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GENETICS AND PHARMACOGENETICS OF PAGET'S DISEASE OF BONE

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SUMMARY

1 INTRODUCTION

Paget's disease of bone (PDB) is a disorder characterized by chronic focal rapid bone remodeling with the formation of bone structurally abnormal. It was first described in 1877 by Sir James Paget as "osteitis deformans". Paget's disease of bone typically results in enlarged and deformed bones in one or more regions of the skeleton [1, 2]. This disease is most often asymptomatic but can cause a variety of medical complications resulting in considerable morbidity and reduced quality of life [3].

1.1 EPIDEMIOLOGY

PDB is most common in Caucasian people of European descent, but it also occurs in African-Americans, while it is rare in people of Asian descent [4, 5]. Clinical, radiological and necropsy data from different countries suggested pronounced geographical variations in the prevalence of the disease. The greatest prevalence rates were described in Britain, followed by Australia, New Zealand and northeastern United States, countries with an high immigration flow of people of British descent in the 19th and 20th centuries [4, 5]. Despite the impact of the disease on the population, limited information about the epidemiology and the true prevalence of PDB in Italy have been available for many years. More recently, the establishment of a registry of Italian PDB cases and the conduction of radiological, biochemical and bone scan surveys extended the knowledge about prevalence, characteristics, genetics and environmental determinants of PDB in Italy.

The first epidemiological observation of PDB in Italy was a radiological survey from Stuart who analyzed more than one-hundred thousand non selected skeletal radiographs in Siena (years 1950-1966) and found 94 PDB cases, consistent with an overall prevalence of 0.09% [6]. An age related increase in the frequency of pagetic cases reaching 0.20% in the 7th decade and a slight male predominance were observed. Conversely, only 4 PDB cases (0.018%) were observed among the 21,777 radiographs from patients under the age of 40. These estimates were

comparable to those reported in similar studies from unselected skeletal radiographs in different populations and in the same period [7]. A re-analysis of selected skeletal radiographs involving pelvis and spine, demonstrated prevalence rates above 1.5-2.0% in subjects older than 40 years [6]. More recently, Detheridge et al. performed an European study on the prevalence of PDB [8]. The study was based on both postal questionnaires and radiological surveys performed in 1982 in different European towns, including 2 Italian towns, Milan in northern Italy and Palermo in Sicily. The prevalence of PDB was higher in Britain (8.3-2.3%) and France (2.0-2.7%) than in any other Western European country. In Italy the prevalence of PDB from abdominal radiographs was reported to be 1.0% in Milan (1.6% in men and 0.4% in women) and 0.5% in Palermo (0.3% in men and 0.7% in women). The sample size, however, was low to obtain a clear picture of the prevalence of PDB in Italy. In order to extend epidemiological knowledge, the prevalence of PDB has been recently estimated in two Italian towns: the district of Siena from radiological, biochemical, and scintigraphic surveys and the city of Turin from pelvic radiological surveys of different decades [9]. Siena is a town of central Italy, located in a rural region that remained fairly isolated until recent times, and with a low immigration flow. In contrast, Turin that is located in northwestern Italy, recognized from the Sixties a strong immigration flow from other Italian regions (mainly from southern and northeastern, but also from central Italian regions); so, only about 50% of actual population was born in this district, and Turin population could be considered sufficiently representative of the whole Italian population. By these studies, the prevalence of PDB was estimated to be within 0.9-2.4% in the general population from the city and the surrounding of Siena and within 0.7-1.0% in the general population from the city and the surrounding of Turin. Differences in prevalence rates between and within the 2 towns were mainly due to the different approaches, radiological in Turin and

radiological, scintigraphic and biochemical in Siena (Fig. 1).

Town	Years	Method	Age (years)	No. of observations	Prevalence of Paget's disease (%)		
					All patients	Males	Females
Siena	1950–1966	Unselected X-rays	≥20	100,268	0.094	0.094	0.093
Milan	1981–1982	Pelvic X-rays	≥55	1,061	1.00	1.60	0.40
Palermo	1981–1982	Pelvic X-rays	≥55	509	0.50	0.30	0.70
Turin	1986–1988	Pelvic X-rays	≥60	2,305	0.43	0.74	0.22
Turin	1992–1993	Pelvic X-rays	≥60	1,101	0.73	1.22	0.43
Turin	1999–2000	Pelvic X-rays	≥60	1,984	0.60	1.00	0.34
Siena	1999–2000	Pelvic X-rays	40–90	1,778	0.89	1.05	0.79
Turin	2001–2002	Pelvic X-rays	≥60	1,219	0.90	1.40	0.56
Siena	2000–2003	Biochemical (ALP)	45–89	7,449	1.75	1.88	1.14
Siena	2000–2004	Bone Scan	43–88	7,906	2.45	3.00	1.93

Fig.1. Age-related prevalence of PDB in Siena from radiological, biochemical and scintigraphic surveys (adapted with permission from Gennari L et al J Bone Miner Res 20:1845-1850, 2005).

In particular, the analysis of pelvic radiographs performed between 1999 and 2000 in Siena showed a radiological prevalence of 16/1778 (0.89%), consistent with an estimated overall prevalence of PDB from 0.98% to 1.48%, considering that the pelvic involvement is commonly described in 60-90% of PDB patients. Slightly lower rates were observed in Turin, with a radiological prevalence of pelvic PDB of 41/6609 (0.62%) and an estimated overall prevalence ranging from 0.69 % to 1.03%. Overall, 296 of the 7449 subjects showed elevated alkaline phosphatase (ALP) levels and normal liver enzymes, with a prevalence of "biochemical hyperphosphatasia" of 3.97%. Interestingly, 87 (36.8%) of the 236 subjects with elevated ALP that accepted to perform a more detailed screening also showed increased bone ALP and radiological or scintigraphic diagnosis of PDB. Based on the observed 3.97% prevalence of elevated ALP levels and the verified diagnosis of PDB in 36.8% of these subjects, the estimated prevalence of PDB from this survey was 1.46%. When prevalence was adjusted to take into account that about 20% of patients with PDB have ALP levels within the normal range the overall prevalence of PDB was 1.75%. Moreover, prevalence rates were higher in males (1.88%) than in females (1.14%) and increased steadily from the 40-49 year age group through the 80-89 year age group. In addition, we also diagnosed PDB in 12 of the 944 subjects (1.3%) with normal ALP levels that accepted to be further

investigated. In those subjects the disease was diagnosed mostly in one bone with only 4 subjects having more than one localization. Prevalence estimates from the bone scan survey in 7906 scan performed in Siena from 2000-2004 were higher (2.4%). Considering these prevalence rates and according to census data (ISTAT, 2002, http://demo.istat.it/pop2002/index.html), the estimated number of PDB patients in Turin and in Siena was 4476 and 1334, respectively. Moreover, even though an extrapolation of these results to the entire Italian population may not be accurate, it can be estimated that there may be currently from 124,788 to 338,576 people (considering the radiographic and scintigraphic prevalence rates, respectively) potentially affected with PDB in Italy. These data confirm PDB to be the most common bone remodeling disorder in elderly people in Italy, excluding osteoporosis, with an estimated prevalence of at least 1%, comparable to that observed in United States and other European countries but lower than that described in high prevalence areas such as Britain, Australia, and New Zealand. Data from several countries support the view that there are important secular trends in the prevalence and severity of PDB. Recent epidemiological observations indicated a decrease in the prevalence of the disease that was particularly evident in those populations of British descent where the highest incidence rates of PDB had been described. A remarkable example is given by a 1999 radiological survey from Cooper et al that replicated a previous study performed in 1974 and showed about a 3-fold decrease in prevalence rates (from 5.0% to 2.0%) over a 20-years period in the same 10 British centres [10]. The decrease was much more marked in the high prevalence area of Lancashire than in towns from different regions. Similar results were reported in New Zealand [11-12]. Moreover, the annual incidence of new cases of PDB declined significantly in Britain and the United States over the past 20 years [13-14]. In Italy there are not similar studies to confirm this trend. However, the comparison of prevalence estimates from different Italian studies performed in different periods does not seem to indicate a clear reduction in

prevalence rates over the past 20-30 years. Together with a reduction in prevalence rates several studies also demonstrated a decrease in clinical severity of PDB, with less extensive skeletal involvement and a reduced incidence of serious complications such as osteosarcoma [11, 15, 16]. This secular trend toward milder disease seems to be maintained in recent years [17]. The analysis of the 147 Italian subjects from Siena did not evidence any significant correlation between birth year and extent of bone involvement or skeletal distribution of lesions [18]. Similarly, no differences in clinical severity were observed when PDB patients were divided into 2 groups according to the birth date cut-off of 1930, that represented the median value of this population. Thus, there was no apparent evidence toward a decrease of severe PDB in Italy over the last decades. However, skeletal extension and the mean number of affected sites were lower with respect to many other studies from different countries. This may reflect a reduced clinical severity of PDB in Italy. Interestingly, the comparison of clinical characteristics of PDB subjects from Siena described by Merlotti et al in 2002-2004 [18] with those reported by Stuart in PDB subjects from the same hospital in 1950-1956 [6] demonstrates a decrease in the number of affected skeletal sites and a reduction of neoplastic degeneration (Table 1).

	Siena 1950–1956 (Stuart[6])	Siena 2002–2004 (Merlotti et al.[10])
Monostotic	35 (33%)	62 (42%)
Polyostotic	71 (67%)	85 (58%)
Three or more affected sites	49 (69%)	42 (49%)
Four or more affected sites	32 (45%)	34 (28%)
Osteosarcoma	4 (3.7%)	0
Giant cell tumor	1 (0.9%)	0
Fractures	18/94 (19.1%)	20 (13.6%)

$p < 0.01$ χ^2 test or Fisher exact test (if $n < 5$).

Table1. Comparison of disease characteristics of patients with Paget's disease in Siena in the 1950-1956 and 2000-2004 studies (adapted with permission from Gennari L et al J Bone Miner Res 20:1845-1850, 2005).

Possibly, the decrease in clinical severity of PDB might be occurred earlier in Italy than in other countries and does not seem to be maintained through recent years. The geographical distribution of PDB has been shown to differ significantly not only between different countries but also within the same country. Areas of remarkable high prevalence of disease have been described in Lancashire (northwest of England), in northeastern United States, and in some delineated territories of central and western Spain [19-22]. Apart the twofold higher prevalence of disease observed in Milan than Palermo in the 1982 European study from Detheridge et al. [8], mild differences were shown in the more recent radiological survey between the towns of Siena and Turin [9]. In addition, two different studies suggested a remarkably localized area of high prevalence of PDB in the Campania region, especially within the surroundings of Naples, Salerno, Avellino and Caserta. In the first study performed in Siena, the analysis of demographic and immigration history (familial provenience, and actual or childhood place of residence) of 147 consecutive PDB patients as compared to age and sex-matched controls, indicated that about 14% of PDB subjects vs. 2% of controls reported actual or previous residency in rural districts of Campania [18]. Moreover, when the analysis was restricted to subjects living in the administrative district of Siena an increased prevalence of PDB was observed within the Chianti area, while a reduced prevalence was observed in the urban area [18]. A similar preliminary analysis of 75 PDB patients in Turin confirmed an increased prevalence of cases with actual or previous residence in Campania with respect to age-matched controls [23]. The reasons for these geographic variations in prevalence rates of PDB are unknown and may be due to either genetic or environmental factors. It was speculated that arsenic pesticide in imported cotton might have been a possible environmental factor underlying the increased prevalence of the disease in Lancashire cotton mill towns [24]. On the other hand, the high prevalence areas located in central and western Spain mainly represent

rural regions that remained fairly isolated until recent times [20, 21, 25, 26]. Such populations could be strongly influenced by their genetic background and their habits of meat consumption without sanitary control [25, 26]. Similarly, the area of Chianti near Siena, and the districts of Caserta, Salerno, and Avellino in Campania, mainly include rural and fairly isolated places where the possibility of animal contacts could be higher than in other parts of Italy. Consumption of unpasteurized milk is also particularly frequent in these areas. Future population-based studies will be needed to confirm these associations and to explore the possible environmental and/or genetic determinants. Indeed, the hypothesis of a high prevalence of PDB in the Italian region of Campania was also supported by studies from other countries that described this region as a parental place of origin of their PDB cases [27-29]. Importantly, a recent study indicated significant differences in clinical severity of PDB patients from the high prevalence area of Campania with respect to PDB cases from other part of Italy [23]. PDB patients from Campania had an earlier onset of disease, an increased skeletal extension, and a higher tendency toward neoplastic degeneration than patients from Turin and Siena. Together with a case of osteosarcoma, five cases of giant cell tumour were observed in the 125 PDB patients living in Campania, consistent with a prevalence of 6/125 (4.8%). The latter association represents a particular and quite original clinical feature. In fact, the association between PDB and giant cell tumour is rare and less than 60 cases have been described worldwide since the first report [30-32]. This complication usually occurs in patients with severe polyostotic disease and may be multifocal, with up to 27 lesions described in a single patient. Remarkably, more than 50% of described PDB cases with giant cell tumour have been observed in subjects originary or descending from the region of Campania, and particularly from the town of Avellino [28, 33, 34]. Familial clustering has been recently described in these subjects [34].

1.2 ETIOPATHOGENESIS

The cause of PDB remains in large part unknown. Research findings suggest that the disorder may be caused by a slow-acting viral infection of bone, a condition which is present for many years before symptoms appear. There are also data supporting a hereditary hypothesis, since the disease may appear in more than one member of a family, and mutations in different genes have been recently associated to classical PDB or PDB-related disorders. Current evidence suggests that both environmental and genetic factors are involved in classical PDB.

a) Genetics factors

Since 1889, two years after the description of the first case of PDB [1], Sir James Paget wrote, "I have tried in vain to trace any hereditary tendency to the disease. I have not found it twice in the same family." In the beginning of this century, however, the first families with more than one pagetic patient were reported and since then several familial cases have been described [35-37]. The possibility that heredity might play an important role in the pathogenesis of PDB was first raised in the 1949 [38]. In support of a genetic disease etiology is the ethnic difference in prevalence of the disease, which persists after migration to other countries [39]. Moreover, several observations from the literature clearly demonstrated that PDB has an important genetic component, with familial PDB accounting from 10-40% of cases in different studies. Familial PDB in Italy has been described since many years [6, 40, 41]. However the difficulty in ascertaining the familial distribution of the disease (due to the late onset of the symptoms and to the high frequency of asymptomatic bony involvement) makes it impossible to obtain a clear estimate of familial and sporadic cases. A recent analysis of 147 consecutive Italian PDB patients from Siena, showed clinically confirmed familial aggregation in 15% of cases [18]. Pedigree analysis indicated an autosomal dominant pattern of inheritance with variable penetrance. No significant differences between familial and sporadic cases were observed concerning age of diagnosis, disease extension

and male to female ratio even if the disease appeared to be diagnosed earlier in

familial than sporadic cases, may be due to a better prevention. Similar estimates

were reported in a survey of 125 pagetic subjects from Campania, showing family

history of PDB in 23 (18.4%) of patients [42]. In this case, however, an increased

clinical severity and a preferential involvement of the skull and the spine was

observed in familial with respect to sporadic PDB. Taken all together, these results

are in keeping with previous studies from different populations of British descent,

where the proportion of familial cases ranged from 12% to 20% [27, 43-45].

