cell membranes, they have acquired additional importance due to their being precursors of molecules with a strong chemotactic capacity, such as sphingosine-1-phosphate (S1P) and ceramide-1-phosphate (C1P)[20,21]. The latter, with significant roles in the function and dynamics of other myeloid populations[22], does not appear to intervene in the migration of the OCs, with, to date, no receptors associated with these cells having been identified.

S1P is the product of the phosphorylation of sphingosine by two kinases, sphingosine-kinase 1 and 2, a reaction which is activated in response to a number of mediators which include various cytokines and hormones. After its synthesis it may be activated in the intracellular environment but also be released into the bloodstream, where it interacts with at least five G protein-coupled receptors, of whichS1PR1 and S1PR2 have been identified in osteoclast precursors[23,24]. After the bonding of S1P to its receptor, this is rapidly internalised in a way very similar to that which happens with the bonding of the ligand to CXCR4, and, at the present time, this is considered to be a highly significant factor in the dynamics of hematopoietic progenitor cells and in the traffic of immune cells between the lymphoid organs and the peripheral tissues. Its role in bone diseases is beginning to be understood, it having been observed that low concentrations of S1P are chemotactic for the osteoclast precursors, while high concentrations have the opposite effect. S1PR2-nul mice develop osteopetrosis, while in ovariectomised rats, the S1PR2 antagonist, JTE013, slows osteoporosis, reducing the number of OCs[24]. Contrarily, the ablation of osteoclast S1PR1 causes osteoporosis[25].

These facts suggest the existence of a fine control of osteoclast migration dependent on the gradient of S1P[26], which may be summarised as follows: in the bloodstream there is a high concentration of S1P, while in the bone tissue it is lower. The skeletal OCs, after the activation of the S1PR1, migrate towards the circulation system, while the activation of S1PR2 exerts an opposite effect, inducing migration in the opposite direction, with OCs accumulating in the bone. We are, therefore, looking at a molecular system of therapeutic interest[27-29], since the stimulus of S1PR1 or the blocking of S1PR2 causes an antiresorptive effect notable in murine models in, respectively, provoking the departure or slowing the arrival of OCs to the resorption sites.

Regulation of osteoclast differentiation

Osteoclast differentiation is a strongly regulated process whose study has been limited due to the necessity of using mixed cultures of osteoblasts and OCs to obtain mature cells[30]. Since the discovery of RANKL, the advance in the knowledge of these mechanisms has been enormous by making possible the culture of isolated osteoclast precursors in the presence of RANKL without the need for the interaction of other cells[31]. It is widely known that the mature OCs are the only cells in an organism capable of reabsorbing bone[32]. Nevertheless, to achieve the development of their complete resorptive mechanism the osteoclasts have to undergo a profound transformation after their arrival in the proximity of the mineralised surfaces, which starts with the initial intervention of M-CSF and the expression in its membrane of RANK (Figure 1). At present, the mechanism by which a sub-group of multipotential mononuclear precursors begin to express RANK in their membranes, and as a consequence, follow the path to differentiation as osteoclasts after being exposed to RANKL[33], is not known.

a) M-CSF signal

After the initial expression of PU-1, a transcription factor required for the generation of the progenitors of the lymphoid and granulocyte-macrophage series, which acts in the very early phases of myeloid differentiation, the expression of c-Fms occurs, the receptor of M-CSF which will characterise the population of the primitive osteoclast precursors[13,34]. After its union with the ligand, the c-Fms, as with other members of the super-family of tyrosine-kinase receptors to which it belongs, is phosphorylated and activated to ERK (extracellular signal-regulated kinase) through GRB-2 (growth factor receptor bound protein 2) and to AKT through Pl3K (phosphoinositide 3-kinase), provoking cell proliferation signals. In addition, through the activation of MITF (microphthalmia-associated transcription factor) the expression of Bcl-2 (anti-apoptotic B-cell leukaemia/lymphoma-associated gene 2) an essential factor for survival, is induced[35-38]. Lastly, the expression of RANK occurs in the membrane of the precursors, which will enable the action of RANKL on these cells and their final differentiation into mature OCs.

b) RANKL signal

RANK lacks intrinsic enzyme activity in its intercellular domain and needs to transduce the signal from the ligand through the recruitment of adaptor molecules, among them TRAF-6, GAB-2 (Grb-2-associated binder-2) and phospholipase C. The last two of these are not indispensable in the initial phase but are necessary in a subsequent amplification phase[39]. However, TRAF-6 is essential to activate the distal signal, in which NFkB, AP-1 and various MAPKs (mitogen-activated kinases), above all JNK (Jun N-terminal kinase), p38 and ERK, are involved.

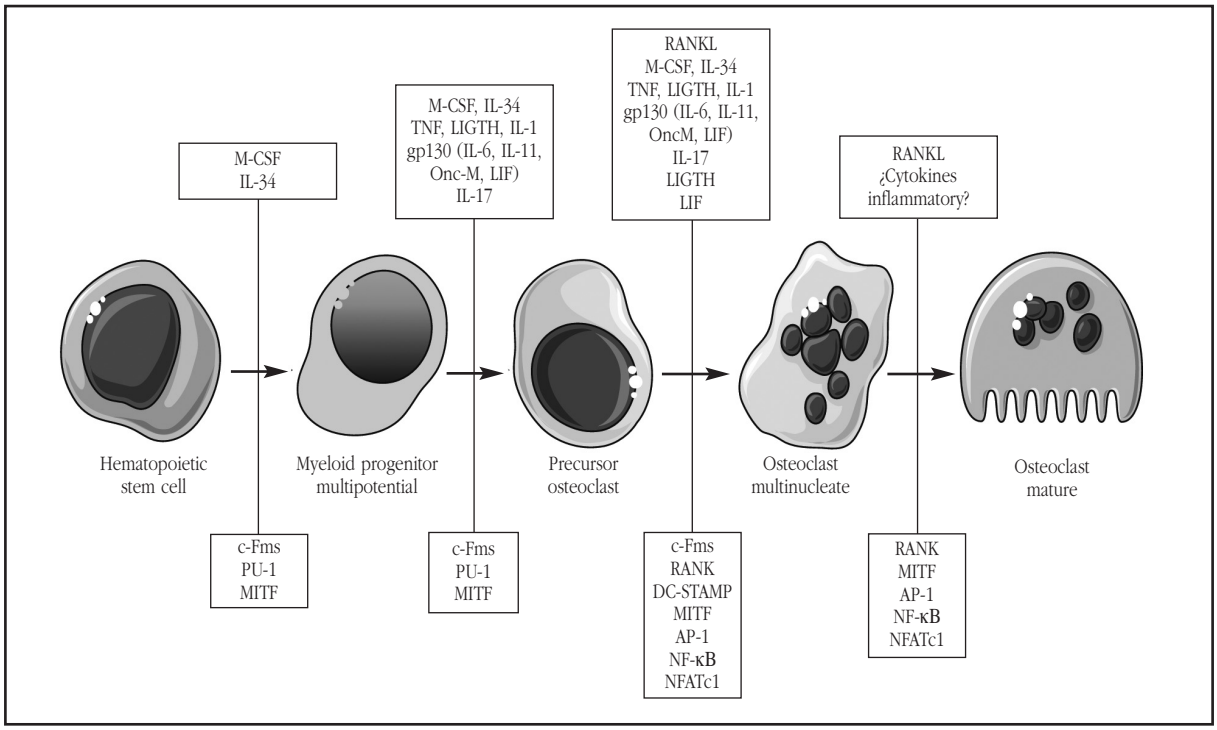
The activation of NF-κB is one of the earliest and most crucial molecular events which occur after the union of the ligand to RANK. NF-κB belongs to a family of dimeric transcription factors which, in the non-activated cell, stays captive in the cytoplasm due to being bonded with inhibitory proteins called IkB (inhibitors of κB kinase). The RANKL/RANK/TRAF6 signal provokes the proteolysis of these inhibitors, which allows the translation to the nucleus of free NFkB, where it bonds with DNA response elements, inducing the transcription of the target genes[40]. This intracellular signalling pathway participates in the regulation of various genes involved in immune and inflammatory responses, which produce cytokines such as IL-1, IL-2, IL-6, IL-7 and TNF, chemokines, interferons and anti-apoptotic proteins, such as BIRC2, BIRC3 and BCL2L1. In humans, the deregulation of NF-κB is associated with various diseases such as diabetes mellitus, Alzheimer's, autoimmune diseases, osteoporosis and arthrosis, and is a potential therapeutic target, partly limited by its non-specificity[41].