Differences between these studies may be due to the lack of detailed clinical

evaluation of the families of affected patients that may reveal more persons with

asymptomatic PDB. Indeed, in a study from Spain, Morales Piga et al, after

detailed clinical analysis of 35 PDB patients and their 128 first-degree relatives,

demonstrated familial PDB in 40% of cases [46]. A similar preliminary observation

in PDB patients from Turin demonstrated familial aggregation of disease in 26% of

cases [23]. Moreover, several studies suggested that PDB is a genetically

heterogeneous disorder with at least 7 genetic loci initially reported to be

associated to a higher risk to develop the disease. In 2002, two positional cloning

studies identified mutations in the *SQSTM1* gene as the cause of late-onset 5q35-

linked PDB in sporadic and familial cases of British and French-Canadian descent

[47, 48]. This gene consists of 2870 nucleotides grouped into eight exons (Figure

6) spanning a 17-kilobase genomic segment [47]. A single P392L mutation at

nucleotide position 1215 in exon 8 was first identified in 16% and 46% of sporadic

and familial PDB subjects of French-Canadian origin, respectively. The same

mutation was also reported by Hocking et al. [48] together with two different

mutations of *SQSTM1* in 18 PDB families predominantly of British descendent

(P392L in 19.1%, E396X in 5.8%, and a splice donor site mutation in intron 7 in 1

family). Moreover, the P392L mutation was reported in 8.9% of the sporadic PDB

cases of British descendent. Following these first reports, *SQSTM1* mutations have

now been identified as an important cause of PDB by a wide variety of investigators in several populations [49-55]. To date, at least 14 different PDB-associated mutations have been identified in this gene, and all affect the ubiquitin-associated (UBA) domain of the protein which is involved in noncovalent ubiquitin binding (Figure 6 and Table 2). Overall, these mutations have been described in up to 50% of familial and 20% of sporadic cases of PDB [56]. The most common disease-causing mutation reported in *SQSTM1* is the proline-leucine amino acid change at nucleotide1215, codon 392 (*P392L*). Recent studies have suggested that this mutation is carried on a common haplotype background in the vast majority of PDB patients from British descent (United Kingdom, Australia, and New Zealand) [57]. This indicates that P392L is a founder mutation that might explain the high incidence of PDB in British migrants to the southern hemisphere. The *SQSTM1* gene encodes for the sequestosome 1 protein, also known as p62. The name sequestosome 1 derives from the ability of the protein to form cellular aggregates know as ''sequestosomes,'' which may be sites of intracellular protein degradation. The p62 protein is highly conserved through evolution and is composed by 440 amino acids that form the following conserved domains [58, 59].The molecular mechanism by which *SQSTM1* mutations enhance osteoclast activity and cause PDB are not well understood. Functional studies using protein binding assays show that all of the PDB mutations in *SQSTM1* gene manifest as loss or alteration of ubiquitin binding *in vitro* [60, 61], indicating that the disease mechanism is likely to involve the inability of mutant p62 to establish regulated protein-protein interactions with an ubiquitinated osteoclast protein(s). This may lead to over-stimulation of the NF-κB pathway. Accordingly, osteoclasts derived from monocytes from *SQSTM1* mutation carrying patients (K378X, truncating) showed increased bone resorption *in vitro* when compared with those derived from control monocytes [55], consistent with the activation of NF-κB-dependent responses. The *SQSTM1* mutations that insert a stop codon (such as A390X, L394X, and E396X)

lead to a truncated protein that lacks all or part of the domain binding multi-ubiquitinated chains [62] and have been associated with the most aggressive cases of classical PDB in some studies [54, 62]. Missense mutations (i.e. P392L, P387L, G411S and M404V) lead to production of the complete native protein. It has been demonstrated that the P392L and G411S mutations modify the secondary structure of the UBA domain without affecting the function of the binding domain for multi-ubiquitinated chains. Selective loss of binding to a specific ubiquitinated substrate, rather than overall loss of ubiquitin binding, may explain the pathogenic effect of these mutations [54]. For the other missense mutations, the amino-acid substitutions may decrease or abolish the ability of the UBA-domain to bind ubiquitinated chains [59]. These mutations may also alter the half-life of the p62 protein or interfere with protein–protein interactions. Moreover, other genetic studies revealed a mutation in the *TNFRSF11A* gene (encoding RANK) in an Asian family with early-onset PDB [63, 64]. In a preliminary analysis in Italian PDB patients, no mutations in the *TNFRSF11A* gene were reported [65], consistent with other studies in Caucasian populations [66, 67]. In contrast, two different studies recently identified 3 different mutations of *SQSTM1* gene in Italian PDB patients. In a first study the P392L mutation was described in 10% sporadic and 30% familial PDB cases [68]. In another similar study in sporadic PDB patients [50], the overall prevalence of *SQSTM1* mutations was lower (4.8%) and 3 different mutations were described (P392L, M404V, and G425R). A more detailed investigation of pedigree of the patient with the M404V mutation provided evidence for a familial form of PDB and the extension of genetic analysis confirmed the mutation in 4 affected subjects and 6 unaffected family members [69]. A different study identified the P392L mutation of the *SQSTM1* gene in 2 polyostotic members of a PDB Italian family [70].

b)Environmental factors

Although the *SQSTM1* mutations have been associated to PDB, they are not sufficient to cause the disease in all subjects and probably other factors are involved in the etiopathogenesis of the disorder such as environmental and infective agents, facilitating the expression of the disease in genetically susceptible subjects. In fact, some patients with the *SQSTM1* mutation do not develop PDB, even at older ages, [71] and the P932L *SQSTM1* mutation, present in 5–10% of PDB subjects, is not sufficient to induce a pagetic phenotype in osteoclasts [72]. This may suggest the involvement of a protective mechanism for certain individuals or the requirement of a "trigger factor" for the disease. Both morphologic and immunocytologic studies also showed the presence of paramyxovirus material in pagetic osteoclasts, suggesting that a latent viral infection may be involved in the etiopathogenesis of the disease [73-75]. In vitro study evidenced that canine distemper virus can infect human osteoclast precursors and create dose dependently increases in osteoclast number and size [76]. Similarly measles virus nucleocapsid protein transfection in human osteoclast precursors induced osteoclast differentiation and activation as well as other typical features of pagetic osteoclast cells [77]. In particular, osteoclast precursors of transfected animals are not hyper-responsive to RANKL as pagetic osteoclasts. Conversely, some other workers have failed to detect paramyxovirus in pagetic bone using in situ hybridization– reverse transcription–polymerase chain reaction [78, 79]. Conceivably, viral proteins may have to interact with mutated forms of SQSTM1/p62 to induce the expression of the full pagetic phenotype. Importantly, the remarkable geographical distribution of PDB and the referred association with animal-related factors in some studies (i.e., maintaining pets or ingestion of contaminated bovine meat) points also to a consistent influence of the environment. Interestingly, the analysis of PDB patients from the Italian Registry evidenced a significant association between PDB and contacts with animals in rural districts

(OR=2.22; p=0.001; Chi-squared test) [18]. In particular, previous or current

contact with animals such as pigs, rabbits, and cattle appeared to represent a

predisposing factor for the disease. In contrast no significant association with dog

or cat ownership was observed, even though when analyses were limited to

subjects living in rural areas an increased prevalence of the disease was also

present in subjects who had contacts with cats and dogs (67% vs. 44%, PDB vs.

controls, respectively). These associations are partly different from those

previously reported in PDB populations from other countries, where an increased

risk due to dog or cat ownership was clearly indicated [25, 80, 81]. Conversely, the

described association with exposure to cattle was also observed in PDB cases from

Spain [25]. All these findings are in keeping with an important role of the

environment in the pathogenesis of PDB, maybe facilitating the expression of the

disease in genetically susceptible subjects. Thus, different infective agents may be

involved in the pathogenesis of PDB, probably canine distemper virus and measles

virus [5, 82]. However, newly recognized paramyxoviruses have been associated

with disease status in several animal species, including horses and swine, and

limited information is actually available on the public health risk of many of these

infective agents [83, 84]. Importantly, while cats and dogs are commonly

vaccinated against most of the common paramyxovirus infections such as

distemper virus [85], swine and cattle are often unvaccinated in rural areas from

Italy and might represent a vehicle for virus transmission to humans. Probably a

latent viral infection may be involved in the aetiology of PDB and that different

viral agents, infecting distinct animal species in different geographical areas or

similar viral agents infecting different hosts could account for the observed

discrepancies. Importantly, both a current and a previous contact with the host

appeared equally effective in conferring susceptibility to the disease.

1.3 CLINICAL CHARACTERISTICS

Paget's disease of bone may be *monostotic*, affecting only a single bone or a proportion of a bone, or may be *polyostotic*, involving two or more bones. Sites of disease are often asymmetric. In most instances, sites affected with Paget's disease at the time of diagnosis are the only ones that will show pagetic change over time. Although progression of disease within a given bone may occur, the sudden appearance of new sites of involvement some years after the initial diagnosis is uncommon. Generally the evolution of the disease follows three major phases. In the early phase, termed "*osteolytic phase*" bone resorption predominates and there is a concomitant increased vascularity of involved bones. In this phase body calcium balance may be negative and the typical radiological picture is represented by an *"advancing lytic wedge"* or "*blade of grass*" lesion in a long bone (i.e. femur or tibia) or by *"osteoporosis circumscripta"*, as seen in the skull. Commonly the excessive resorption of pagetic bone is followed closely by formation of new bone. During this second phase of the disease the new bone that is made is structurally abnormal, presumably because of the accelerated nature of the remodeling process. Newly deposed collagen fibers are laid down in a disorganized rather than a linear fashion, creating the so called "*woven bone*" (Fig. 2). Such a woven-pattern is not specific for PDB but it just reflects a high rate of bone turnover. With the time, the hypercellularity at the affected bone may diminish leading to development of a sclerotic, less vascular pagetic mosaic without evidence of active bone turnover.
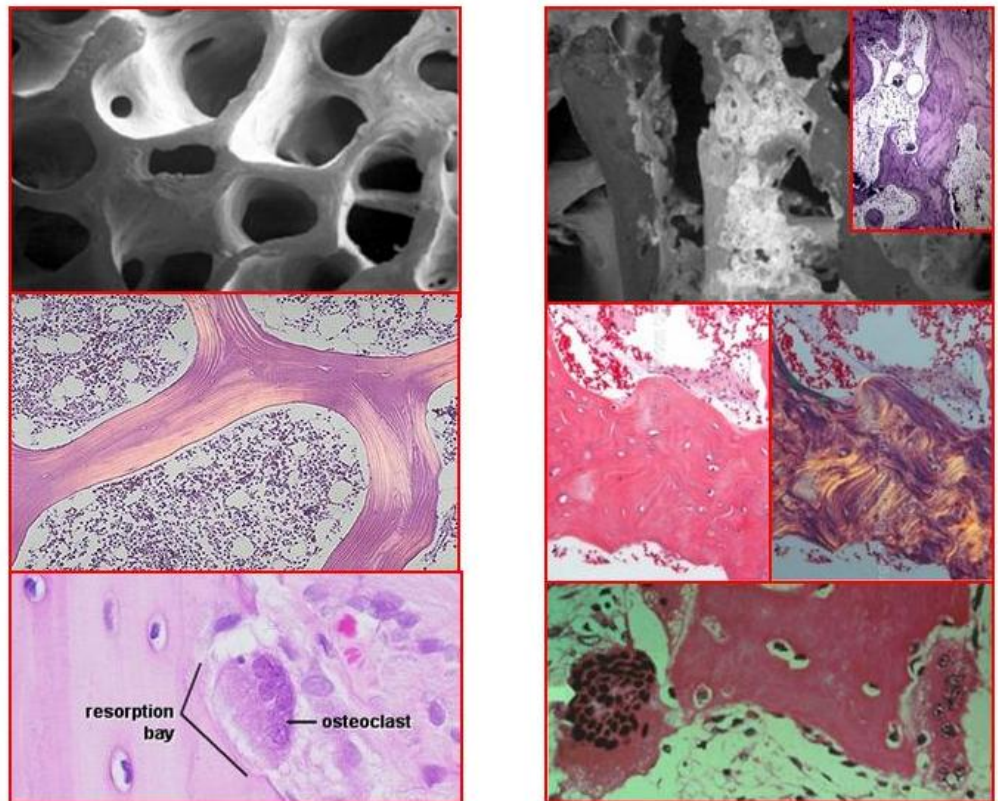
Fig.2. Microscopic structural representation of normal bone (left) and pagetic bone (right) with typical "woven bone" (up) and multinucleated osteoclasts (down)