RANK also induces the activation of NFATc1 (nuclear factor of activated T-cells, cytoplasmic 1), currently considered to be the master regulator for osteoclast activation[42]. NFATc1 belongs to the family of NFAT transcription factors, identified initially in nuclear extracts of activated T-lymphocytes[43]. In subsequent studies it was shown that its role in osteoclast activation was significant when it was observed that the monocyte-macrophage precursor cells in bone marrow stimulated by RANKL had a selective and marked overexpression of NFATc1[44]. The activation of this factor is dependent on NFkB and c-Fms, probably in this order[45].

c) Co-stimulation and amplification of the RANKL signal

Coordinated with the RANKL signal other transduction pathways for inductor signals for NFATc1 have been observed in the OC (Figure 2), whose role could be decisive in pathological states[46]. At least two Ig-like receptors are known: OSCAR[47] (osteoclast-associated receptor) and TREM-2[48] (triggering receptor expressed in myeloid cells). Both are associated with adaptor proteins which contain ITAM (immunoreceptor tyrosine-based activation motifs) motifs such as DAP-12 (DNAX-activation protein 12) or FcRγ (Fc receptor common γsubunit). Although the ligand for these receptors is not known with any certainty

Figure 1. Maturation stages of the osteoclast. In the upper section are shown the principle cytokines involved, and in the lower section, the transcription factors and transmembrane proteins. The PU-1 and MITF expression is the initial event which characterises the population of myeloid precursors which will go on to differentiate into osteoclasts. These two transcription factors provoke the expression of the M-CSF receptor which, after its bonding with the ligand, induces the expression of RANK. This fact is definitive for the formation of the mature osteoclasts, after the cytoplasmic, but not nuclear, fusion, governed by DC-STAMP



MITF: microphthalmia-associated transcription factor; DC-STAMP: dendritic cell-specific transmembrane protein; LIF: leukemia inhibitory factor; Onc-M: oncostatin M.

(recently OSCAR has been associated with specific motifs expressed in fibrillar collagen)[49], when activated, the phosphorylation of the ITAMs by tyrosine-kinase occurs and, in collaboration with other molecules such as BLNK (B cell linker protein) and SLP76 (Src homology 2 domain-containing leukocyte protein of 76 kD), the activation of PLCγ2 is then provoked, contributing to the amplification of the RANK signal. It is not known whether these pathways are significant in physiological states, although in pathological situations such as osteoporosis, arthritis or cancer, it is highly probably that their over-activation contributes to the state of marked osteoclast stimulation which they exhibit[47-52].

NFATc1 is a regulator central to osteoclast activation, both in the sense of being a stimulator of the RANK signal and in the opposite sense, as a target for different molecules which inhibit its expression. In the positive sense, the expression of NFATc1 induced by RANK/NFkB/c-Fos is dependent on the signalling pathway p38. Other signals, coming from Ig-like receptors associated with adaptor factors such as FcRγ y DAP12, act in a coordinated way with the above signals through the transitory increase in intracellular levels of calcium, due to mechanisms not yet clarified which could also involve PLCγ2, which then activates calcineurin. This enzyme dephosphorylates the cytosolic NFATc1, which allows its translocation to the nucleus, where, in concert with PU.1 and MITF, it goes on to activate the promoter regions of various genes which code for molecules essential for osteoclast function such as cathepsin K, OSCAR, DC-STAMP, TRAP and V-ATPase-d2. In addition, there is an increase in its own synthesis through a process of auto-amplification described in 2005 by Asagiri et al.[45]. However, these secondary activation pathways of NFATc1 are dependent on the main pathway and, in the absence of RANKL, and no stimulus occurs in isolation from these receptors, leading to an absence of osteoclast activation[53].

To avoid unchecked osteoclast formation which would result from the NFATc1 pathway, there is a series of negative regulators which act on this factor, generally indirectly through the proximal signal[54]. Within the group of cytokines, IL-4 and IL-13, products of the Th2 cells, perform pleiotropic functions, among which is a powerful anti-osteoclast action which is executed in way which is dependent on STAT-6 (signal transducer and activator of transcription 6) with the final result being the expression of NFATc1. Other cytokines such as IL-10, IL-27 or IFN-γ inhibit the formation of OCs from their precursors or their activation, through mechanisms dependent on the RANK/NFkB/NFATc1 signal[55].

The activation of various TLRs (toll like receptors) reduces the rate of formation of mature OCs induced by RANKL through IFN-β-dependent mechanisms, although independent mechanisms have also been observed. On the other hand, the activation of TLRs is one of the most powerful inductors of inflammatory cytokines, such as TNF and IL-1, which act synergistically with RANKL in the production of inflammatory osteolysis in diseases such as rheumatoid arthritis or periodontal disease[56].

In brief, we may intuit that the TLRs, as key elements in the innate immune system, have an antagonistic role strongly dependent on context. On the one hand, by initiating the inflammatory response, the transformation of precursors into OCs would reduce, which would increase the pool of cells available for transformation into macrophages. However, in a more advanced stage, if their activation persisted in a sustained way, they would act as inductors for osteoclastogenesis, indirectly by means of inflammatory cytokines. The confirmation of this attractive hypothesis would constitute one more element to support the idea of the OCs' significant participation in the immune response.

There are other factors which inhibit the formation or activation of the OCs in addition to those already cited: cytokines such as TRAIL[57] (TNF-related apoptosis inducing ligand), IL-12 and IL-18[58], different intracellular signalling molecules such as SHIP1[59] (Src homology 2-containing inositol-5-phosphatase 1), NF-κB p100[60] and some components of the Notch pathway[61], various transcriptional repressors such as MafB (v-maf musculoaponeurotic fibrosarcoma oncogene family protein B)[62], C/EBPβ (CCAATenhancer-binding protein β)[63], IRF-8 (Interferon regulatory factor)[64], and BcL6 (B cell lymphoma)[65]. All these molecules are potential targets of therapeutic interest, but their detailed analysis is beyond the scope of this review.

d) Osteoclast activation pathways independent of RANKL

The RANKL signal is the most important osteoclast activation pathway and its annulment in murine models results in the complete disappearance of the OCs, which means that the role of pathways independent of activation appear, in theory, to be unimportant. However, in 2005 Kim et al. demonstrated that the presence of cofactors such as TGF-β, the hematopoietic precursors in mice null for RANKL, RANK and TRAF-6 would succeed in being differentiated into OCs[66]. It is evident that the interest in this topic is enormous, since there could be, at least in pathological circumstances, non-canonical osteoclast activation pathways which could be modulated to achieve different therapeutic responses to the complete annulment of OCs.

Within the TNF superfamily, given the structural homology between its members, various ligands and receptors have been investigated. One of the most interesting is LIGHT (also known as TNFSF14 and CD258). This type II transmembrane protein is expressed primarily in activated T-cells, NK cells, dendritic cells and macrophages, performing key biological functions in the innate and adaptive immune responses through the homeostasis, differentiation and activation of the T-lymphocytes[67]. It joins three receptors which share a structural similarity in their cytoplasmic stem: TNFRSF14/HVEM (herpes virus entry mediator), LT-βR (lymphotoxin β receptor) and DcR3 (decoy receptor 3)[68]. Although the role of LIGHT in bone resorption is not known, it has been observed that it causes a powerful osteoclastogenetic action independent of RANK and OPG, through AKT, NFkB and JNK in human and murine monocytes, using TRAF-2 and TRAF-5. Its function in bone diseases has not been clarified, but it is, without a doubt, an interesting target of potential therapeutic interest[69,70].