This is the so-called "sclerotic" or "burned-out" phase of PDB. Typically all these three phases of the disease can be seen at the same time at different sites in a single pagetic patient. Many patients who have PDB do not know they have it, since the disease may be so mild that is not detected. Sometimes, the patient's doctor is alerted to the possibility of PDB when physical deformities appears (i.e. enlargement of the skull or bowing of the tibia) or when a blood test reveals an elevated level of alkaline phosphatase In other cases, the diagnosis is made only after complications have developed. Possible complications of the disease include bone deformities, nerve compression syndromes, bowing limbs, fractures, hearing loss, secondary osteoarthritis, cardiovascular manifestations (i.e., high output failure, valvular and blood vessel calcifications), and in rare occasions, osteosarcoma. Pain, and namely localized bone pain, is the most common symptom that brings a patient with PDB to a physician. The first phase of PDB involves

thinning of the bone, which is being aggressively resorbed away; this is called *"lytic disease"*. This process can cause small breaks (microfractures) in the bone that are painful, especially when they involve weight-bearing bone. Alternatively, another source of pain may be from irritation of nerves covering affected bones. Osteoarthritis is common among patient with PDB and can be quite painful [13, 18]. Pathologic fractures may occur at any stage even though are more common in the lytic phase of the disease. They particularly involve long bones with active area of advancing lytic disease (i.e. the femoral shaft or the subtrocanteric area) and may occur spontaneously or follow slight trauma. One of the most serious complications of PDB is neoplastic degeneration of pagetic bone with an increased incidence of sarcomas, especially in polyostotic cases of the disease. The majority of these tumors are classified as osteosarcomas, although fibrosarcomas and condrosarcomas may be also seen. Approximately 1% of pagetic patients develop osteosarcoma, an increase in the risk that is several thousand-fold higher than in the general population. It has been estimated that 20% of the patients with osteosarcoma over the age of 60 have PDB as a predisposing condition [86]. This significantly contributes to the mortality and morbility of PDB patients. The sarcomas most frequently arise in the femur, tibia, humerus, skull, mandibula, and pelvis while rarely occur in vertebrae. Typically pagetic osteosarcoma is osteolytic in contrast to the sclerotic appearance of radiation-induced osteosarcomas. Death from massive local extension or from pulmonary metastases occurs in the majority of cases in 1 to 3 years. Benign giant-cell tumor also may occur in pagetic bone [28]. Radiographic evaluation of lesion as well as bone biopsy may be useful in the diagnosis. For the biochemical point of view, PDB is characteristically associated with an increase in bone turnover but normal concentrations of serum calcium, phosphate, parathyroid hormone, and vitamin D metabolites. Over the past years various markers of bone turnover have been indicated for the diagnosis of PDB. Among those, bone specific alkaline phosphatase seems to have the best diagnostic

accuracy as a measure of increased bone turnover of pagetic bone. Considering its simplicity and low cost, total serum alkaline phosphatase concentration is still a valid alternative. Other markers of bone formation, such as the aminoterminal (PINP) and carboxyterminal (PICP) extension peptides of type I collagen that are released into circulation during the conversion of type I procollagen into collagen have proved to be differently sensitive in PDB. Among the various biochemical markers of bone resorption, the most sensitive ones in PDB are collagen type I related peptides [87, 88]. In extensive and active disease most markers of bone turnover will be abnormal and the choice of resorption marker can then be based on cost and availability. In addition to their use in diagnosis, all these measurements are important tools for monitoring a patient's response to treatment for PDB. PDB is diagnosed primarily by radiological examination [89]. Radiographs of painful or deformed bones usually show the characteristic mixed appearance of areas of lysis due to increased osteoclastic resorption with sclerosis from excessive osteoblastic bone formation. A characteristic appearance that distinguishes PDB from other conditions is the increased diameter of affected bones, particularly those of the spine (Fig. 3) or the shafts of long bones.
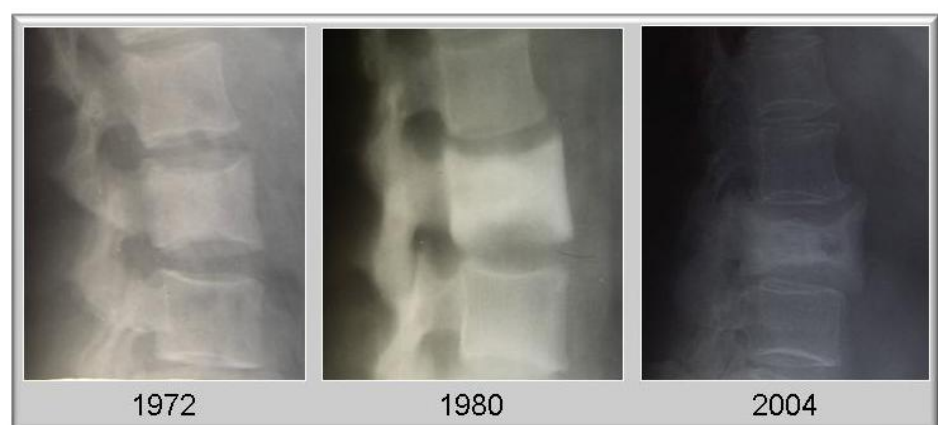
Fig.3. Radiological characteristic of a dorsal untreated pagetic vertebra with increased diameter and density (centre) and the occurrence of fracture (right) along time.

Scintigraphy is a sensitive but non-specific method of detecting areas of skeletal abnormality and is the best way for assessing the skeletal distribution of PDB (Fig. 4). Although some sites may be asymptomatic, it is important that they are

identified because they may be susceptible to complications, such as fracture. Newer imaging modalities such as CT and MRI have improved the ability to evaluate neurologic symptoms in the context of PDB. They can also result useful to establish the extent and the character of the neoplastic degeneration of pagetic tissue.
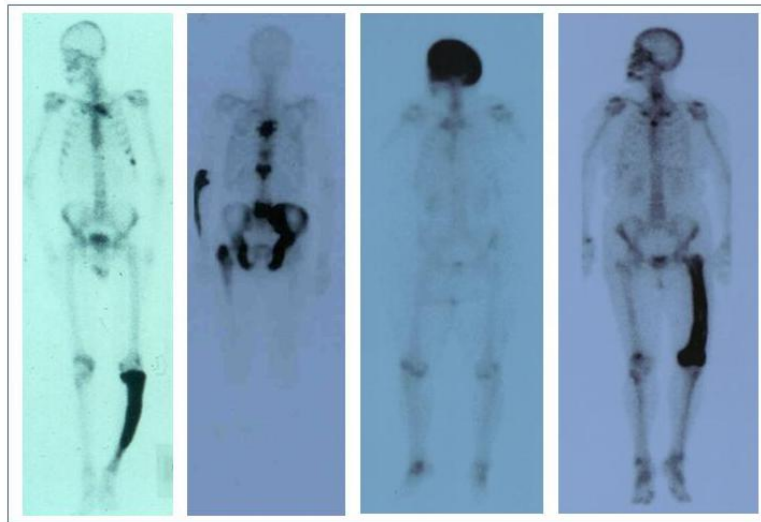
Fig.4. Bone scan characteristics of monostotic and polyostotic PDB.

1.4 THERAPY

The primary goal of PDB treatment is to restore normal bone turnover in order to relieve symptoms such as bone pain and prevent complications that result from the abnormal resorption and overgrowth of pagetic bone. Treatment can be also indicated for PDB patients with orthopaedic complications, undergoing elective surgery at affected bone sites. In this case the normalization of bone turnover is able to reduce blood flow in pagetic bone and thus decrease blood loss during surgery. In elderly polyostotic patients with advanced disease, treatment is also indicated for the management of immobilization hypercalcemia. However, almost any patient (particularly those with the involvement of the skull, weight bearing bones, and bones adjacent to major joints) may benefit from antiresorptive treatment, even if there are no symptoms, because of the potential to reduce disease progression, bone deformity and related complications. Indeed, even though it has not been proven conclusively that restoring normal bone turnover effectively

reduces the risk of later complications, in untreated PDB the progression of disease

usually occurs with extension of osteolitytic changes and bone deformity [90].

Conversely, suppression of bone turnover with antiresorptive therapy is associated

with normal lamellar patterns of new bone deposition as seen on bone biopsy

specimens [91], and there are isolated case reports showing improvement of

deformity or hearing loss after treatment, immobilization (leading to

hypercalcemia) [92, 93]. Since in PDB the increased activity of osteoclasts leading

to increased bone resorption remains coupled to a parallel increase in osteoblast

activity and bone formation, it is sufficient to treat the osteoclast to restore bone-

remodelling rates towards normal. Currently, all agents used to treat PDB are

antiresorptive in nature, and include calcitonin and bisphosphonates (Table 2).

| BP	Dose	ALP Normalization
Etidronate	400mg die for 6 months	20%
Tiludronate	400mg die for 3 months	35%
Clodronate	1600mg die for 6 months	>70%
Alendronate	40mg die for 6 months	60-80%
Risedronate	30mg die for 2 months	>70%
Pamidronate	Os (600mg) or iv. at different doses	40-90%
Neridronate	200mg i.v. in 2 days or 25mg i.m/week for 2 months	>60%
Olpandronate	40mg i.v. in 5-10 days or 200 mg/die per os for 15 days	80-90%
Ibandronate	2mg i.v. single infusion	>50%
Zoledronate	5mg i.v. single infusion	>90%

Table 2. Available Bisphosphonate treatment for PDB. In blue are indicated compounds actually available in Italy.

Moreover, since new bone formation usually occurs during treatment in order to

repair pagetic bone, and since hypocalcemia and hyperparathyroidism are common

after the suppression of bone turnover, daily supplements of calcium and vitamin D

should be also recommended to PDB patients in addition to antiresorptive therapy.

Bisphosphonates are the treatment of choice of PDB, as well as of many other

conditions characterized by increased bone resorption such as osteoporosis and

bone metastases. Numerous studies have shown the efficacy of bisphosphonates in the management of patients with PDB. These treatments are associated with a reduction in plasma alkaline phosphatase (ALP) activity and an improvement in radiological and scintigraphic appearance [94]. In addition to improvement in bone turnover, bisphosphonate therapy has also been associated with a reduction in bone pain and bone deformity, whereas the effects of treatment on the development or progression of other complications are poorly understood. Recently, the availability of newer, more potent nitrogen containing bisphosphonates has improved treatment outcomes. These compounds have a greater binding affinity to hydroxyapatite and increased potency in terms of inhibition of bone resorption[95, 96]. Their ability to achieve optimal control of bone turnover at lower doses than the previous compounds has opened the way to intravenous administration regimens. Moreover their greater binding affinity offers the potential for sustained remission. However, there have been few head to head randomized trials comparing these intravenous bisphosphonate regimens, and it is not shown if these drugs differ in therapeutic efficacy. During the last decades, the development of potent amino-bisphosphonates such as alendronate and risedronate has led to substantial improvement in clinical management of patients with PDB [94, 97-101]. However, these drugs require daily oral dosing for up to 6 mo, with patients required to fast before and after treatment because of the very low bioavailability of these compounds and to remain upright for at least 30 min after dosing to reduce the risk of upper gastrointestinal complications. All these aspects significantly affect compliance of PDB patients. Similarly, available intravenous regimens, such as pamidronate, can also be inconvenient for patients because they are usually given as a series of slow intravenous infusions each lasting a few hours, thus requiring multiple visits and treatment courses. Moreover, pamidronate resistance has been described in a substantial proportion of patients [102-105]. The recent development of more potent intravenous aminobisphosphonates might address these problems,

allowing a more effective and convenient management of PDB. In a recent randomized trial, a single infusion of zoledronic acid (5 mg) showed a greater and long-lasting effect than oral risedronate [106, 107]. In short-term studies, intravenous neridronate was well tolerated and effective in decreasing bone turnover markers in a dose-related manner (from 25 to 200 mg) in patients with active PDB [108-110].

We performed a 15 months randomized study specifically to compare different intravenous bisphosphonate regimens in 90 subjects with active PDB [111]. At baseline, patients were randomly assigned to receive pamidronate (30 mg, i.v., for 2 consecutive days every 3 months) or zoledronate (4 mg, i.v.). After 6 months, non-responders patients to pamidronate were crossed over to zoledronate (4 mg, i.v.) or neridronate (100 mg, i.v., for 2 consecutive days). Among non-responders patients to pamidronate, a single treatment course with either neridronate or zoledronate led to the achievement of therapeutic response in more than 90% of subjects (Fig. **5**). Normalization of alkaline phosphatase levels was observed after 6 months in 80% and 83% of patients treated with neridronate or zoledronate, respectively and was maintained in most patients at 9 months. A slightly increased efficacy on the reduction of bone pain was described with both zoledronate and neridronate over pamidronate [111].
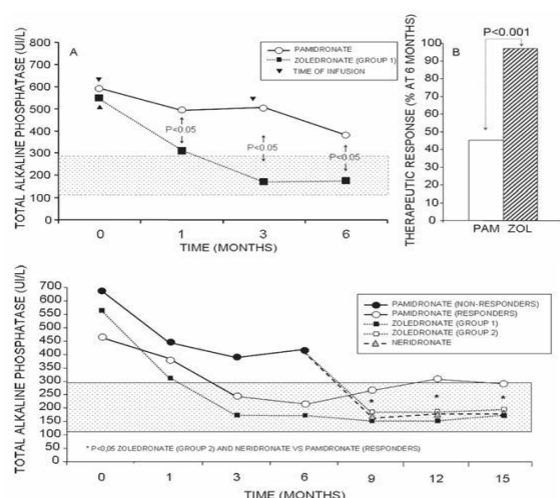
Fig. 5. Total ALP levels and therapeutic response to different intravenous bisphosphonates treatments in PDB (from Merlotti D et al. J Bone Miner Res 2007;22:1510-17)

All nitrogen-containing bisphosphonates administered intravenously can induce an acute phase reaction with fever, musculoskeletal pain and other flu-like symptoms. These effects are transient and occur predominantly on first exposure to the drug in most patients who has not previously been exposed to a nitrogen-containing bisphosphonate. In fact, previous treatment with a bisphosphonate appears to provide some protection from acute phase reactions with zoledronic acid or other aminobisphosphonates [112]. Some reports have documented hypocalcemia occurring in patients treated with intravenous amino-bisphosphonates. This complication is generally asymptomatic and mostly occurs if patients do not take calcium and vitamin D supplements. Osteonecrosis of the jaw (ONJ) has been identified as a potential complication, particularly with long-term, high dose intravenous bisphosphonate therapy in malignant diseases [113]. However this complication seems extremely rare in patients with PDB treated with a bisphosphonate (with less than 10 cases reported to date). Moreover, the efficacy and safety demonstrated in the recent trials with neridronate and zoledronate in PDB constitutes a real progress and a cost-effective approach. Their rapid suppression of bone turnover, ease of administration, long-term effects on disease remission, as well as their good tolerance currently support the use of these aminobisphosphonates as a first-line therapeutic option in patients suffering from PDB, and particularly in those with severe polyostotic disease.

2. AIMS OF THE STUDY

Based on our previous epidemiological and clinical observations [9, 18, 23, 65, 68, 111], and the recruitment of a large cohort of familial and sporadic PDB subjects from the Italian Registry of PDB patients, the aims of our research have been the following:

1) to perform a genetic screening of *SQSTM1* mutations in Italian PDB patients and to explore possible interactions with environmental factors;

2) to identify new susceptibility genesi and to explore their potential interaction with *SQSTM1* on PDB phenotype;

3) to identify a new susceptibility gene causing giant cell tumour in PDB patients;

4) to investigate the long term efficacy of bisphosphonate treatment in PDB and the possible pharmacogenetic implications.

3. MATERIALS AND METHODS

The Italian cohort of PDB patients of the Italian Registry analyzed in our research projects consisted of 654 unrelated and consecutive PDB patients from different Italian regions recruited at the Bone Disease Units of Turin, Siena, and Naples. These are the three main national centres for the diagnosis and treatment of PDB, located, respectively, in northern, central, and southern Italy (www.pagetitalia.com). All patients were born in Italy, and all but three patients were of Italian origin (as assessed from parental history). Diagnosis of PDB was based on biochemical evaluation, bone scintigraphy, and subsequent X-ray examination of areas of increased isotope uptake. For all subjects, a detailed medical history was obtained, including family history, place of birth, place of residence during childhood, occupation, age at diagnosis, skeletal extent, complications, age at onset of PDB symptoms, dietary habits, and animal contacts. When available, clinical data were collected to evaluate the presence of PDB complications. In particular, the presence of neoplastic degeneration, cranial nerve disorders, hearing loss, hip or knee replacements, osteoarthritis, fractures, back pain, hypertensive disease, hyperparathyroidism, renal stones, or heart failure was recorded. The study was approved by local ethical committees, and all subjects had given informed consent to being included. All data were collected through common questionnaires shared by all participating centres.