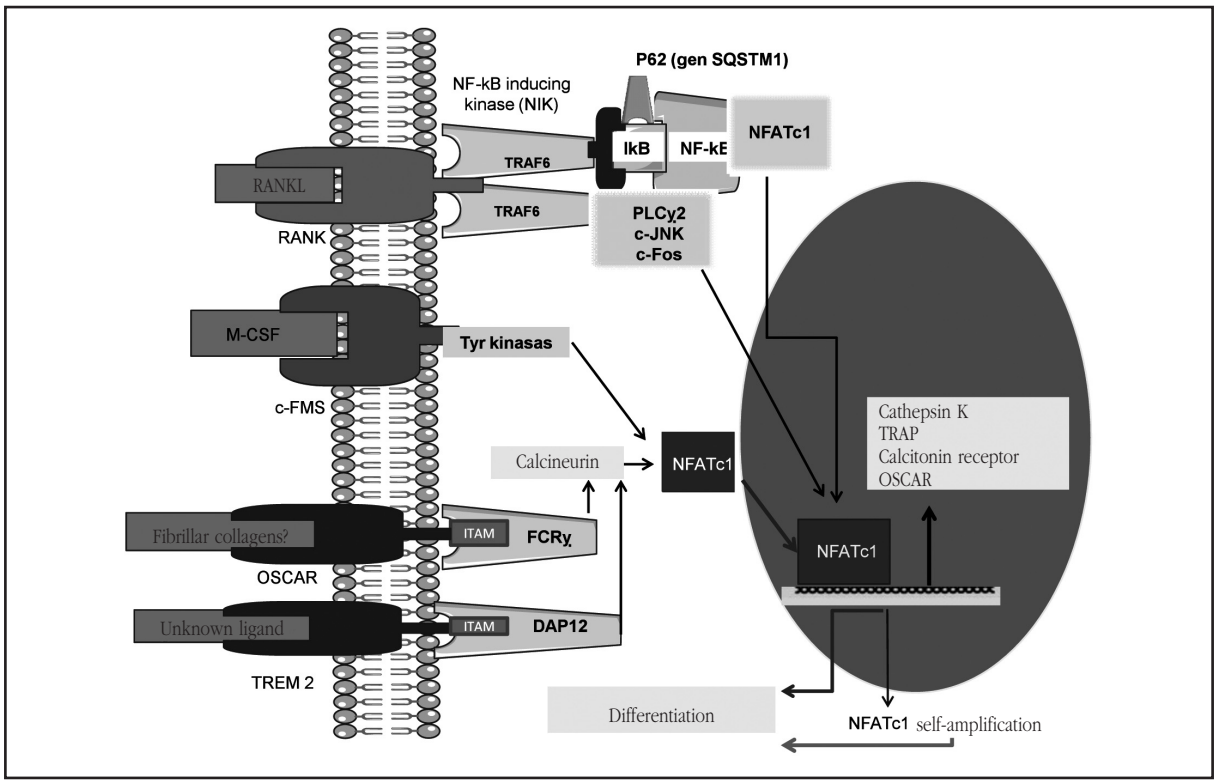
Two other members of the TNF superfamily have shown an osteoclastogenetic capability independent of RANKL. APRIL (a proliferation inducing ligand, TNFSF13) and BAFF (cell activating factor belonging to the TNF, also known as BLyS and TNFSF 13b), are capable, in *in vitro* culture, of inducing cells with the osteoclast phenotype from mononuclear precursors, although of a smaller size and with a lower number of nuclei and resorptive capacity than those induced by RANKL or LIGHT[71].

e) Origin of RANKL in osteoclast activation

Although the origin of RANKL which is involved in bone remodelling is classically thought to be the OBs, there have been a number of experimental findings which have cast doubt on this idea. In a pioneering study, Corral et al.[72] showed that the ablation of osteoblast progenitors by the administration of ganciclovir in mice bearing a thymidine-kinase transgene under the control of the osteocalcin promoter, did not cause any effects on the osteoclastic surfaces or on the markers for resorption, even after several weeks of follow up, in those in which the population of osteoblasts had disappeared from the bone surfaces. More recently, and using a similar transgenic murine model, Galli et al. observed that the absence of osteoblasts did not affect the levels at the baseline, or after being stimulated by PTH, of RANKL mRNA[73]. These studies indicate that the classic paradigm, which is that the RANKL which governs osteoclast activation comes from OBs or their precursors, should be revised[74].

OCs are formed in different locations in the skeleton with different purposes and with a variety of support cells charged with synthesising the RANKL necessary for their activation. For example, the femurs of mice which lack osteocytic RANKL develop a normal morphology, which indicates that the cortical modelling of the long bones is controlled by cells other than the osteocytes; whereas during chondral ossification, the main source of RANKL, which enables the reabsorptive action of the osteoclasts on the calcified

Figure 2. Canonic osteoclast activation and co-stimulatory signals. In addition to the canonic signals for proliferation and activation the osteoclast may receive other types of signals whose role could be highly important in inflammatory states



TRAF6: receptor associated factor TNF 6; PLC: phospholipase C; c-JNK: N-terminal kinase c-Jun; ITAM: immunoreceptor tyrosine-based activation motifs; DAP12: death associated protein 12; TREM 2: triggering receptor expressed on myeloid cells 2.

cartilage, are the hypertrophic chondrocytes[75]. The OC is also the effector cell for the erosion which characterises rheumatoid arthritis[76,77], and its activation is supported by the collaboration of the synovial cells of fibroblast lineage of the lymphocyte subclass Th17[78]. These facts suggest that the role of RANKL derived from the osteocytes could be limited to bone remodelling.

The osteocyte is a cell which provides a large amount of RANKL during physiological remodelling[79]. This fact is even more plausible from the biological point of view due to the known role of these cells in the detection of both mechanical and hormonal signals, which enables them to act at true regulators of bone remodelling, at least in physiological conditions. Using Cre-LoxP technology, which allows the modification of DNA in specific types of cells, Xiong et al.[74] caused the deletion of the osteocyte RANKL gene in mice and observed a reduction in OCs, with an increase in bone mass and of the markers for resorption, without alterations in the development of the skeleton or in dental eruption. In the laboratory of Takayanagi[79] the same results were obtained using similar technology. In summary, these studies demonstrate that osteocytes are the main producer cells for RANKL in physiological bone remodelling.

The RANKL which comes from the osteocyte is, therefore, the cytokine which controls physiological bone remodelling in response to mechanical and hormonal signals. The mechanism by which RANKL accesses OCs has not yet been sufficiently clarified. There is experimental evidence that the presence of soluble RANKL in the medium is sufficient to produce osteoclast expansion[80] and that the osteocytic projections express RANKL from the membrane and reach the bone surface where they make contact with the OCs and their precursors[64,81]. Finally, there is evidence that, both through the production of soluble RANKL and through that expressed in the membrane by the dendrites, the osteocytes control osteoclast activation. It has a dual role, since it also possesses the capability of producing sclerostin through the activation of its gene, SOST, and so contribute to the regulation of osteoformation[82].

Osteoclastic fusion

The osteoclast precursors are mononuclear cells which express TRAP, with no resorptive capability in *in vitro* cultures. The first step by which they acquire their functionality is through cell fusion, which then enables the formation of mature OCs. Understanding the intimate mechanisms which control this critical event in the physiopathology of remodelling is fundamental to the development of the new therapies.

In physiological conditions, the pre-OC TRAP cells + and the mature OCs are only found on the bone surfaces, which indicates that the fusion occurs in these locations. Using techniques of DNA subtraction in precursor cells stimulated by isolated M-CSF or M-CSF and RANKL, it was observed that DC-STAMP (dendritic cell-specific transmembrane protein) is an essential molecule for the fusion of mononuclear cells as a first step for the formation of active mature OCs. This transmembrane protein, discovered in 2000[83], is also expressed in dendritic cells and macrophages[84]. Its annulment in murine models provoked osteoporosis associated with a complete absence of fused mononuclear OCs as well as foreign-body giant cells. In these mice there persisted a moderate degree of resorptive activity in the mature cells, which indicates that their fundamental role is performed at fusion[85]. The regulation of DC-STAMP is complex and depends not only on the RANKL/RANK pathway but also on other independent factors, such as IL-32[86], Tal1 (T-cell acute lymphocytic leukemia 1)[87], LDLR (low-density lipoprotein receptor)[88], CCN2/CTGF (CCN family 2/connective tissue growth factor)[89] and vitamin E[90], among others, whose role is even less well known but which could be future targets of therapeutic interest.

OC fusion is promoted by other molecules such as the inflammatory cytokines. Among these, in addition to the actions already mentioned of RANKL, both TNF-α and LPS (lipopolysaccharide) are capable of inducing OC fusion under certain circumstances. For example, the action of TNF-α is specifically blocked by Ac anti-TNF-α, while the effect of LPS is partly blocked by these drugs, and completely blocked by polymyxin B[91]. The activation of these pathways is accompanied by intracellular signals dependent on kinases, and when inhibitors are used specific to these pathways OC fusion is reduced, while levels of DC-STAMP are not altered. These findings indicate that there are alternative pathways which regulate OC fusion independently of DC-STAMP, although it is not known if they exert physiological functions or only interfere with pathological processes[92].

Additional roles for osteoclasts

In addition to their function as the only cells capable of reabsorbing calcified bone matrix, OCs participate in other processes which we summarise below.

1. Stimulation of bone formation

Bone remodelling is a coupled process in which the osteoclast activity is followed by the action of the osteoblasts. The pharmacological inhibition of the former provokes a reduction in the latter, while the osteoforming stimulus is followed by a secondary increase in resorption. In principle, the model would appear to be simple, attributing to factors released from the matrix reabsorbed by the OCs a role in the recruitment of osteoblast[93,94]. However, in a study published in 2001, the Molecular Biology Group of the University of Hamburg demonstrated that, in some murine models of osteopetrosis and in a patient with the malignant infantile form, in spite of a functional alteration in the resorptive mechanism in the presence of a normal number of OCs, such as is produced with the annulment of the chloride channels ClC-7 C, there was normal bone formation[7]. This fact suggests that there are factors independent of the matrix reabsorbed by the OCs whose role in the coupling is probably more significant.

Among the mechanisms in which OCs intervene directly stimulating osteoformation, the following have been proposed[95]: on the one hand, ephrin B2, expressed in the osteoclast membrane, is capable of provoking an activation signal by bonding with its osteoblast receptor EphB4; also, sphingosine-1-phosphate is capable of causing the recruitment of osteoblast precursors to the remodelling sites[96], although treatment with analogues of this molecule has not shown significant results in the mending of fractures[97]. OC expresses, in addition, regulatory factors negative to osteoblasts, such as Atp6v0d2 (a subunit of the V-ATPase proton pump)[98]. Even though the physiological role of these molecular signals is not known, the findings which have been commented on suggest that the intervention of the OCs in remodelling is not limited to bone resorption, but that they also play a significant role in the coupling through molecular signals which participate in the recruitment, activation and inhibition of the osteoblasts.

2. Immune cells

Both OCs and OBs have the capability of responding to a wide variety of cytokines produced by the cells of the innate and adaptive immune systems[78,99-101]. The OCs contain all the mechanisms necessary for endocytosis and the processing of exogenous proteins coming from the material generated during resorption and in pathological situations such as osteomyelitis. In 2009, Kiesel et al.[102] demonstrated that the OCs could recruit T CD8+ FoxP3+ cells and present their antigens. These cells would play a regulatory role, whose function in non-inflammatory situations is unknown. A very attractive but non-proven hypothesis relates this capacity of the OCs as presenters of antigens to the existence of a large reservoir of CD8+ central memory T-lymphocytes in the bone marrow, the former participating in the latter's recruitment and maintenance[103].

The extraction of necrotic bone during a bacterial infection is another of the mechanisms in which OCs play a part in the immune response. In fact, in an elegant study in which murine models which emulated the biology of osteomyelitis and of periodontal implants were used, Li et al.[104] demonstrated that the functional inhibition of the OCs by bisphosphonates and by osteoprotogerin was associated with an increase in the quantity of necrotic cortical bone around the implant which acted as nests for the bacterial colonisation, while at the same time reducing the size of the drainage

orifice through which the opsonised bacteria were expelled to the exterior of the lesion. These data are highly significant since they suggest that the pharmacological inhibition of osteoclasts could be contraindicated in bone infections, as well as in the pathogenesis of osteonecrosis of the jaw, where bacterial colonisation is very important, and where OCs would play a key role, at least in its initial phases.

3. Articular cartilage

In those process in which the destruction of hyaline articular cartilage occurs, giant multinucleated cells have been observed which express the osteoclast phenotype (TRAP+, cathepsin K+, MMP9+, CD14−, HLA-DR−, CD45+, CD51+ and CD68+). These cells, called "chondroclasts" in some publications, have the capability of reabsorbing the cartilaginous matrix and have been implicated in the pathogeny of diseases such as rheumatoid arthritis or arthrosis[105]. Their specific role has not been established with any certainty, although there is various indirect evidence to suggest that they may play a significant role on articular damage. It is known that 30% of the total RANKL which is produced in arthritic joints is synthesised in the cartilage, essentially through the chondrocytes[106]. The soluble part of this cytokine acting like a paracrine, may participate, through osteoclast activation in locations of chondral-sinovial contact, in the pathology of erosion and of juxtaarticular osteopenia, which characterise rheumatoid lesions. Furthermore, even though it has not been demonstrated with sufficient certainty, chondrocytic RANKL may contribute to the transformation and activation of the mononuclear precursors, resulting in chondroclasts capable of degrading the cartilage. The mechanisms through which this action would occur is not yet known, but there is, undoubtedly, an interesting question to be asked based around about the possible therapeutic role of the inhibitors of RANKL in processes such as arthrosis.

4. Energy metabolism

Osteocalcin, a small peptide produced by osteoblasts, stimulates the secretion of insulin by the beta pancreatic cells, a finding of enormous importance in decisively implicating bone tissue in the hormonal control of energy metabolism[107]. This molecule has a number of the characteristics of a hormone: it is a specifically cellular product, synthesised in a pre-propeptide form and secreted into the circulation after a process of vitamin K-dependent gamma-carboxylation. This fact explains its great affinity for the bone matrix, which causes it to be released during bone resorption and converted into its active form after exposure to the acid pH of the resorption lacuna. In transgenic mice which lack V-ATPase activity, hypoinsulinemia and glucose intolerance associated with reduced levels of osteocalcin are observed[108]. A study which analysed the effects of alendronate in a small sample of patients showed reduced levels of infra-carboxylated osteocalcin which is associated inversely with an increase in body weight and of fat mass[109]. However, a review of the results of the FIT, HORIZON and FREEDOM studies did not show any alteration in these parameters, nor in glucose metabolism[110]. In summary, while animal models suggest a role for bone remodelling in the control of energy metabolism, the studies carried out in humans show discordant results which need to be clarified in the future[111].

Conclusions

The OC has been considered classically to be a cell whose function is exclusively that of bone remodelling, and which exhibits gregarious behaviour. However, in the last decade experimental findings have drastically transformed this over-simplistic view. The OC shares common origins with the cells of the immune system, both in the myeloid and the lymphoid series. Its role in articular inflammatory diseases such as rheumatoid arthritis is probably highly significant, since, to its well-known function as the only cell capable of dissolving the calcified bone matrix, are added new roles due to its capacity to secrete cytokines and as an antigen presenter cell. OCs, as extraordinarily dynamic cells, are therapeutic targets of enormous interest (Table 1) due to their participation in processes such as osteoporosis, arthrosis or cancer.