Detailed description of methods and techniques concerning the clinical, genetic and statistical analyses for each study are indicated in each specific publication attached in the results section .

4 RESULTS

STUDY AIM #1

Genetic screening of *SQSTM1* mutations in Italian PDB patients and possible interactions with environmental factors

Eleven different mutations (Y383X, P387L, P392L, E396X, M401V, M404V, G411S,D423X, G425E, G425R, and A427D) were observed in 34 of 92 (37%) and 43 of 441 (10%) of familial and sporadic PDB patients, respectively.

Among the population of PDB patients, we identified a subset of patients who developed giant cells lesions at one or more pagetic sites. All patients with giant cell tumor were negative for *SQSTM1* mutations. Evidence of possible gene-environment interaction was also observed. In fact, patients reporting animal contacts showed an increased number of affected sites (2.54±2.0 versus 2.19±1.9, p<.05) over patients without animal contacts. This difference also was evidenced in the subgroup of patients with *SQSTM1* mutations (3.84±2.5 versus 2.76±2.2, p<.05). Overall, these data suggest that animal-related factors may be important in the etiology of PDB and may interact with *SQSTM1* mutations in influencing disease severity (publication A).

Moreover, we performed a small epidemiologic and *SQSTM1* genetic screening in a different population of cases from a rural area of Calabria: In that area, the crude radiographic prevalence of pelvic PDB was 0.74% (8/1068; male:female 5:3, mean age 71.6±13.1 yr) leading to an estimated overall prevalence of PDB between 0.82% and 1.21%. PDB patients from Calabria showed clinical characteristics similar to those reported in patients from Campania. The disease was also frequently complicated by osteoarthritis and the right side of the body was more affected than the left. The *SQSTM1* gene analysis revealed the presence of a novel missense mutation (M401V) in exon 8 in one subject with a familial and aggressive form of PDB (publication B).

Publication A

JBMR

SQSTM1 Gene Analysis and Gene-Environment Interaction in Paget's Disease of Bone

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ABSTRACT

Even though *SQSTM1* gene mutations have been identified in a consistent number of patients, the etiology of Paget's disease of bone (PDB) remains in part unknown. In this study we analyzed *SQSTM1* mutations in 533 of 608 consecutive PDB patients from several regions, including the high-prevalence area of Campania (also characterized by increased severity of PDB, higher number of familial cases, and peculiar phenotypic characteristics as giant cell tumor). Eleven different mutations (Y383X, P387L, P392L, E396X, M401V, M404V, G411S, D423X, G425E, G425R, and A427D) were observed in 34 of 92 (37%) and 43 of 441 (10%) of familial and sporadic PDB patients, respectively. All five patients with giant cell tumor complicating familial PDB were negative for *SQSTM1* mutations. An increased heterogeneity and a different distribution of mutations were observed in southern Italy (showing 9 of the 11 mutations) than in central and northern Italy. Genotype-phenotype analysis showed only a modest reduction in age at diagnosis in patients with truncating versus missense mutations, whereas the number of affected skeletal sites did not differ significantly. Patients from Campania had the highest prevalence of animal contacts (i.e., working or living on a farm or pet ownership) without any difference between patients with or without mutation. However, when familial cases from Campania were considered, animal contacts were observed in 90% of families without mutations. Interestingly, a progressive age-related decrease in the prevalence of animal contacts, as well as a parallel increase in the prevalence of *SQSTM1* mutations, was observed in most regions except in the subgroup of patients from Campania. Moreover, patients reporting animal contacts showed an increased number of affected sites (2.54 ± 2.0 versus 2.19 ± 1.9, $p < .05$) over patients without animal contacts. This difference also was evidenced in the subgroup of patients with SQSTM1 mutations (3.84 ± 2.5 versus 2.76 ± 2.2, $p < .05$). Overall, these data suggest that animal-related factors may be important in the etiology of PDB and may interact with SQSTM1 mutations in influencing disease severity. © 2010 American Society for Bone and Mineral Research.

KEY WORDS: SQSTM1; PAGET'S DISEASE OF BONE; ENVIRONMENT; GENETICS; GIANT CELL TUMOR

Introduction

Paget's disease of bone (PDB; OMIM 167250, 602080) is a chronic disease that typically results in enlarged and deformed bones in one or more regions of the skeleton.[1,2] Excessive bone breakdown and formation disrupt normal bone architecture and strength. As a result, bone pain, arthritis, noticeable deformities, and fractures can occur.

The etiology of PDB has remained largely unknown for several decades. Both morphologic and immunocytologic studies demonstrated the presence of paramyxovirus material in pagetic osteoclasts, suggesting that a latent viral infection may be involved in the pathogenesis of this disorder.[2,3] However, PDB also has a clear hereditary component. Familial clustering has been recognized to occur in PDB in 10% to 40% of cases, and epidemiologic studies have indicated that the relative risk of PDB

in first-degree relatives of patients is about 7 to 10 times greater than in the general population.[4–6] Genome-wide scan in families with PDB identified at least 7 potential susceptibility loci for the disease, even though some of these gene assignments turned out to be false-positives.[7] In 2002, Laurin and colleagues identified a recurrent C→T transition at position +1215 leading to a proline-to-leucine substitution at codon 392 (P392L) on the SQSTM1 gene (within the 5q35 PDB3 locus) as a cause of PDB in about 50% and 20% of familial and sporadic French-Canadian patients, respectively.[8] This gene encodes the p62/sequestosome 1 protein, which acts as a scaffold protein in the NFκB pathway as well as an intermediate protein in the proteosomal degradation of polyubiquitinated proteins. The same P392L mutation was identified subsequently in familial and sporadic PDB subjects from different countries.[9–19] Currently, at least 20 further mutations in the SQSTM1 gene have been identified, all of which are clustered within or near the ubiquitin-associated (UBA) domain of the protein and lead to increased NFκB signaling and enhanced bone resorption. In some but not all patient samples, truncating mutations (where all or part of the UBA domain is deleted) were associated with a more severe phenotype than missense mutations.[11,16,17,19–21] Despite the fact that SQSTM1 mutations have been associated with a consistent number of familial PDB cases, incomplete penetrance has been described, and the prevalence of these mutations is low in sporadic PDB.[20–22] Moreover, even in PDB families with SQSTM1 mutations, some affected relatives without the mutation were described, suggesting that additional factors (either genetic or exogenous) may be associated with disease expression.[19] This is in keeping with results from experimental animal models of PDB.[2,23,24]

Marked geographic differences in the distribution of PDB also have been described, with a higher prevalence of the disease in populations of British descent.[25] Moreover, increased-prevalence areas have been described in different countries. We recently characterized an area of increased prevalence of PDB in the region of Campania, in southern Italy. Patients from this region also showed increased severity of disease often associated with peculiar phenotypic characteristics (ie, giant cell tumor) and an increased number of familial cases.[26–28] In this study we compared the clinical characteristics and prevalence and type of SQSTM1 mutations in a large sample of unrelated PDB patients from several Italian regions, including patients from the high-prevalence area of Campania. This sample also included three families with PDB associated with giant cell tumor. The large number of SQSTM1 mutations detected in our sample and

the detailed clinical and anamnestic information collected from each patient have allowed us to better characterize genotype-phenotype correlation as well as to explore potential interactions between genetic and environmental factors.

Material and Methods

Subjects

The participants in this study consisted of 608 unrelated and consecutive PDB patients from different Italian regions recruited at the Bone Disease Units of Turin, Siena, and Naples. These are the three main national centers for the diagnosis and treatment of PDB, located, respectively, in northern, central, and southern Italy (www.pagetitalia.com). General characteristics of recruited patients are reported in Table 1. All patients were born in Italy, and all but three patients were of Italian origin (as assessed from parental history). Diagnosis of PDB was based on biochemical evaluation, bone scintigraphy, and subsequent X-ray examination of areas of increased isotope uptake. For all subjects, a detailed medical history was obtained, including family history, place of birth, place of residence during childhood, occupation, age at diagnosis, skeletal extent, complications, age at onset of PDB symptoms, dietary habits, and animal contacts. The latter included either pet ownership or a lifestyle shared with animals in rural districts (ie, working or living on a farm). Specific questions were asked to assess the presence of animal contacts in different decades of life in each patient following a detailed questionnaire, as described previously.[26] When available, clinical data were collected to evaluate the presence of PDB complications. In particular, the presence of neoplastic degeneration, cranial nerve disorders, hearing loss, hip or knee replacements, osteoarthritis, fractures, back pain, hypertensive disease, hyperparathyroidism, renal stones, or heart failure was recorded. The study was approved by local ethical committees, and all subjects had given informed consent to being included. All data were collected through common questionnaires shared by all participating centers. The cohort of PDB patients from Turin partially overlapped (54 of 186 patients) with a cohort examined in two previous studies on SQSTM1 mutations.[13,29]

The patients having a previous report of at least one other family member affected with PDB were defined as familial cases. If the presence of relatives with suspected clinical features of PDB (ie, focal bone pain and/or bone deformity or deafness) was referred, these relatives were invited to undergo a specific

Table 1. General Characteristics of Patients

	Turin	Siena	Naples
Number (n)	186	275	145
Age (years)	71.4 ± 10.3	70.9 ± 11.8	67.7 ± 10.4*
Age at diagnosis (years)	61.5 ± 10.7	60.1 ± 12.8	57.1 ± 10.7**
Familial patients (n)	30	38	31
Affected sites (n)	2.24 ± 2.2	2.35 ± 0.9	3.13 ± 2.1**
Polyostotic patients (n)	86	154	109***
Patients from Campania (n)	15	29	145

*p < .001; **p < .005; ***p < .0001.

GENNARI ET AL.

28

diagnostic test for PDB. Patients with a negative history were classified as sporadic PDB.

First-degree relatives of all recruited patients also were invited to undergo biochemical evaluation of total alkaline phosphatase to uncover new familial PDB cases, which were confirmed by radiologic and bone-scan analyses. However, since we did not perform a detailed evaluation of all the first-degree relatives, we cannot exclude the possibility that sporadic patients may have had relatives with asymptomatic disease.

Pedigrees of the three families from Campania with PDB and giant cell tumor are shown in Fig. 1. All members from these families had polyostotic disease, with a high number of affected skeletal sites (7.6 ± 2.3, 5.0 ± 3.0, and 6.5 ± 2.1 in families 1, 2, and 3, respectively) and an early age of diagnosis (36.0 ± 9.5, 46.3 ± 1.5, and 32.5 ± 10.6 years in families 1, 2, and 3, respectively). Overall, five patients developed giant cell tumor. The mean ages at PDB diagnosis and giant cell tumor onset in these patients were 43.2 ± 9.3 and 58.8 ± 7.7 years, respectively, and the mean number of affected skeletal sites was 7.8 ± 3.5 (range 4 to 12). In two of these patients, multiple giant cell tumors were observed. After 12.0 ± 3.8 years from the diagnosis of giant cell tumor, four of these patients died owing to cardiovascular complications.

Genetic analysis

Genomic DNA was extracted from peripheral blood leukocytes using standard procedures. We conducted mutation screening of exons 7 and 8 of SQSTM1 and their intron-exon boundaries using PCR, followed by automated DNA sequencing. We performed PCR in reactions ($25\,\mu L$) using Taq DNA polymerase (1 U; Fermentas, Glen Burnie, MD), 1X buffer, deoxynucleoside triphosphate (dNTP, $0.2\,mM$; Amersham, Uppsala, Sweden), primers ($0.5\,\mu M$), and DNA ($50\,ng$). PCR conditions were as follows: initial denaturation at $94^{\circ}C$ for 3 minutes, followed by 35 cycles of $94^{\circ}C$ for 30 seconds, 30 seconds at $62^{\circ}C$, and extension at $72^{\circ}C$ for 45 seconds, and a final extension for 10 minutes at $72^{\circ}C$. Exons 7 and 8 of the SQSTM1 gene were amplified by

using, respectively, two pairs of primers located in the flanking introns: 5'-CATGCGTGCTCCCCGACTGT-3'/5'-GCCCTGCAGTGG-AGAACATC-3' for exon 7 and 5'-CTCTGGGCAGGCTCGGACAC-3'/5'-CTTGCACCCTAACCCCTGAT-3' for exon 8. Samples then were ExoSap-digested (Amersham) and sequenced using the Big Dye Terminator Ready Reaction Kit (Applied Biosystems, Foster City, CA). Sequencing reactions were performed on a 9700 Thermal Cycler (Applied Biosystems) for 25 cycles of $95^{\circ}C$ for 10 seconds, $60^{\circ}C$ for 5 seconds, and $60^{\circ}C$ for 2 minutes. After the sequencing, each reaction was column-purified (Amersham) to remove excess dye terminators. Sequencing of the products was performed on the ABI Prism 3710 Genetic Analyser (Applied Biosystems). All the remaining exons and intron-exon boundaries of SQSTM1 also were screened in familial PDB patients who did not show mutations in exons 7 and 8.

To analyze the genetic background of mutated patients, we genotyped four single-nucleotide polymorphisms (SNPs) located in exon 6 (C916T, G976A) and the 3' untranslated region of SQSTM1 (C2503T, T2687G) as performed in previous studies.[8,22,30,31] The software program PHASE was used to reconstruct haplotypes (www.stat.washington.edu/stephens/).

In order to estimate the haplotype frequencies in the Italian population, as well as to differentiate the presence of SQSTM1 mutations from polymorphisms, we also analyzed DNA samples from 100 control subjects without any history of PDB or other skeletal disorders.

Statistical analysis

Continuous variables were compared by ANOVA. Comparisons between the groups were analyzed by chi-squared test or the Fisher exact test for categorical variables, whichever was appropriate. Analysis was performed using Statistica 5.1 (Statsoft, Tulsa, OK, USA) and SPSS (Release 6.1, SPSS, Chicago, IL, USA). All data are expressed as means $\pm$ SD. Adjustment for multiple comparisons was not performed.

Results

General characteristics of patients

A family history of PDB in at least one relative was evidenced in 99 of the 608 recruited patients (16.3%). In keeping with our previous observations,[27,28] PDB subjects from Campania showed an earlier age at diagnosis (56.6 ± 11.3 versus 61.4 ± 12.1, $p < .0001$) and an increased clinical severity with respect to PDB patients from other Italian regions. In particular, an increased proportion of polyostotic cases (72.5% versus 50.4%, $p < .0001$) and an increased number of affected skeletal sites (3.1 ± 2.2 versus 2.2 ± 1.9, $p < .0001$) were observed in PDB patients from Campania than in patients from other regions. No correlation was observed between year of birth and number of affected sites in the overall sample of PDB patients. Conversely, the year of birth was negatively correlated with the age at diagnosis of PDB ($r = -0.31, p < .0001$). The latter association was highest in PDB patients living outside Campania ($r = -0.65, p > .0001$) and decreased in magnitude in PDB patients from Campania ($r = -0.22, p < .005$).
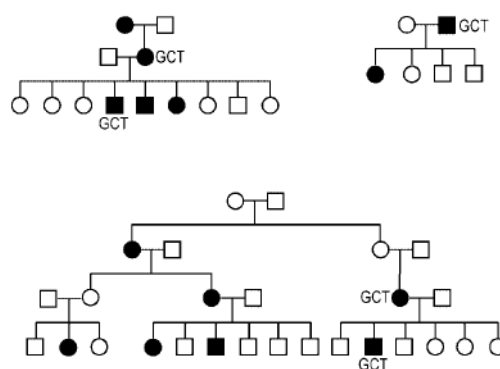
Fig. 1. Pedigrees of the three kindreds from Campania with giant cell tumor (GCT) and Paget's disease of bone. Pagetic patients are indicated in black.

29

Table 2. Prevalence of *SQSTM1* Gene Mutations in Italy

Mutation	Northern Italy ($n = 132$)	Central Italy ($n = 169$)	Southern Italy ($n = 212$)	Islands ($n = 15$)
Y383X	0	0	7 (3.30%)	0
P387L	2 (1.51%)	0	1 (0.47%)	0
P392L	7 (5.30%)	14* (8.28%)	19 (8.96%)	0
E396X	1 (0.76%)	0	3 (1.41%)	0
M401V	0	0	1 (0.47%)	0
M404V	6 (4.55%)	5 (2.96%)	1 (0.47%)	1 (6.67%)
G411S	0	1* (0.59%)	0	0
D423X	1 (0.76%)	0	0	0
G425E	0	1 (0.59%)	3 (1.41%)	0
G425R	0	0	2 (0.94%)	0
A427D	0	0	2 (0.94%)	0

*With the inclusion of 1 patient with a G411S/P392L mutation, 5 patients without *SQSTM1* mutations were excluded from analysis owing to the inability to assign their region of origin.

Mutation screening of the *SQSTM1* gene in the overall sample

Genetic analysis was completed in 533 of the 608 patients. Eleven different mutations in the *SQSTM1* gene were observed in 34 of 92 (36.9%) and 43 of 441 (9.7%) of familial and sporadic PDB patients, respectively (equivalent to 14.4% of the overall cohort) (Table 2). DNA analysis from 100 control subjects failed to detect the reported *SQSTM1* mutations. A significantly higher prevalence of mutations was observed in polyostotic than monostotic patients (21.0% versus 5.7%, $p < .0001$).

Two of these mutations, M401V (A1241G) and A427D (C1320A), were novel and have not been described previously. Moreover, we disclosed for both amino acids a high degree of conservation across species from fish to humans (data not shown), which argues in favor of an important role of these amino acids in the function of p62 protein. The other mutations (Y383X, P387L, P392L, E396X, M404V, G411S, D423X, G425E, and G425R) were described previously in other populations. One subject carried a double P392L and G411S mutation. He had a polyostotic form of disease (with three affected skeletal sites) diagnosed at 55 years of age.

The overall prevalence of *SQSTM1* mutations was higher in younger than in older PDB patients. In fact, 64.9% of the mutations were observed in subjects with a birth age above the median (corresponding to 1937). The *SQSTM1* mutation rate was 9.5% versus 19.2% in PDB patients below or above the median age ($p < .01$). A similar trend was observed when patients were grouped according to the decades or quartiles in relation to year of birth. Overall, 31 of 77 *SQSTM1* mutations (40.26%) were observed in subjects in the upper quartile of age (year of birth after 1946).

After the analysis of exons 7 and 8, all the remaining exons and intron-exon boundaries of *SQSTM1* were screened in the 58 familial PDB patients who did not show mutations in exons 7 and 8. No further mutations in *SQSTM1* gene were found. In particular, all the three families with giant cell tumor complicating PDB were negative for *SQSTM1* mutations.

Genotype-phenotype correlation

As shown in Table 3, PDB subjects with *SQSTM1* mutation showed an increased number of affected skeletal sites, an increased prevalence of polyostotic disease, and earlier age of onset than PDB patients without mutation. Conversely, no differences were observed in the occurrence of major complications of PDB between patients with or without mutation. A similar pattern also was observed in familial versus sporadic PDB patients. With respect to sporadic patients, familial patients were younger (63.5 ± 12.0 versus 69.3 ± 11.6 years, $p < .005$) and showed an earlier age at onset (54.6 ± 12.1 versus 60.7 ± 11.3 years, $p < .0001$), a higher number of affected skeletal sites (3.56 ± 2.7 versus 2.33 ± 1.8, $p < .0001$), and an increased proportion of polyostotic disease (73.8% versus 54.9%, $p < .005$). When familial patients with and without *SQSTM1* mutation were compared, there were no differences in age at onset (53.9 ± 10.9 versus 54.9 ± 12.8 years, $p = 0.7$), and ther was a mild but not significant variation in the number of affected skeletal sites (4.07 ± 2.7 versus 3.23 ± 1.9, $p = 0.08$) or in the prevalence of polyostotic disease (67.2% versus 85.1%, $p = .06$). Conversely, PDB patients with *SQSTM1* mutations considered to be sporadic cases showed an earlier age at onset (55.8 ± 11.5 versus 61.4 ± 11.2 years, $p < .005$), an increased number of affected skeletal sites (3.31 ± 2.3 versus 2.18 ± 1.8, $p < .0005$), and a

Table 3. Clinical Characteristics of Patients With or Without *SQSTM1* Gene Mutations

	Subjects (n)	Familial patients (n)	Age (years ± SD)	Age at diagnosis (years ± SD)	Polyostotic patients (n)	Affected sites (n ± SD)
WT	456	58	69.7 ± 21.5	60.6 ± 11.6	238	2.31 ± 1.9
SQSTM1	77	34	67.8 ± 12.2	55.0 ± 11.2	64	3.60 ± 2.6
p Level	—	<.0001	.74	<.0005	<.0001	<.0001

higher prevalence of polyostotic disease (82.2% versus 50.9%, $p = .0001$) than sporadic PDB patients without SQSTM1 mutations.

In order to better characterize genotype-phenotype correlation, we analyzed 98 first-degree relatives of subjects with SQSTM1 mutations, and we detected 20 additional mutations in 18 affected and 2 unaffected subjects. Interestingly, the mutation was not observed in 2 affected PDB relatives of two familial PDB patients with SQSTM1 mutations, indicating phenocopy. The first kindred was composed of two affected brothers from southern Italy, of whom only one had the M404V mutation. They had a similar polyostotic phenotype with three or four affected skeletal sites and a similar age at diagnosis (around 50 years). The second kindred included three affected patients from central Italy, of whom two had the P392L mutation. The two mutated patients, an 87-year-old woman and her 85-year-old brother, had polyostotic PDB with two and three affected sites, respectively. Their affected brother (82 years old) without SQSTM1 mutation had monostotic PDB of the left pelvis.

Phenotype characteristics of subjects according to the type of SQSTM1 mutation are shown in Table 4. Overall, slight differences in the number of affected skeletal sites or age at disease onset were observed among patients with different mutations. A higher number of affected skeletal sites was observed in the two patients with A427D mutations (7.00 ± 2.8, range 5 to 9) and in the seven unrelated patients with Y383X mutations (4.84 ± 3.8, range 1 to 12). The latter mutation also was associated with the lowest age at diagnosis (mean 48.1 ± 9.3 years, range 40 to 60 years). Similar trends were observed when familial and sporadic patients were considered separately or when affected relatives with SQSTM1 mutations were included (bringing the overall number of mutated patients to 95). In this latter case, marked differences in clinical severity of disease also were observed, even within each single family with P392L, M404V, or E396X mutations. In fact, even in the case of a family with an E396X mutation, causing the truncation of most of the UBA domain, the number of affected skeletal sites in three PDB patients varied from two to seven, with an estimated onset of disease at between 38 and 64 years of age.

To further explore possible genotype-phenotype correlations, we grouped patients according to the type or site of SQSTM1 mutation: truncating versus missense and outside versus inside the structured region of the UBA domain (amino acids 392 to 431). As shown in Table 5, an earlier age at diagnosis was observed in patients with truncating mutations than in those with missense mutations, whereas the number of affected skeletal sites and the frequency of polyostotic disease did not differ significantly. Mutations outside the UBA domain (Y383X and P387L, $n = 10$) did not differ significantly from mutations inside the UBA. Moreover, we observed a negative correlation between year of birth and severity of disease, expressed as number of affected sites, in patients with missense mutations ($r = -0.22$, $p < .05$) but not in patients with truncating mutations. The correlation between birth year and age at PDB diagnosis observed in the overall sample remained statistically significant independent of type (truncating or missense) or site (inside or outside UBA) of mutation.

Regional distribution of SQSTM1 mutations and gene-environment interactions

The prevalence of SQSTM1 mutations was higher in southern (18.4%) than in central (11.8%) and northern (12.9%) Italy (Table 2). Moreover, an increased heterogeneity and a different distribution of mutations were observed in southern Italy (showing 9 of the 11 mutations) than in central and northern Italy (where only 4 and 5 of the reported mutations were observed, respectively). Interestingly, all seven patients with the Y383X mutation were from southern Italy and specifically from Campania. In contrast, the M404V mutation was more frequent in northern (4.6%) and central (3.0%) Italy than in southern Italy (0.5%).

Given the reported characteristics of PDB patients from Campania, we performed a subanalysis of patients from this region. Differences in terms of age at diagnosis and severity between familial and sporadic patients or between patients with or without SQSTM1 mutations were milder in PDB patients from Campania than in patients from other regions. Moreover, despite

Table 4. Genotype-Phenotype Correlation in Patients with SQSTM1 Gene Mutations

Mutation	Subjects (n)	Familial patients (n)	Age (years ± SD)	Age at diagnosis (years ± SD)	Polyostotic patients (n)	Affected sites (n ± SD)
Y383X	7	4	63.5 ± 4.6	49.0 ± 8.8	5	4.28 ± 3.8
P387L	3	0	80.0 ± 14.0	64.3 ± 9.3	3	3.33 ± 2.3
P392L	39	12	69.5 ± 11.0	56.7 ± 11.8	33	3.56 ± 2.6
E396X	4	3	55.0 ± 13.6	50.5 ± 17.0	4	3.25 ± 2.5
M401V	1	1	78	48	1	10
M404V	13	9	68.2 ± 16.5	53.5 ± 11.5	9	3.07 ± 2.1
G411S/P392L	1	0	68	55	1	3
D423X	1	0	61	57	1	2
G425E	4	1	59.2 ± 7.2	49.7 ± 5.4	3	2.25 ± 0.9
G425R	2	2	59.5 ± 2.1	52.0 ± 5.6	2	3.50 ± 0.7
A427D	2	2	78.0 ± 7.0	62.0 ± 7.1	2	7.00 ± 2.8
p Level	—	—	.04	.46	.75	.20

31

Table 5. Genotype-Phenotype Correlations According to the Type (Truncating versus Missense) or Site (Outside versus Inside the Structured Region of the UBA Domain) of *SQSTM1* Mutation

	Truncating	Missense	Outside UBA	Inside UBA
Number (*n*)	12	65	10	67
Age (years)	60.2 ± 9.8[*]	68.8 ± 12.1	69.0 ± 11.5	67.9 ± 12.2
Age at diagnosis (years)	50.5 ± 11.3[**]	56.3 ± 10.7	52.8 ± 11.6	55.7 ± 11.1
Familial patients (*n*)	7/12	27/65	4/10	30/67
Affected sites (*n*)	3.77 ± 2.9	3.45 ± 2.4	4.22 ± 3.1	3.41 ± 2.0
Polyostotic patients (*n*)	10/12	54/65	8/10	56/67

[*]$p < .05$ and

[**]$p = .05$, truncating versus missense mutation.

a higher prevalence of *SQSTM1* mutations in the group of patients from Campania, a consistent number of analyzed PDB families from this region (26 of 35) did not have the mutation. Genotype-phenotype analysis in PDB patients from Campania did not evidence any significant difference in relation to the type or site of mutation. Moreover, the prevalence of *SQSTM1* mutations did not differ significantly based on decades or quartiles of age in patients from Campania, whereas marked age-related differences in the prevalence rates of *SQSTM1* mutations were observed in patients from the other regions (20.9% versus 8.4%, $p < .001$ in patients above or below the median age, respectively).

Overall, 342 of 533 (64.2%) PDB patients indicated animal contacts for at least 10 years before onset of the disease. These included pet ownership (mainly cats and dogs) and previous or current contact with animals such as pigs, rabbits, sheep, and cattle in rural districts. The prevalence of subjects with these contacts did not differ between patients with or without *SQSTM1* mutations in the overall sample (58.4% versus 65.2%, $p = 0.2$) but became significant in patients living outside Campania (48.9% versus 64. 2%, $p < .05$). Conversely, patients from Campania had a high prevalence of animal contact (69.3% versus 62.1% in the other regions, $p = .09$) but without any difference between patients with or without *SQSTM1* mutations. When familial patients from Campania were considered, animal contacts were observed in 90.1% of families without *SQSTM1* mutations. Interestingly, a progressive age-related decrease in the prevalence of animal contact was observed based on the median age as well as the decade of age in the overall sample of patients. This decrease was not observed in the subgroup of patients from Campania but increased in magnitude in patients from other regions. In particular, in younger patients (with a birth year after 1950), 70.4% of patients from Campania reported animal contacts with respect to 48.9% of patients from other regions ($p < .05$).

Overall, PDB patients reporting animal contacts showed an increased number of affected sites (2.19 ± 1.9 versus 2.54 ± 2.0, $p < .05$) and a higher prevalence of familial disease (23.0% versus 14.1%, $p < .05$) than patients without animal contacts. A significant difference in the number of affected skeletal sites in relation to animal contacts also was seen in the subgroup of patients with *SQSTM1* mutations (3.84 ± 2.5 versus 2.76 ± 2.2, $p < .05$). This difference also was seen when missense or truncating mutations were considered separately. All the preceding differences became milder and not significant when only pet ownership was considered instead of animal contacts.

SQSTM1 haplotypes in PDB patients and controls

We genotyped four SNPs in exon 6 and the 3′ untranslated region of the *SQSTM1* gene. The four selected SNPs were analyzed in all mutation carriers as well as in 100 control individuals and 100 PDB patients without *SQSTM1* mutations. Genotype distribution for these SNPs followed a Hardy-Weinberg equilibrium. There was no significant difference in distribution of the genotypes between patients and controls for any of the SNPs studied.