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Table 1. Summary of potential osteoclastic molecular targets

Molecular target	Characteristics	Consequences of pharmacological intervention	Citation
CX3CL1 (fractalkine)	Chemokine expressed in the osteoblast membrane with chemotactic and pro-adhesive action	Its blocking reduces the recruitment of osteoclast precursors	112
CX3CR1	CX3CL1 receptor expressed in the osteoclasts	Its blocking reduces the recruitment of osteoclast precursors	113
CXCL12/ CXCR4	Chemokine and its receptor both expressed in the osteoblasts	Its blocking reduces the arrival of OCs to the bone	114
S1P	Lipid mediator which controls the dynamics of the migration of the osteoclast precursors	The S1P agonists promote the arrival of the osteoblasts by means of the receptors S1PR1 and 2	29
CSF-1R (c-fms)	CSF receptor expressed in osteoclast precursors	Reduces osteoclast migration and activation in experimental arthritis	115
MAPK MK2	One of the most specific MAP kinases in the transduction of the intracellular osteoclastic signal	Inhibition of osteoclast activation without effecting osteoformation	116
NFATc1	Nuclear factor key to osteoclast activation	Inhibition of osteoclast activation	117
TGF-β	Multifunctional cytokine which regulates proliferation in different cell lines, very abundant in the bone	Blocking the TGF-β signal inhibits RANKL-induced osteoclastogenesis	118
Gα11 protein	Osteoblastic G protein involved in osteoclast activation	Its over-expression provokes osteopenia through a dual mechanism	119
PKC-δ	Central role in differentiation, fusion and function of OCs participating in the ERK signalling pathway of M-CSF and RANKL	Its inhibition alters the intracellular osteoclastic signal	120
DC-STAMP	Transmembrane protein which functions as an essential regulator of osteoclast fusion	Functional blocking of the mature osteoclasts	121

S1P: sphingosa-1-phosphate; CSF-1R: colony stimulator factor receptor 1; MAPK: mitogen-activated protein kinase; TGF-β: transforming growth factor beta; PKC-δ: protein kinase C delta; DC-STAMP: dendritic cell-specific transmembrane protein.

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Doctors handwriting

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Introduction

Doctors are famous for their poor handwriting. It is not for nothing that the expression "doctor's handwriting" exists, referring to handwriting which is almost illegible, and which, in all cases, only the art and sagacity of the pharmacist can decipher. In fact, if one looks for the definition of "legibility" in some online dictionaries phrases such as: "the legibility of this prescription is nil" are given as an examples[1]. Popular culture considers this fact as an almost inherent quality of the medical profession.

However, from a legislative point of view, Royal Decree 1718/2010 of 17th December, regarding medical prescriptions and dispensary orders, states: *"All the data and instructions given on medical prescriptions should be clearly legible"*[2]. Doctors therefore have a duty to write their prescriptions clearly.

Is there any truth to all this? It may be that doctors' handwriting is as legible as that of the rest of the population and that what we have here is an urban legend. Or if it is indeed the case that doctors have worse, often illegible writing, what impact could this fact have on the health of their patients? These questions have led us to investigate what has been published about this matter in the scientific literature, with the aim of finding firm answers.

Materials and methods

In order to research this article we carried out a bibliographic search in the following databases:

a) In Spanish: Google Academic, SciELO, Dialnet, Freemedicaljournals and Latindex, using different combinations with the following key words: letra, médico, legible, ilegible, legibilidad, prescripción, doctor,

b) In English: PuMed, Google Scholar, DOAJ, Freemedicaljournals, Open J-Gate, Electronic Journals Library, EBSCO, EMCARE and Academic Keys. The terms used were: Writing, medical, illegible, legibility, prescription, doctor.

Results

History

There have been references to the handwriting of doctors since the time of Molière. Hence, in his play "The Doctor in Spite of Himself" (in Spanish, "El médico a palos") the author satirised doctors who wrote in a Latin which was illegible to all but themselves[3]. A century ago, in The Lancet, in January 1915, an editorial condemned poor handwriting, reproducing "the most atrociously illegible prescription ever seen", as well as the arbitrary way in which it was interpreted by the pharmacist. They concluded that "unless there may be an understanding or private code between the prescriber and the pharmacist, the only thing that could be said of this prescription is that the doctor wrote it should have been ashamed of themselves"[4]. Forty years later, the topic appears again in a letter from J.J. Conybeare, who comes to the defence of bad handwriting, saying that he considered it to be a mistake to penalise an examinee for poor handwriting, maintaining that "trying to decipher handwriting was a question of honour, and that deliberate penalisation should be avoided"[5]. A debate then ensued between defenders and detractors of the illegibility of doctors' handwriting. Hence, a month later, a letter from W.W Kaye supported the opposite position, expressing the view that it was not enough to penalise exams or written tests by reducing marks for poor handwriting, but even proposing that disapproval should be shown. He considered that "poor handwriting could only be considered to be no less an example of bad manners than presenting oneself at a social occasion poorly-dressed, dirty and with muddy boots"[6]. One week later, the debate had turned to whether or not the defect could be corrected or not, and E.W. Playfair held that "yes, this was possible up to the age of 60, and that he had achieved it at 22…". And his elegant signature appears as proof of this. The author was annoyed with his complacent colleagues and agreed with the view that "poor handwriting appears to

be more like bad manners than ugliness because, unlike from the latter, it is easy correctible". Other, more cynical letters commented that, among other things, poor handwriting is inversely proportional to knowledge, or that it serves to hide spelling mistakes 6.

The debate. Do doctors have worse handwriting than the rest of the population?

Given the history presented above, studies have been carried out to try to answer this question. Thus, in a study conducted by a group of researchers at the University of Kansas, 20 workers (10 women and 10 men) were chosen from 7 different professions (accountants, lawyers, builders, scientists, doctors, mechanics and engineers) who were asked to write a certain sentence in a period of not more than 17 seconds. Then four researchers independently evaluated the legibility of the different sentences, awarding a score of 1 to 4 (deficient, passable, good and excellent) without knowing anything about who had written them. After adjusting for age and educational level the only truly significant difference was between men and women but not between professions. Out of all the cases 40% of the sentences written by men were illegible (a score lower than 2 being considered to be illegible), as opposed to 20% of the women[7]. In this study the writing of the doctors was neither more nor less legible than that of other professions.

The British Medical Journal published a similar study, but on this occasion only health sector workers were selected from: clinicians, managers and administrators. They had to write the sentence "Quality is the best thing since sliced bread" and were obliged to stop writing after 10 seconds. The evaluation of the writing was made by four non-clinical volunteers using the same scale as was used in the aforementioned case (Figure 1). With the aim of summarising the scores given by all the evaluators in a single figure these were added together and then 3 points subtracted: this created a scale which ranged from 1 to 13. 209 samples of writing managed to be collected. The average score was around 7. Again, it was impossible to find any statistically significant difference between doctors and non-doctors (p=0.074) (Table 1). The rest of the results agreed with the earlier study, reaffirming that the women had better handwriting than the men (average of 6.3 as opposed to 8.5, $p<0.0001$)[8].

In the medical literature, however, there also appear publications which affirm that doctors do have worse handwriting than other health workers. In a study, also published in the British Medical Journal by Lyons et al. in 1998, 92 workers from different hospital departments were recruited and divided into three groups: 1) doctors, 2) nurses and other health workers, and 3) administrators. Each of them was asked to complete a form with their name, the 26 letters of the alphabet and the numbers 0 to 9 as clearly as possible. Subsequently, the forms were analysed with "Teleform", a computer programme which when unable to recognise a character gives it an error score. All the statistical work was carried out using the SPSS® statistics software.

In general, there were no differences in terms of non-recognised numerical characters between the three groups. However, the doctors had the worse average score in terms of the recognition of each letter. The difference was statistically significant both if compared individually with each group as well as if these were combined. The same result was obtained when males were excluded from the study. Possible confusion factors were controlled, such as the department in which the individuals worked and their ages[9].