Consistent with previous studies in different populations,[8,22,30,31] the H_1 (916T-976A-2503C-2687T) and H_2 (916C-976G-2503T-2687G) haplotypes accounted for the largest proportion of patients (94.3%) and controls (91.5%). The remaining patients were accounted for by six rare haplotypes with individual frequencies of between 0.2% and 3.6%. The presence of H_1 and H_2 haplotypes was observed in 75.0% (including two H_1/H_1 homozygous subjects) and 90.0% (including seven H_2/H_2 homozygous subjects) of patients with the P392L mutation, respectively, compared with 76% and 80% observed in control individuals. Since we did not perform allele-specific PCR, we could not unambiguously assign the mutation to one of the two haplotypes in the 26 H_1/H_2 heterozygous subjects, except than in three familial patients, in whom genetic analysis of affected family members was able to assign the P392L mutation to the H_2 haplotype. An increased prevalence of the H_2 haplotype also was observed in patients with M404V and Y383X mutations. In particular, the Y383X mutation was associated with the H_2 haplotype in 100% of patients. In fact, only one heterozygous H_1/H_2 subject with Y383X was observed, and subsequent genetic analysis of affected family members (with the identification of one H_2/H_2 mutation carrier) demonstrated that the mutation is carried with the H_2 allele. Conversely, 3 of 4 and 4 of 4 unrelated PDB patients with the G425E and E396X mutations, respectively, were negative for the H_2 haplotype, suggesting that in this case the mutation is carried with a different haplotype, probably the H_1, which was present in 100% of these patients (with a frequency of 87.5% and 75.0% in G425E and E396X mutation carriers, respectively).

Discussion

Despite the significant progress that has been made in recent years, the etiology of PDB is not completely understood.[2,3,24] Mutations in the *SQSTM1* gene have been described worldwide in consistent proportion of patients with PDB from different ethnic groups, suggesting that functional differences in this gene are a direct cause of the disease, particularly in familial PDB. However, several clinical and experimental observations raised the hypothesis that other genes and/or environmental triggers are necessary to cause the disease, at least in some cases.[2,20,21,23,32–34] In this study we report the results of the largest *SQSTM1* mutation screening performed to date in consecutive familial and sporadic PDB patients.

In our sample, we identified 11 different *SQSTM1* mutations in 14.4% of patients. This percentage is comparable with the results of mutational analysis studies performed in other countries, such as Great Britain (13.9%), France (12.8%), and Canada (19.8%).[8,9,11,17] Consistent with previous findings, the prevalence of mutations was highest (36.9%) in patients with a clear family history. Since we were not able to clinically exclude the presence of PDB in all first-degree relatives of recruited patients, we cannot exclude that a proportion of patients reporting no family history and thus classified as sporadic patients may have familial PDB. In particular, in sporadic patients with *SQSTM1* mutations, we observed an increased severity of disease that is remarkably similar to that seen in familial patients. The latter observation may suggest that a consistent proportion of sporadic PDB patients with *SQSTM1* mutations may indeed represent familial patients. This is in keeping with a recent report in a well-characterized sample of PDB patients from the United States showing absence of *SQSTM1* mutations in sporadic patients.[18] Of interest, we also observed a higher heterogeneity of *SQSTM1* mutations in our sample of patients of Italian ancestry with respect to patients from other countries. In fact, we detected 11 different mutations. Together with the results from a recent additional study in an Italian population,[29] 15 different *SQSTM1* mutations have been described in more than 800 Italian patients analyzed to date. An additional mutation (P364S) has been described in a single PDB family of Italian descent living in Australia.[19] This heterogeneity is higher than observed in populations of British descent or in other European populations and might reflect the complex history of Italy as well as the several foreign invasions and dominations that occurred between sixth and nineteenth centuries. Moreover, in our study, a different distribution and a higher heterogeneity of mutations were particularly observed in southern Italy (showing 9 of the 11 mutations) than in central and northern Italy. While the M404V mutation was more frequent in northern and central Italy than in southern Italy, the Y383X mutation was observed in seven unrelated patients from Campania and was absent in patients from other regions. This mutation also was described in a previous Italian study in a family from Campania and in two sporadic patients of unreported origin[29] but was not observed in more than 1000 PDB patients from other countries analyzed in previous studies. The mutation is one of the two known truncating *SQSTM1* mutations located outside the UBA domain and seems to be associated with a severe phenotype in most

patients. A similar phenotype has been described in one familial patient with the other mutation (K378X), and functional analysis confirmed that this mutation, leading to the complete elimination of the UBA domain, is associated with potentiated osteoclast formation and bone resorption in human primary cell cultures.[16]

Despite the severe phenotype and the earlier age at onset of disease, we did not find *SQSTM1* mutations in the three kindreds with giant cell tumor. This complication represents a quite unusual clinical feature of PDB (described in fewer than 100 patients worldwide) and occurs mainly in patients with severe polyostotic disease, with a remarkably higher prevalence in patients from Campania.[35–37] In fact, more than 50% of the patients described originated in or descended from ancestors who lived in this Italian region.[27] Thus it can be speculated that a different gene is responsible for this particular variant of familial PDB, alone or in combination with an environmental trigger.[38]

Phenotype-genotype associations in PDB have not been investigated extensively, and the results from available reports are conflicting. This may reflect the limited number of patients with *SQSTM1* mutations, except the P392L mutation, available in previous studies. Consistent with recent observations in two populations of British descent and in the French population, we confirmed that PDB patients with *SQSTM1* mutations have a more extensive disease and an earlier age at diagnosis than patients without *SQSTM1* mutations.[11,17,19] This observation is in contrast to a previous report in a smaller sample of patients from Italy that did not show significant phenotypic differences in relation to the presence of *SQSTM1* mutations, as well as between familial and sporadic patients.[29] The prevalence of *SQSTM1* mutations, however, was lower in that study (8.7%) than in our or in previous studies, most likely reflecting the reduced number of familial patients (12 of 357, equivalent to 3.4%). When we compared clinical characteristics of patients with different *SQSTM1* mutations, we did not observe major differences in the number of affected skeletal sites or in the age at diagnosis, even though a trend for a more severe phenotype clearly was observed in most patients with the Y383X and A427D mutations. Since all patients with these mutations were from Campania, we cannot exclude the possibility that this finding is related to the overall increased clinical severity of disease observed in patients from this region. Moreover, a variable disease severity was observed among affected members of kindreds with the same *SQSTM1* mutation for all mutations. These findings are in keeping with two previous detailed analyses of large PDB kindreds with *SQSTM1* mutations of diverse racial or ethnic background showing high variability in intrafamilial expressivity of disease as well as incomplete penetrance.[20,21] Moreover, in one of these studies, offspring who inherited an *SQSTM1* mutation from their parents were diagnosed with PDB later in life and had less extensive disease than their parents.[21] Even though a slight reduction in clinical severity and a higher variation in the number of affected skeletal sites among family members were observed in missense with respect to truncating mutations, we can conclude that there are no major genotype-phenotype differences in relation to the type or site of *SQSTM1* mutation. Together with the described cases of incomplete penetrance[20–22]

33

and the examples of phenocopy observed in this and other previous studies,[19] these findings further reinforce the hypothesis that additional factors may be required to cause the disease (at least in a group of patients) as well as its skeletal extension in subjects with or without SQSTM1 mutations. In this context, the presence of somatically acquired SQSTM1 mutations in the pagetic bone cannot be excluded, even though this hypothesis remains controversial and probably restricted to a limited number of patients.[39,40]

We and others have previously evidenced an association between PDB and animal-related factors, as well as a significantly higher prevalence of the disease in rural than in urban districts.[26,41–43] In this study, we also demonstrated that patients reporting persistent animal contacts for at least 10 years before the onset of disease have an increased number of affected skeletal sites and an increased prevalence of polyostotic disease. Interestingly, this association also was evidenced in patients with SQSTM1 mutations, suggesting an interaction between genetic and environmental factors. The observed differences, however, were small, and their clinical impact remains to be addressed in future prospective studies with larger numbers of patients with SQSTM1 mutations. In fact, even though adjustment for multiple comparisons generally is not required in this kind of study,[44,45] with a more conservative approach (ie, with Bonferroni's correction), some of these differences will no longer be significant.

Nevertheless, together with different previous epidemiologic reports, these data provide further evidence that infective agents may be involved in PDB, not only promoting the occurrence of the disease in some patients but also influencing its clinical severity. In this context, the progressive age-related decrease in the number of patients reporting animal contacts observed in this study is consistent with secular trends showing a decrease in both the prevalence and severity of PDB over time[25,46] and also might explain, at least in part, the parallel age-related increase in the prevalence of SQSTM1 mutations observed in our sample. In fact, it cannot be excluded that in patients without SQSTM1 mutations, the disease also may originate from contacts with an environmental factor (alone or in combination with additional genetic causes). Thus a reduced exposure to these animal-related environmental agents in recent years could have led to a relative increase in the frequency of PDB patients owing to SQSTM1 mutations. Consistently, this phenomenon was not observed in PDB patients from Campania, where a higher prevalence of animal contacts was observed even in recent years, reaching 90% in familial PDB patients without SQSTM1 mutations. To date, the nature of the possible environmental trigger remains unknown, but several reports suggested that viral infections of the paramyxovirus family may infect the osteoclast, inducing most of the cellular abnormalities of PDB.[32,33,47–49] Indeed, in one of these studies, canine distemper virus not only induced NFκB activation and bone resorption but also markedly increased sequestosome 1/p62 gene expression in human osteoclast cells.[33]

Finally, results from our analysis evidenced an increased occurrence of the P392 mutation with the H_2 than H_1 haplotypes. This is consistent with the notion of a "founder effect" for this mutation, even if it was not possible to unambiguously assign the mutation to one of the two haplotypes in most of the heterozygous subjects, because we did not perform allele-specific screening. Moreover, we also observed two H_1-H_1 homozygous patients carrying the P392L mutation, suggesting that the same mutation has occurred independently at least twice, as observed previously in the French-Canadian population.[8,22] An increased prevalence of the H_2 haplotype also was seen in patients with the M404V mutation, whereas the Y383X mutation was carried with the H_2 haplotype in all patients. This also strongly supports the presence of a founder effect for this mutation. Conversely, most patients with G425E and E396X mutations were negative for H_2 haplotype, suggesting that in this case the mutation is carried with a different haplotype, most likely H_1.

In conclusion, while the clinical impact of most of the reported differences remains to be addressed, our findings further underline the complex etiology of PDB that cannot be explained solely by the presence of SQSTM1 mutations. It is likely that both genetic and environmental factors may cause PDB or most likely interact with each other to cause the disease and its variable phenotypes. Moreover, our results also indicate that the increased severity of PDB cases from Campania observed in this and other previous studies seems to be related to concomitant factors: (1) an increased heterogeneity of SQSTM1 mutations (with higher prevalence of truncating mutations such as Y383X), (2) an increased persistence of the environmental trigger (probably related to a lifestyle shared with animals), and (3) the presence of additional predisposition genes (including a gene causing familial PDB with giant cell tumor). Further genetic studies in this population, particularly in familial patients negative for SQSTM1 mutation, might be extremely useful for a real understanding of the complex etiology of this disorder.

Disclosures

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34

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36

Epidemiological, clinical, and genetic characteristics of Paget's disease of bone in a rural area of Calabria, Southern Italy

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ABSTRACT. *Background:* The prevalence of Paget's disease of bone (PDB) is unknown in peninsular Southern Italy, although an elevated clinical severity of the disease was reported in patients from Campania. *Aim:* This study was performed to evaluate the epidemiological and genetic characteristics of PDB in a rural area of Calabria, the southernmost region in the Italian peninsula. *Subjects and methods:* We examined 1068 consecutive pelvic radiographs of patients older than 40 yr referred for any reason to the "Spinelli" Hospital, Belvedere Marittimo, from January 1st 2004 to December 31st 2006. In subjects with radiological findings of pelvic PDB, a ^{99m}Technetium methylene diphosphonate bone scan and the sequence analysis of the *sequestosome 1* (SQSTM1) gene were subsequently performed. *Results:* In the examined geographic area, the crude radiographic prevalence of pelvic PDB was 0.74%

(8/1068; male:female 5:3, mean age 71.6±13.1 yr) whereas the estimated overall prevalence of PDB between 0.82% and 1.21%. PDB patients from Calabria showed clinical characteristics similar to those reported in patients from Campania. The disease was also frequently complicated by osteoarthritis and the right side of the body was more affected than the left. The *SQSTM1* gene analysis revealed the presence of a novel missense mutation (M401V) in exon 8 in one subject with a familial and aggressive form of PDB. *Conclusion:* The study results confirmed that patients with PDB from rural districts of Southern Italy show an earlier onset and an increased clinical severity of the disease that appears mostly independent from the presence of germinal *SQSTM1* mutations.
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INTRODUCTION

Paget's disease of bone (PDB) (OMIM 602080) is a chronic disorder of the adult skeleton featuring one or more areas of aggressive osteoclast-mediated bone resorption preceding imperfect osteoblast-mediated bone repair (1). In these areas, pagetic osteoclasts appear microscopically markedly increased in number and size, can have 100 nuclei per cell, and contain paramyxoviral-like nuclear and cytoplasmatic inclusions (2). Thirty years ago, the observation of these inclusions suggested the involvement of a latent paramyxoviral infection in the PDB pathogenesis (3). On the other hand, PDB also has a clear hereditary component and several genetic loci have been linked to familial and sporadic PDB (4). Most likely, environmental and genetic factors interact in the pathogenesis of PDB, and this phenomenon could explain the variable phenotypic presentation and the peculiar geographic distribution of the disease (5). Epidemiological observations indicate that PDB is most common in Europe, North America, Australia, and New Zealand (6), while is rare in sub-Saharan Africa and Asia (6, 7). In countries such as South America and Israel, the

disease occurs predominantly in patients of European descent (8, 9).
In Italy, epidemiological observation from 2 studies (including 4 towns: Milan and Turin in Northern Italy, Siena in Central Italy, and Palermo in Sicily) evidenced a PDB prevalence between 0.5 to 1.5% in subjects ≥60 yr, allowing us to estimate that 150,000-300,000 subjects may be affected by the disease (10, 11). Although an increased clinical severity of the disease has been observed in subjects from Campania region, specific data on the prevalence of PDB in peninsular regions of Southern Italy are not actually available (12). Therefore, the present study was performed to evaluate the prevalence of PDB in a rural area of Calabria (2 million inhabitants, 15,080 per square km) the southernmost region in the Italian peninsula, using a radiographic survey. Moreover, considering the role of the mutations in *sequestosome 1* gene (SQSTM1; also known as *p62*, OMIM 601530) in the PDB pathogenesis, we also performed the sequence analysis of this gene in all patients identified by this radiographic survey and in 100 healthy control subjects without clinical evidence of metabolic bone diseases (1, 4, 5, 13).

MATERIALS AND METHODS

Study design and clinical analysis

All white Caucasian patients consecutively referred for any reason to the "Spinelli" Hospital, Belvedere Marittimo (Cosenza, Italy) from January 1st 2004 to December 31st 2006 were considered for possible participation in the study. We evaluated on-

ly the subjects ≥40 yr of age. The radiographic sample consisted of 1068 consecutive pelvic radiographs. The selected X-ray films, taken from films stored at the Radiology Department, showed (a) the entire pelvis and sacrum, (b) the lumbar vertebrae, and (c) the femoral heads, sites that are commonly affected by PDB. Radiographs were sequentially mounted on multiview X-ray view boxes and reviewed for the presence of PDB. The finding of PDB on the pelvic radiograph was based on the following standardized radiographic criteria: (a) expansion of bone size; (b) thickened, disorganized trabeculae; (c) thickened, expanded cortex; (d) osteosclerosis; and (e) deformity (14).

Subjects with radiological findings of pelvic PDB, underwent a ⁹⁹ᵐTechnetium methylene diphosphonate (⁹⁹ᵐTC-MDP) bone scan to evaluate the extent of the disease. The diagnosis of PDB on the bone scan was based on the following standardized criteria: (a) areas of intensively increased uptake, often showing a "V" or "flame-shaped" leading edge; (b) ring of increased activity in the margins of the skull; (c) deformity and expansion of bone size; and (d) tracer accumulation throughout one or more vertebrae, affecting the body and posterior elements, including the spinous and transverse processes (15, 16). The extension of PDB, expressed as percentage of skeleton involved by the disease, was evaluated according to the criteria proposed by Davie and co-workers (17).

A fasting venous blood sample was also drawn and serum levels of total alkaline phosphatase were determined (normal range 80-265 U/l). Serum samples were separated within 1 h of collection and kept frozen at –80 C until biochemical analysis. Each parameter was measured in duplicate for all patients.

Using a fixed-sequence questionnaire previously validated (12), a detailed medical history was obtained for all PDB subjects, including personal and family history, place of birth, place of residence during childhood and adolescence, housing, occupation, age at PDB diagnosis, dietary habits, pet ownership, and animal contacts. Clinical data were also collected to evaluate the presence of PDB complications. In particular, the presence of neoplastic degeneration, cranial nerve disorders, hearing loss,

hip or knee replacements, osteoarthritis, fractures, back pain, hypertensive disease, hyperparathyroidism, renal stones, and heart failure were recorded.

The revision of X-ray films, ⁹⁹ᵐTC-MDP bone scans, and biochemical and genetic analyses were performed at the Department of Clinical and Experimental Medicine of the "Federico II" University Medical School and at the Institute of Genetics and Biophysics "Adriano Buzzati-Traverso", in Naples.

All subjects enrolled gave their informed written consent to participate in the study and accepted to perform the additional analyses. The study was conducted according to the Declaration of Helsinki, following approval by the local Ethics Committee. All the examined subjects were born and lived in the same geographical area, the territory of the Local Health Authority Cosenza 1, belonging to the Calabria region (Fig. 1).

Genetic analysis

DNA was extracted from whole-blood samples with the salting-out procedure described by Miller et al. (18). We initially conducted mutation screening of exons 7 and 8 of *SQSTM1* gene and their intron-exon boundaries using PCR, followed by automated DNA sequencing. We performed PCR in reactions (25 μl) using Taq DNA polymerase (1 U; Fermentas), 1X buffer, dNTP (0.2 mM) (Amersham), primers (0.5 μM) and DNA (50 ng). PCR conditions were as follows: initial denaturation at 94 C for 3 min followed by 35 cycles of 94 C for 30 sec; 30 sec at 62 C and extension at 72 C for 45 sec, and a final extension for 10 min at 72 C. Exons 7 and 8 of the *SQSTM1* gene were amplified by using, respectively, two pairs of primers located in the flanking introns: 5'-CATGCGTGCTCCCCGACTGT-3'/5'-GCCCTGCAGTG-GAGAACATC-3' for exon 7 and 5'-CTCTGGGCAGGCTCGGA-CAC-3'/5'-CTTGCACCCTAACCCCTGAT-3' for exon 8. Samples were then ExoSap digested (Amersham) and sequenced using the Big Dye Terminator Ready Reaction Kit (Applied Biosystems). Sequencing reactions were performed on a 9700 Thermal Cycler (Applied Biosystems) for 25 cycles of 95 C for 10 sec, 60 C for 5 sec, and 60 C for 2 min. After the sequencing, each reaction was
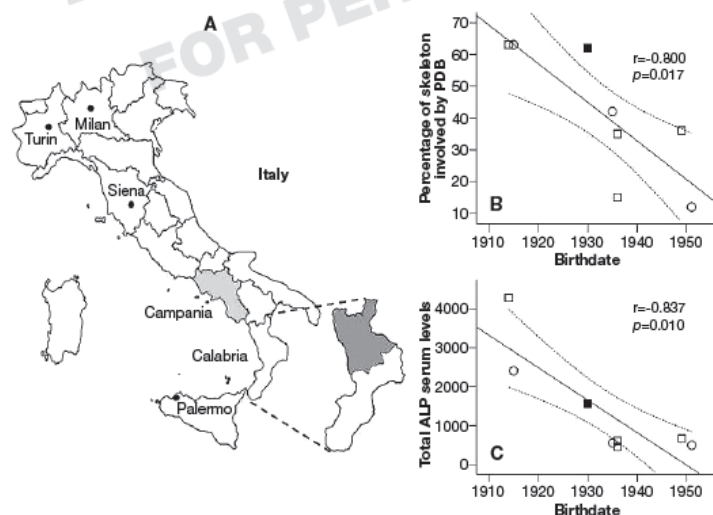

Fig. 1 - A) Map of Italy with identification of cities (Turin, Milan, Siena, and Palermo) where radiographic survey for Paget's disease of bone (PDB) has been performed (10, 11). The high-prevalence region Campania is identified in light gray (12). The territory of Calabria (pertaining to the Local Health Centre, Azienda Sanitaria Locale, ASL Cosenza 1) where this survey was performed, is indicated by dark gray shading in the insert map. B-C) Relationship between birthdates of PDB patients and extension of disease, expressed as percentage of skeleton involved at ⁹⁹ᵐTechnetium methylene disphosphonate bone scan (B) and serum levels of total alkaline phosphatase (ALP) at diagnosis (C). Males: □; females: O; SQSTM-1 ᴹ⁴⁰¹ⱽ: ■.

38

column purified (Amersham) to remove excess dye terminators. Sequencing of the products was performed on the ABI prism 3710 Genetic Analyser (Applied Biosystems) (19). All the remaining exons and intron-exon boundaries of *SQSTM1* gene were also screened in PDB patients without mutations in exons 7 and 8.

Statistical analysis

Statistical analysis was performed using the SPSS© (SPSS Inc., Chicago, IL) statistical package (version 11.5). Pearson's correlation coefficient was used to determine relationships between different parameters. Data are expressed as mean±SD.

RESULTS

We examined the X-ray pelvic films of 1068 consecutive subjects aged >40 yr (mean age 67.3±15.4 yr; body mass index 26.4±3.4 kg/m²). According to Clinical Classification Software [CCS (20)], 113 subjects (10.6%; 68 females) were admitted to the "Spinelli" Hospital for endocrine, nutritional, and metabolic diseases (CCS diagnostic category 3), 262 (33.9%, 211 females) for heart diseases (CCS diagnostic category 7.2), 65 (6.3%, 30 females) for cerebrovascular diseases (CCS diagnostic category 7.3), 30 (2.8%, 20 females) for diseases of veins and lymphatics (CCS diagnostic category 7.5), 238 (22.3%, 94 females) for respiratory diseases (CCS diagnostic categories 8), 178 (16.7%, 98 females) for gastrointestinal, liver, biliary tract, and pancreatic diseases (CCS diagnostic categories 9), and 82 (7.7%, 45 females) for signs, symptoms, factors influencing health care (CCS diagnostic categories 17).
Eight of the 1068 radiographs examined showed evidence of PDB [male:female (M:F) 5:3, mean age 71.6±13.1 yr, range 55-90 yr, body mass index 26.3±2.1 kg/m²]. No patients had a previous diagnosis of PDB. The clinical characteristics of these patients are summarized in Table 1. Pelvic PDB was evident in 7 radiographs (87.5%, M:F 5:2) and unilateral involvement of the pelvis was present in 5

patients (62.5%, M:F 3:2, 4 with involvement of the right pelvis). The only patient without pelvic involvement was a female with PDB involving the 3rd lumbar vertebra and the proximal right femur. All radiographic lesions were predominantly sclerotic in nature. All PDB patients had radiological evidence of osteoarthritis of the hip, and the sacroiliac joints were fused in 1 male. Two PDB cases (25%, 1 M and 1 F) also had vertebral fractures. The crude radiographic prevalence of the disease in subjects aged >40 yr from the analysis of pelvic radiographs was 8 out of 1068 (0.74%). Given that in previous studies the pelvic involvement is commonly described in 60-90% of PDB patients (11, 12), the estimated overall prevalence of PDB in the geographical area examined ranged from 0.82% to 1.21%. The crude prevalence of pelvic PDB in subjects >60 yr of age was 6 out of 723 (0.83%), with an overall estimated prevalence of PDB ranging from 0.92% to 1.38%. Accordingly, the pelvic PDB prevalence in subjects between 40 and 60 yr of age was 2 out of 345 (0.58%), leading to an estimated prevalence of PDB in this age range between 0.64% and 0.97%.
99mTC-MDP bone scan indicated that all patients had polyostotic PDB. The number of affected sites was 4.0±2.2 and PDB patients from Calabria showed a prevalent involvement of the skull (4/8, 50%) and the spine (5/8, 62.5%). The mean serum levels of total alkaline phosphatase in these patients were 1381±1359 U/l (median value 638 U/l; range 458-4285 U/l).
From the analysis of questionnaires, only 1 patient had a positive family history of PDB was (namely the sister of 1 male participant). However, his clinical data were not utilized in this study because the diagnosis was formulated *post mortem*, based on case file analyses. All patients referred animal contacts and the recurrent use of un-pasteurized milk and of fresh, homemade meat products without sanitary control.
As reported in Figure 1, we found a negative relationship between the birthdates of pagetic patients and the

Table 1 - *Clinical characteristics of patients with Paget's disease of bone (PDB) from Calabria, Southern Italy.*

P	Birthdates	S	tALP U/l	PDB extension	Familial PDB	SQSTM1 mutation	PDB complications	Co-morbidity
1	1914	M	4285	63%	No	No	Hearing loss, bone deformity, osteoarthritis	Hypertension, DM, atherosclerosis
2	1915	F	2410	63%	No	No	Bone deformity, osteoarthritis	Hypertension, DM, atherosclerosis
3	1930	M	1567	62%	Yes	M401V	Hearing loss, bone deformity, osteoarthritis	Hypertension, MI, Prostate cancer, HCV infection
4	1935	F	556	42%	No	No	Osteoarthritis, nephrolithiasis	Hypertension, DM, atherosclerosis, HCV infection
5	1936	M	458	35%	No	No	Osteoarthritis, vertebral fracture	DM
6	1936	M	611	22%	No	No	Osteoarthritis	Multinodular non-toxic goiter
7	1949	M	665	36%	No	No	Osteoarthritis	Hypertension, WPW syndrome
8	1951	F	498	15%	No	No	Osteoarthritis, vertebral fracture	Hypertension, obesity

P: patient; S: sex; M: male; F: female; tALP: total alkaline phosphatase serum levels (normal range 98-275 U/l); *SQSTM1*: sequestosome 1; DM: diabetes mellitus; MI: myocardial infarction; WPW: Wolf-Parkinson-White; HCV: hepatitis C. PDB extension was evaluated based on 99mTechnetium methylene diphosphonate bone scan according to criteria proposed by Davies et al. (17); PDB complication were evaluated according to criteria proposed by White (1).

extension of the disease, expressed as percentage of skeleton involved at the ^{99m}TC-MDP bone scan (r=—0.800; p=0.017), and as serum levels of total alkaline phosphatase (r=—0.837; p=0.010).

In the PDB patient with familial disease (PDB localization: skull, right scapula, right and left humerus, lumbar vertebrae L3-L5, pelvis, sacrum, right and left femur, and right tibia) we detected a novel mutation (M401V) in the exon 8 of the gene (Fig. 2). The mutation caused a missense changing an ATG encoding methionine at codon 401 to GTG that encoded a valine (M401V). This mutation was confirmed in two separate analyses for this patient and was not observed in controls subjects. No further mutations in the *SQSTM1* gene were evidenced.

DISCUSSION

The PDB epidemiological surveys in Europe, mostly based on populations of British descent (21-23), have shown that the disorder is more prevalent in Britain and France than in other European countries, with localized areas of high prevalence being described in Lancashire, United Kingdom, Sierra de La Cabrera district, Spain, and Campania, Southern Italy (24-26). Moreover, PDB patients living or who had lived in rural areas of Campania also show an earlier onset of the disease, an increased skeletal involvement, and a higher tendency to neoplastic degeneration compared to PDB cases from other parts of Italy (12). In particular, the evidence of a familial predisposition for the occurrence of neoplastic complications in this disease (osteogenic sarcoma, giant cell tumor) is virtually limited to PDB patients with family disease from Campania (12, 27-33).