In analysing these studies it does not appear to have been established unequivocally that doctors had worse handwriting than the rest of the population. In these studies professionals from various fields were required to write something at a given speed. The result was that there were no statistically significant differences, but the sample size of the Schneider et al.[7] study was very small, and both in this and the work by Berwick et al.[8], the way of evaluating the legibility was somewhat subjective. On the other hand, there have also been published articles[9] which confirm the hypothesis that doctors write illegibly on occasion. Thus, a group of Spanish researchers took a representative sample of clinical histories, which included numbers, at a hospital in the southeast of Spain. Certain specialities such as intensive care, haematology, gynaecology and paediatrics were excluded for having peculiarities in their systems for recording data. Understood as "clinical history" were any documents written by a doctor which included the name of the patient, the age, the reason for the consultation and the medical situation. Then, two resident doctors who were recent arrivals at the hospital and not involved in the control of admissions or the drafting of clinical cases evaluated the legibility of the documents on a score of 1 to 4:

1) Illegible (all or almost all words impossible to identify).

2) The majority of the words are illegible, the meaning of the text is confused.

3) Some of the words are illegible, but the writing could be understood by a doctor.

4) Legible, all the words could be clearly read.

In cases of disagreement between the two doctors, a third adjudicated the scoring. Thus, 117 reports were examined, of which 18 (15%) had a score of 1 or 2. In the study the results were given for each speciality individually. This showed that the worst scores belonged to the surgery department[10].

Another study, carried out in a university hospital in Switzerland, evaluated the legibility of medical prescriptions. The results were that 52% had poor legibility and that 4% were totally illegible. And it was not only that they could not be read: those with the worst legibility usually also had a number of errors[11].

Figure 1. Representative image of each category of writing (receiving the same score from all the evaluators. A deficient, B passable, C good, D excellent). Berwick et al.[8]

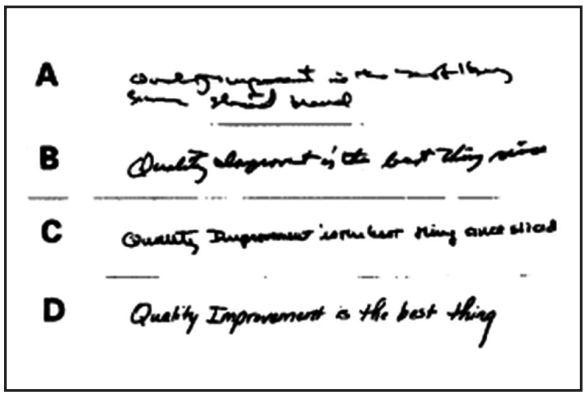
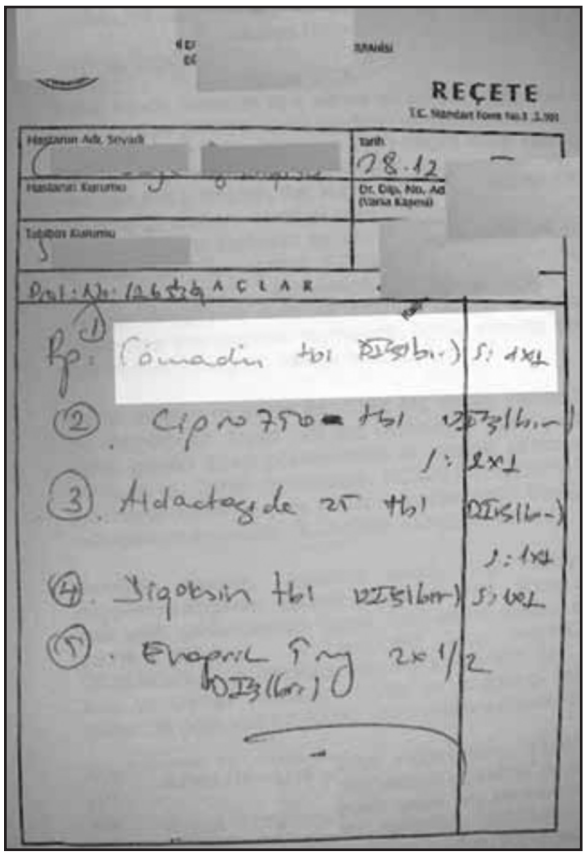


Figure 2. Difficult to read medical prescription, as a result of which the pharmacy dispensed Famodin instead of Coumadin. Yilmaz et al.[14]



Finally, a study carried out at the Cook County Hospital in Chicago showed that 16% of doctors had illegible handwriting and in 17% it was barely legible[12].

Repercussions

A further step is the legal prosecution of this problem and the sentencing of doctors and/or pharmacists for not writing clearly on the prescription for a drug, with a different drug being dispensed, with the end result being the death of the patient. Although many cases have been published, we will comment on only a few. Thus, a doctor had to pay a fine of 225,000 dollars to the family of a patient who died because a prescription which prescribed 20 mg of Isordil (isosorbide dinitrate) was interpreted by the pharmacist as Plendil (felopidine), a calcium antagonist used in the treatment of high blood pressure and whose maximum dose is 10 mg/day. After 6 days of taking an overdose of felopidine, the patient died of a myocardial infarction. The professionalism of the doctor and the attention paid by him to the patient was not called into question by the judge, who blamed the illegibility of the prescription for causing the death of the patient[13]. The pharmacist had to pay a fine equivalent to the doctor's.

On another occasion, a patient of 65 years of age had surgery to carry out the replacement of the mitral valve, and was prescribed Coumadin (Figure 2). However, the pharmacist did not dispense Coumadin because he interpreted the prescription as having been written for Famodin. The patient therefore did not take the anticoagulant that had been prescribed to him and in the follow up visit a month later had an INR (International Normalised Ratio) of 0.7 and a mitral thrombosis visible in the echocardiogram. He was perfused with heparin and when the INR reached 3.6 the surgery was carried out. A large number of thromboses were extracted from the left auricle as well as from the prosthetic valve. During surgery the patient developed bradycardia and it was necessary to install a pacemaker. The patient subsequently had severe hypotension and died during the operation. His family sued the pharmacist for being responsible for the occurrence. In order to establish possible responsibilities a study was conducted to try to evaluate the legibility of the prescription: the prescription was sent to 113 pharmacists with different levels of experience. It was interpreted correctly in only 70.8% of cases (75.6% among those most experienced and 43.7% of the most novice apprentices) (Table 2)[14].

Few professionals expose their handwriting more than doctors, and in very few situations is that which is written so important to the life of a person. When a patient is seen in an emergency clinic and he is given a copy of his clinical history written by us, they usually read because they are concerned and want to know in greater detail what the doctor told them.

The question of whether doctors write better or worse is important if we take into consideration the risks which are taken when a prescription or clinical record is illegible. A doctor should have perfectly legible (if not nice) handwriting always. It is not acceptable that, out of 117 reports written by doctors 18 (15%) can simply not be read[10], and even worse, if that were possible, that 4% of the prescription evaluated as illegible and 52% as difficult to read, as seen in the study by Hartel et al.[11].

We have already seen the consequences which inappropriate writing may have. On occasion,

Table 1. Average number of handwriting errors by group and character type. Berwick et al.[8]

	Median errors			Value of p	
	Doctors	**Nurses and other health professions**	**Administrative**	**All**	**Medical versus the rest**
All	**N=38**	**N=32**	**N=22**		
Letters*	7 (0-10)	3 (1-6)	4 (2-5)	0.006	0.001
Numbers#	1 (0-1)	1 (0-2)	0 (0-1)	0.15	0.60
Only women	**N=13**	**N=28**	**N=16**		
Letters*	6 (3-10)	3 (1-6)	3 (1-5)	0.10	0.036
Numbers#	1 (0-1)	1 (0-1)	0 (0-1)	0.29	0.82

* Maximum possible error in the alphabet = 26
\# Possible error numbers = 10

Table 2. Percentage of pharmacists according to level of experience who interpreted the same prescription, associating it with different medications. Yilmaz et al.[14]

	Pharmacist		Apprentice with experience		Rookie apprentice		Total	
Business name	N	%	N	%	N	%	N	%
Coumadin	34	75.6	39	75	7	43.7	80	70.8
Famodin	10	22.2	13	25	9	56.3	32	28.3
Famoser	1	2.2	-	-	-	-	1	0.9

patients die because of this type of negligence. The pharmacist should not be having to guess what it was the doctor wanted to write. In the final study[14], around 30% of the pharmacists end up providing an incorrect drug due to the ambiguity of the prescription.

Why, it should therefore be asked, does this type of thing happen? Too much work and too little time in which to carry it out could explain it, but it could also be related to a lack of awareness of this issue. Socially, it does not appear to be something which is worth addressing, but rather as seen as an amusing aspect of doctor's writing.

Be that as it may, handwriting which involves the health of patients should be able to be read without any difficulty, however long it takes to be written. Having said all this, there is no excuse for not writing correctly. Therefore, this requires a commitment and an awareness of the risks which are taken when notes are written unclearly and in a hurry. In the end it is the patients who suffer.

Possible solutions

One solution which, at least in the field of medical prescriptions appears to be happening, is the computerisation of documentation. This possibility has already been suggested in many of the aforementioned articles. The same is happening with clinical records. The electronic prescription may be an important aid in facilitating the legibility of prescriptions as well as clinical reports. However, this also runs the risk that, again with the excuse of being in a hurry and having too much work, computerised jargon will start to be used based on abbreviations and invented words, plagued with spelling mistakes, above all with accents, which could eventually make the text legible but difficult to understand or interpret correctly. An example could be that shown in Table 3.

Conclusions

It seems clear that a significant percentage of doctors have illegible handwriting which, on the one hand, results in significant difficulties in understanding medical reports written by hand, above all for people not related to the health sector, and on the other, is the cause of the dispensing and administering of the wrong medication.

Conflicts of interest: The authors declare that they do not have conflicts of interest of any kind.

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Table 3. Abbreviated and full text in an electronic clinical history

Abbreviated text
Pat male 73y with PH of DM II, AH and IC who attended 4 pain in tx
Full text
Male patient of 73 years of age with personal history of diabetes mellitus type II, arterial hypertension and ischemic cardiomyopathy, who attended due to pain in the thorax

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Clinical case debate: therapeutic holidays, yes or no?

DEAR EDITOR:
The wake-up call from the Food and Drug Administration (FDA), the European Medicines Association (EMA) and the Spanish Agency for Medicines and Health Products (AEMPS) regarding the relationship between the use of bisphosphonates (BP) and the incidence of atypical femoral fractures has caused people to start to consider the option of a break in the continuous use of BP, so-called "therapeutic holidays".
The American Society for Bone and Mineral Research (ASBMR) quickly initiated a working group which published a position statement on the theme of atypical fractures, above all to describe the criteria by which to define them[1,2].
Since then, there have been various authors who have reviewed the prevalence of atypical fractures in their records and their possible relationship with the use of BP[3-5]. The increased risk due to their continuous use appears to be clearly related to time, with the relative risk increasing substantially from the fourth year[3], although the absolute risk is in the region of 11 fractures for each 10,000 years.
These data have led to a serious debate about over how long a period BP should be administered and if therapeutic holidays might be opportune. To date, the most commonly accepted opinion seems to be not to allow these holidays in patients who remain at high risk of suffering a new fragility, while for the rest there should be a strict evaluation of whether the BP has achieved the therapeutic objectives for which it was prescribed[6]. The situation is not that clear-cut, however, what all the societies and experts are agreed on is that treatments should not be withdrawn indiscriminately from patients with osteoporosis for fear of atypical fractures. The risk of suffering an osteoporotic fragility fracture in patients who have already suffered a fracture, for example, is much higher if the treatment is withdrawn than the risk of suffering an atypical fracture.
There are no dogmas in medicine but, at the moment, it seems that we should focus therapeutic holidays on low risk patients, but act much more cautiously with the rest. In other diseases such ischemic cardiomyopathy no patients with a history of angina or myocardial infarction should be left without treatment with hypolipidemics.
Above all, decisions should rest on scientific evidence, avoiding the temptation to take advantage of certain situations to try to achieve non-scientific objectives.

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DEAR EDITOR:
I read with interest the special document publish recently in your review in which, on the one side Drs Sosa Henríquez and Gómez de Tejada and on the other, Dr Malouf Sierra, debated the appropriateness or otherwise of therapeutic holidays for bisphosphonates, based on a clinical case[1].
The authors present a case of a woman of 63 years of age with a history of early menopause and vertebral fracture at 53, for which she had received treat-

ment with alendronic acid for 10 years with good tolerance and compliance. In a current densitometry the patient had a T-score of -2.5 in the lumbar spine and -1.5 in the femoral neck, which means a significant increase in bone mineral density in both areas relative to that at the start of the treatment. The authors told us that she had experienced no falls or fractures during this 10 years, and posed the question as to whether or not a therapeutic holiday for bisphosphonate should be given.

In any disease, before we consider whether to continue, change or withdraw a treatment, we should ascertain whether or not this treatment has worked during the period it has been used. In the case presented to us we are told that the patient had not suffered new fractures, but it appears that this refers to clinical fractures. To ensure that no morphometric vertebral fractures had occurred we would have to carry out a dorsal-lumbar X-ray or a vertebral morphometry. Only then would we be able to say that there were no fractures, given that a high percentage of vertebral fractures are asymptomatic[2]. So, once the presence of new morphometric vertebral fractures in the patient has also been discounted, I think that this more than justifies proposing therapeutic holidays for a drug which has a residual effect in the bone, meaning that it would continue to act in spite of not being administered, and which is not without well-known complications. Some of the arguments Drs Sosa and Gómez de Tejada use to defend the continuation of the treatment are debatable. For example, they compare the discontinuation of treatment with bisphosphonates with that of antibiotics or anti-inflammatories used, respectively, to resolve an infection or reduce inflammation. However, alendronate has a terminal half-life in the skeleton of more than 10 years[3], which allows continued activity in the target tissue for a long time after it is discontinued, something which does not occur with antibiotics or anti-inflammatories, which have half-lives of only a few hours.

Sosa and Gómez de Tejada defend the maintenance of treatment with alendronate in the patient because, to their understanding, the patient continues to be a high risk patient simply for having suffered an earlier vertebral fracture. It is true that patients with a previous vertebral fracture have a higher risk of fracture than those without fracture[4], but this risk diminishes with time[5], and after 10 years without the appearance of new fractures the risk is already much lower, even more so if the fact that the patient has been receiving antiresorptive treatment with alendronate for all those years is taken into account. With bisphosphonates we achieve not only an increase in BMD, which is already associated with a reduction in the risk of fracture, but also an improvement in other bone parameters more related to quality, and which explains more than 80% of its anti-fracture effect[6].

Finally, the authors Sosa and Gómez de Tejada comment that the bisphosphonates are quite safe drugs, and this is completely true, given that the risk of serious complications such as osteonecrosis of the jaw or atypical fracture are extremely low in patients with osteoporosis treated with oral bisphosphonates. But to take this risk, low as it may be, is only justifiable in patients in whom the expected benefits of the drug are clearly greater than this risk, as could be the case in of a patient just after the fracture, but not 10 years after. Furthermore, there is a clear association between these complications and the period of exposure to bisphosphonates[7-8].

It would be more difficult to decide on the discontinuation of bisphosphonate in the case of a patient with a T-score in the spine of <-3. As the T-score at the start of treatment was -3.7 and the patient had not had fractures during these 10 years, we could say that the alendronate had worked, but possibly the patient's current risk remains sufficiently high that the risk of osteoporotic fracture clearly outweighs the risk of complications. This would justify maintaining the antiresorptive treatment with bisphosphonates or with another drug with better reversibility in the bone such as denosumab.

But returning to the case presented to us, I believe that it is more than reasonable to propose therapeutic holidays for bisphosphonates. This does not mean leaving the patient without any antifracture effect, since we know that their skeleton will "ooze" alendronate, neither should we forget their bone fragility. The new challenge will be to avoid holidays for eternity, as well as to know how to monitor the patient to decide the right moment to reinitiate the treatment, which should always happen before the patient develops a new fragility fracture.

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DEAR EDITOR:

The optimum duration of a treatment for osteoporosis is not defined, the exception being the use of teriparatide whose administration is limited to two years. We know that for other drugs it should be longer, but we do not understand well on what criteria this decision might be based. Undoubtedly, these criteria should include the persistence of the therapeutic indication, but other aspects should also be taken into account.

The debate published in this review[1], based on a clinical case by, on the one side Dr Sosa et al. and on the other Dr Malouf, is very interesting. It deals with the question of whether or not to continue with bisphosphonate (BP) in a patient after 10 years of treatment.

In this work they analyse in depth the appearance of adverse effects in relation to the period of time for which the BP has been taken. On the one hand, the appearance of osteonecrosis of the jaw (ONJ), an infrequent complication whose risk does not justify the cessation of long-term treatment, and on the other, the appearance of atypical femoral fractures in these patient, a complication whose incidence could be related to the duration of use of these drugs.

The Spanish Agency for Medicine and Health Products (AEMPS), on April 15th 2011 published an information briefing in which it recommended that patients treated with BP be periodically evaluated (especially after the first 5 years). As Dr Sosa comments, many doctors have started to withdraw treatment with BP from their patients without evaluating whether this withdrawal was appropriate or not. In practice, this translates into leaving a large number of patients with a high risk of fracture without therapeutic protection. We know: firstly, that exposure to BPs increases the incidence of atypical femoral fracture; secondly, that this incidence increases with the duration of exposure to this drug; and thirdly, that in any case, in patients with osteoporosis the incidence of atypical femoral fracture is very low compared with that of osteoporotic fractures.

The consideration of the aforementioned works allow one to deduce that the decisive factor in deciding whether a treatment with BP should be continued or not, is the risk of osteoporotic fracture which the patient has at the time of proposing the discontinuation of the therapy.

The risk is considered to be high when the patient has a bone mineral density (BMD) in the femoral neck lower than -2.5 T, or when they have a history of a previous osteoporotic (vertebral or hip) fracture. This therapeutic approach has become particularly clear from various recent works in the literature[2,3] and is picked up in a recently published work in the review, the American Journal of Medicine[4]. Here the patients are classified in three categories: a) high risk (T-score in the hip lower than -2.5; previous vertebral or hip fracture; treatment with high doses of corticoids); b) moderate risk (T-score in the hip higher than -2.5; absence of previous hip or vertebral fractures; c) low risk (lack of therapeutic criteria at the start of treatment, meaning: treatment inappropriate from the start). In the first category the withdrawal of treatment is not considered to be justified, but with periodic re-evaluation of the therapeutic indications. In the second, the consideration of a temporary withdrawal ("therapeutic holiday") is advised after 3-5 years of treatment. In the third category, logically, the treatment should be discontinued.

The Spanish Society for Bone and Mineral Metabolism Research (SEIOMM) includes these recommendations in a document which takes the aforementioned criteria, adding that if the withdrawal of treatment with BP is desired for some reason from a patient who still has the criteria for being at high risk of osteoporotic fracture, the therapeutic approach should not simply be to discontinue it but to substitute it with another therapeutic agent which acts in a different way[5]. In the clinical case with which we are concerned, the patient had, after 10 years of treatment, osteoporosis in the lumbar spine and a history of vertebral fracture, which means that they ought to be considered as a high risk patient, and that the treatment should be continued or changed for another treatment, since the incidence atypical fractures in patients who have had treatment with BP for more than 10 years is not known.

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DEAR EDITOR:

We have read with interest the clinical case debate regarding therapeutic holidays[1], which clearly reflects the positions for and against the cessation of treatment with bisphosphonates after a period of 5-10 years for alendronate, and perhaps 3-6 years

for zoledronate. We have less evidence in relation to risedronate and even less regarding the risks and benefits of maintaining treatment or not beyond 10 years[2], as is raised by the case under debate.

It is probable that much of the argument that lies behind this subject stems from the lack of incontestable evidence on how to proceed in a case like this, and can only be understood after the appearance of rare complications associated with chronic treatment with bisphosphonates – and other powerful anti-catabolics – such as osteonecrosis of the jaw or atypical fractures[3,4]. These possible complications have caused the medical equivalent of a "social panic", although their risk is really low compared to the benefits, due to the efficacy of these drugs when used in patients with a real risk of osteoporotic fractures[5].

Therefore, as stated in SEIOMM's recommendation document[5], the type of patient who most benefits from continuing the treatment beyond 5 years seems clear. However, we should not forget that among women treated for osteoporosis for 5 years with alendronate and monitored for a further 5 years without treatment, new fractures appear in 22% of cases and, more significant, the vast majority of these will appear during the first year that we have no markers to help us identify these patients[6].

Although scarce, and methodologically questionable, this is the best evidence for treatment with bisphosphonates for up to 10 years. In any case, what is striking is the preoccupation the medical community has with this specific issue when compared with other therapies employed in other pathologies such as, for example, myopathy, diabetes, nephrotoxicity, cataracts, cognitive deterioration or erectile dysfunction, among others, associated rarely with statins (although its benefits in relation to overall and cardiovascular mortality continue to be clear)[7]. The same may be said of the proton pump inhibitors, with which have been associated pneumonia, *clostridium difficile* infection, osteoporotic fractures, thrombocytopenia, iron, vitamin B12 and magnesium deficiency, rhabdomyolysis and interstitial nephritis, and which continue to be widely used drugs[8].

What might happen in the risk-benefit balance in the treatment with bisphosphonates beyond 10 years is reminiscent of one of the best-known sequences in the film "Out of Africa" in which the protagonist, Karen Blixen, played by Meryl Streep, says: "When in the past explorers arrived at the limits of the known world they were afraid to continue and wrote" "There be dragons!"". Until we have solid proof – and this seems unlikely – we can continue to discuss this *ad infinitum*. Hopefully, at least, we will soon have alternative therapies which have been proven to be efficacious in this context, before, as it appears, for fear of dragons we stop treating ever more patients at risk.

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DEAR EDITOR:

I have read the clinical case debate "Therapeutic holidays: yes or no?" published recently in this Journal[1] and, having recognised the excellent arguments and wide literature reviews of those putting the case for and against continuing treatment, dare I, as a doctor, give my opinion on the question raised?

Focusing on the case: it concerns a patient in whom treatment was initiated at 53 years of age due to a vertebral fracture and bone mineral density (BMD) in the range for osteoporosis. The risk factors were corrected and treatment initiated with alendronate and vitamin D, which seems to me a correct approach. This treatment has been maintained for 10 years and is now being assessed as to whether to continue with it or to take what is called a "therapeutic holiday". In terms of the comment about the BMD in the hip not being in the range for osteoporosis, one should take into account that, at 53 years of age, bone loss occurs primarily in the spine. This means that studies to evaluate hip fractures are carried out in older populations, when this fracture starts to appear[2,3].

The situation of the 63 years old patient after 10 years of treatment with alendronate and vitamin D is the following: she has not suffered new fractures (the risk of fracture is greater in the year following the appearance of a fracture); her BMD has increased and she currently has a T-score of -2.5, having reached a plateau in the last two years; and

lastly, the marker for bone resorption has reduced with treatment.

Given the residual effects of the disphosphonates after their withdrawal[4], and the fact that the bone markers may be elevated for 6 months, or even up to a year and a half, after treatment stops, depending on the type of disphosphonate used, in my opinion, this patient may discontinue the alendronate and stop the intake of the vitamin D necessary, since after 10 years there have been no new fractures and the BMD has increased to become stable in the last two years. On the other hand, I would suggest carrying out a new assessment after a year or a year and a half to see how it is developing and, depending on the clinical situation at that time, I would make an assessment as to whether to continue with the therapeutic break or to reinitiate treatment with the same drug, or a different one.

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RESPONSE OF THE AUTHORS:

We have read the various letters to the Editor which have been submitted by readers, some in favour and some against, in the debate around whether therapeutic holidays are appropriate or not.

This is a controversial topic, about which we have no scientific evidence. Hence the difference of opinion, although it is clear that above all there is, in all those who have written (as well as in ourselves), an underlying fear of harming the patient in any way.

We believe the debate enriches our knowledge, and so we thank those readers who have expressed their opinions, encouraging others to continue this type of discussion on any subject published in the Journal.

Manuel Sosa Henríquez
Mª Jesús Gómez de Tejada Romero
Jorge Malouf Sierra