To the best of our knowledge, the present epidemiological survey is the first specifically performed in a rural area of Southern Italy. The radiological prevalence rates observed in this study range between those previously observed in Italy (10, 11). Indeed, the overall age of population analyzed in the current study was lower than in the previous studies, despite a similar or slightly higher estimated prevalence of disease. This might indicate an higher prevalence of PDB in this rural area of Calabria. Moreover, prevalence rates in younger individuals (age range 40-60 yr) were consistently higher in this study than in the previous surveys in Turin and Siena (11), suggesting an earlier onset of disease in this region, even in PDB subjects without *SQSTM1* mutations. Importantly, an increased clinical severity, with a more prevalent involvement of the skull and the lumbar spine, was observed in the 8 identified subjects with PDB of the present study, with respect to PDB cases from different Italian areas (10-12, 26, 34, 35). Moreover, all patients were affected by polyostotic PDB. All the above clinical characteristics appear remarkably similar to those previously described in rural areas of Campania (12). From the early 12th century, Campania and Calabria share the same historical events. Both areas are characterized by a virtual lack of immigration: only in the second half of the 15th century Albanian mercenaries were recruited by the king of Naples and settled in several villages of Southern Appenines (36, 37). Another immigration flux to Calabria, represented mostly by people coming from the former Communist block and northern Africa, has been observed only in the last two decades (data available at: http://www.istat.it/english/annualreport.html). The geographical area analyzed shares also a similar lifestyle and dietary
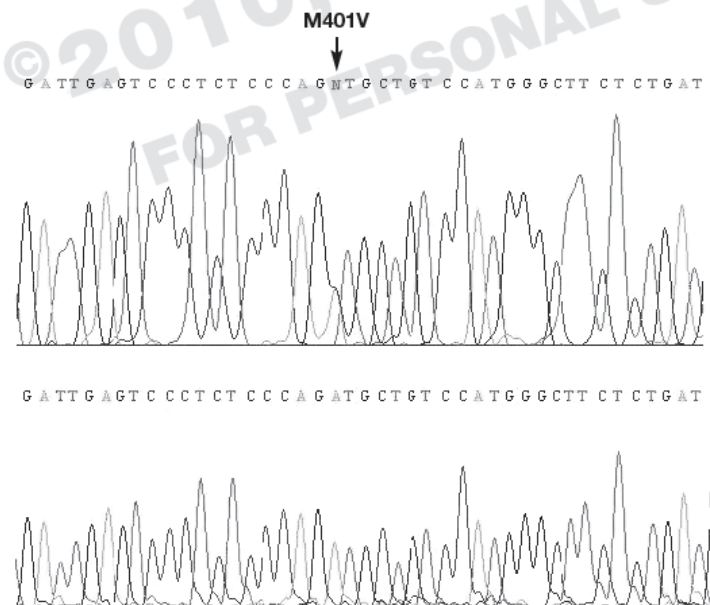

Fig. 2 - *Sequence chromatogram of the sequestosome 1 gene mutations. The mutation is indicated by a black arrow.*

Homo sapiens	GLKEAALYPHLPPEADPRLIESLSQMLSMGFSDEGGWLTRLLQTKNYDIGAALDTIQYSK
Pan troglodytes	GLKEAALYPHLPPEADPRLIESLSQMLSMGFSDEGGWLTRLLQTKNYDIGAALDTIQYSK
Pongo pygmaeus	GLKEAALYPHLPPEADPRLIESLSQMLSMGFSDEGGRLTRLLQTKNYDIGAALDTIQYSK
Macaca mulatta	GLKEAALYPHLPPEADPRLIESLSQMLSMGFSDEGGWLTRLLQTKNYDIGAALDTIQYSK
Bos taurus	GLKEAALYPHLPPEADPRLIESLSQMLSMGFSDEGGWLTRLLQTKNYDIGAALDTIQYSK
Equus caballus	GLKEAALYPHLPPEADPRLIESLSQMLSMGFSDEGGWLTRLLQTKNYDIGAALDTIQYSK
Ovis aries	GLKEAALYPHLPPEADPRLIESLSQMLSMGFSDEGGWLTRLLQTKNYDIGAALDTIQYSK
Mus musculus	GLKEAALYPHLPPEADPRLIESLSQMLSMGFSDEGGWLTRLLQTKNYDIGAALDTIQYSK
Rattus norvegicus	GLKEAALYPHLPPEADPRLIESLSQMLSMGFSDEGGWLTRLLQTKNYDIGAALDTIQYSK
Gallus gallus	GLKEAALYPHLPPEADPRLIESLSQMLSMGFSDEGGWLTRLLQTKNCDIGAALDAIQYSK
Monodelphis domestica	GLKEAALYPHLPPEADPCLIESLSQMLSMGFSDEGGWLTKLLQTKNYDIGAALDAIQYSK
Ornithorhynchus anatinus	GLKEAALYPHLPPEADPRLIESLSQMLSMGFSDEGGWLTRLLQTRNCDIGAALDAIQYSK
Danio rerio	GLKEAALYPHLPQDADPRLVESLSQMLSMGFTDEGGWLTRLLHTKNYDIGGALDTIQYSK
Tetraodon nigroviridis	GLKEAALYPHLPKEADPRLVESLAQMLSMGFTDEGGWLTRLLQAKNFDIGAALDAIQFAR
Salmo salar	TLAKAAAYPHLPQDADPRLVESLSQMLAMGFTDEGGWLTRLLHTKDCDVGAALDTIHYSK
Xenopus laevis	GLKEAALYPHLPPEADPRLIETLSQMLSMGFSDEGGWLTRLLEAKQYDIGSALDAMQSIR

Fig. 3 - *Alignment of the amino-acid sequences of the ubiquitin-associated domain (residues 387-436) of the sequestosome 1 protein along the evolutive scale. The conserved methionine is indicated by a black arrow.*

habits with Campania region: both showed a similar gross domestic product for each habitant (GDP/head), expressed as purchasing power parity (PPP) (data available at: http://ec.europa.eu/regional_policy/newsroom/pdf/map_gdp2005.pdf).

Importantly, an high severity of disease was observed in all patients in our study, independently of the presence of *SQSTM1* mutations. Conversely, other studies evidenced a reduced clinical severity of disease in PDB subjects without *SQSTM1* mutations (38). This might imply the presence of mutations in different genes as well an increased persistence of a possible environmental trigger in our cases, or both these conditions. The latter hypothesis is further supported by the analysis of lifestyle and dietary habits, since all PDB patients identified in this study reported frequent animal contacts and the use of unpasteurized milk or fresh, homemade meat products without sanitary control. Even though we did not specifically perform our questionnaires in a control group in these study, previous Italian reports indicated a lower frequency of animal contacts or use of homemade products (10-12). All these findings are in keeping with an important role of the environment in the pathogenesis of PDB. It is likely that exposure to an environmental trigger (i.e., viral infection) may facilitate (and even anticipate) the expression of the disease as well as increase its severity. On the other hand, the inverse relationship between the extension of the disease and the birthdates of PDB patients which suggests a slight decrease in the clinical expressiveness of the disease, could be due to a reduced exposure to this environmental trigger (i.e., increased prevalence of anti-paramyxovirus vaccination in humans and animals, or decreased use of food products without sanitary control, or improved sanitary standards). The number of PDB cases analyzed in this study, however, was low, and the mean number of affected sites remained above the mean value observed in other Italian regions even in the youngest PDB cases. Further studies are clearly needed to address these issues.

Our radiological survey also confirmed that PDB is slightly more common in men, that osteoarthritis is a frequent complication of PDB and that the right side of the body appears affected by PDB more than the left, in keeping with previous observations (39). The reasons underlying these trends are actually unknown and deserve further investigation in larger samples.

Although only 8 PDB patients were investigated, genetic analysis confirmed the association between disease and mutations in the *SQSTM1* gene, particular in familial cases, in agreement with previous evidences from other geographical areas (38, 40-43). Importantly, we detected a new *SQSTM1* missense mutation (M401V) that is associated with a remarkably aggressive form of PDB, involving 12 affected sites. The observed clinical severity of disease in this subject, as well as the high degree of conservation of the methionine in position 401 (from fish to humans, as shown in Figure 3), strongly argues in favor of an important role of this amino-acid in the function of p62 protein. It is likely that the first helix of the 3-helix bundle that comprises the globular ubiquitin-associated domain, would be perturbed by the valine substitution (Fig. 4).

The major strengths of our study are the large sample of pelvic radiographs, the restricted geographical area,
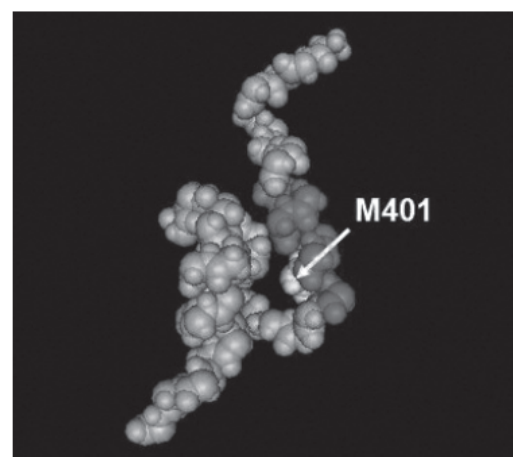

Fig. 4 - *Nuclear magnetic resonance structure of the ubiquitin-associated domain of the sequestosome 1 protein showing missense mutation site. The M401V aminoacid substitution is localised in first part of an alpha-helix.*

D. Rendina, F. Gianfrancesco, G. De Filippo, et al.

and the homogeneous origins and lifestyles of the study population. The Italian National Health Service is organized in local health units, each corresponding to restricted geographical areas. The "Spinelli" Hospital operates within the local health unit Cosenza 1 (Fig. 1), and offers therapeutic health care services for hospitalized patients in general medicine and cardiology. In the examined local health unit, the density population is almost 140 inhabitants/km², and the hospitalization rates (no. of in-hospital admissions/1000 inhabitants/yr) were 155, 299, and 390, respectively for adults <64 yr, from 65-75 yr and >75 yr old (data available at: http://www.ministerosalute.it/servizio/sezSis.jsp?label=usl). From the analysis of in-hospital admissions (reported in Table 1) and the hospitalization rates in this geographical area, it can be assumed that our study population is to a reasonable extent representative of the entire middle-aged and elderly population living within the area of local health unit Paola-Cosenza 1 from 2002 to 2006 (44).

In conclusion, this study confirmed and extended our preliminary reports, indicating that patients with PDB from rural districts of Southern Italy (such as Campania and Calabria) show an earlier onset of disease and an increased clinical severity that appear in great part independent from the presence of *SQSTM1* mutations. Extended clinical and genetic analysis of a larger number of PDB patients from these areas may prove extremely useful in the near future for a better understanding of the environmental and genetic mechanisms underlying PDB.

ACKNOWLEDGMENTS

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43

STUDY AIM # 2:

New susceptibility genes and their potential interaction with *SQSTM1* on PDB phenotype

We identified two non synonymous single nucleotide polymorphisms (SNPs) (C421T, H141Y and T575C, V192A) in the *TNFRSF11A* gene, associated with PDB and with the severity of phenotype in a large population of 654 unrelated patients that were previously screened for *SQSTM1* gene mutations. The largest effect was found for the T575C variant, yielding an odds ratio of 1.29 (p=0.003), with the C allele as the risk allele. Moreover, an even more significant p-value (p=0.0002) was observed in the subgroup of patients with *SQSTM1* mutation, with an odds ratio of 1.71. Interestingly, patients with the C allele also showed an increased prevalence of polyostotic disease (68%, 53%, and 51% in patients with CC, CT, and TT genotypes, respectively; p=0.01), as well as an increased number of affected skeletal sites. These differences increased when analyses were restricted to cases with *SQSTM1* mutation. In human cell lines, cotrasfection with mutated *SQSTM1* and TNFRSF11AA192 produced a level of activation of NFkB signaling greater than cotrasfection with wild-type *SQSTM1* and TNFRSF11AV192, confirming our genetic and clinical evidences. Overall, these results provide the first evidence that genetic variation within the OPG/RANK/RANKL system influences the severity of PBD in synergistic action with *SQSTM1* gene mutations (Publication C). These result were also presented as an oral communication at the 32nd Annual Meeting of the American Society for Bone and Mineral Research in Toronto in 2010 [D Merlotti, F Gianfrancesco, L Gennari, D Rendina, M Di Stefano, G Mossetti, S Gallone, T Esposito, S Magliocca, D Formicola, A Mingione, P Fenoglio, A Criasia, R Muscariello, P Strazzullo, G Isaia, R Nuti. *TNFRSF11A* gene allelic variants are associated with Paget's Disease of bone and interact with *SQSTM1* mutations to cause the severity of the disorder. 32nd Annual

Meeting of the American Society for Bone and Mineral Research, Toronto, Canada, 15-19 October 2010. Abstract 1033. J.BoneMinerRes 25 (Suppl.1)].

Within specific aim 2, we recently initiated a collaboration to perform a genome wide association study in a large PDB cohort of cases from different Countries worldwide (Genetic Determinants of Paget's Disease Consortium). In a first analysis we identified three new loci and confirmed their association with PDB in 2,215 affected individuals (cases) and 4,370 controls from seven independent populations. The new associations were with rs5742915 within PML on 15q24 (odds ratio (OR) = 1.34, P = 1.6×10^{-14}), rs10498635 within RIN3 on 14q32 (OR = 1.44, P = 2.55×10^{-11}) and rs4294134 within NUP205 on 7q33 (OR = 1.45, P = 8.45×10^{-10}). These data also confirmed a previous association of TM7SF4 (rs2458413, OR = 1.40, P = 7.38×10^{-17}) with PDB. The previous associations between variants at the *CSF1*, *OPTN* and *TNFRSF11A* loci and PDB were also confirmed by the meta-analysis of the combined dataset for these loci (Fig. 6).
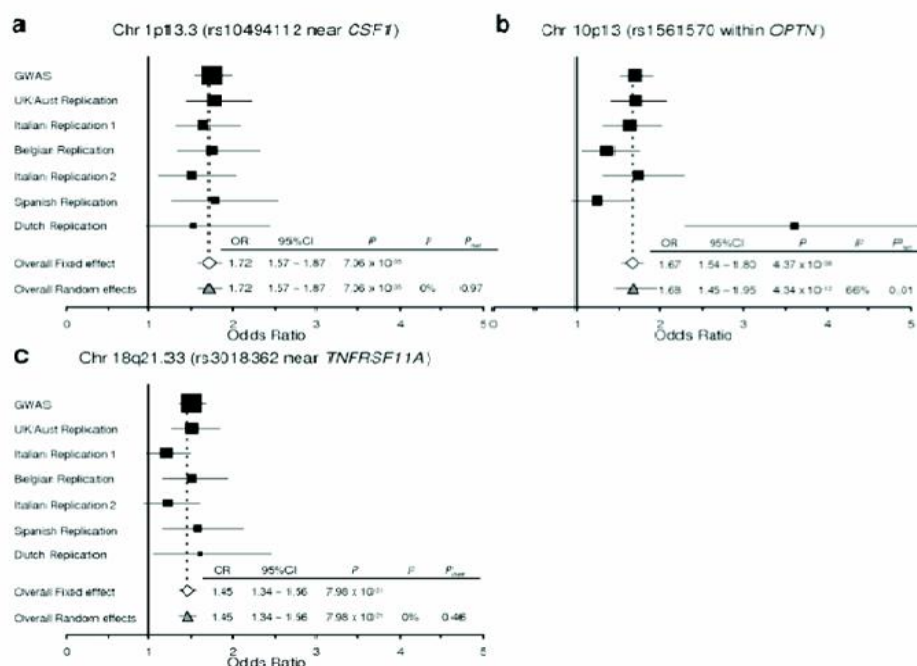
Fig. 6. Replication of association for the identified loci at *CSF1* (a), *OPTN* (b) and *TNFRSF11A* (c): forest plots of overall effect size for SNPs associated with PDB risk estimated by meta-analysis of the GWAS sample and six replication cohorts.

Overall, these seven loci explained 13% of the familial risk of PDB. (Publication D).

Since the results from this recent genome-wide-association study evidenced a particularly higher association between a polymorphic variation (rs1561570) in *OPTN* gene (Fig. 7) and PDB in 2 different replication cohorts of patients from Italy we performed an additional study in a larger cohort 680 Italian cases previously screened for *SQSTM1* mutations of Italian PDB cases . 200 age and sex-matched controls were also genotyped for comparison.
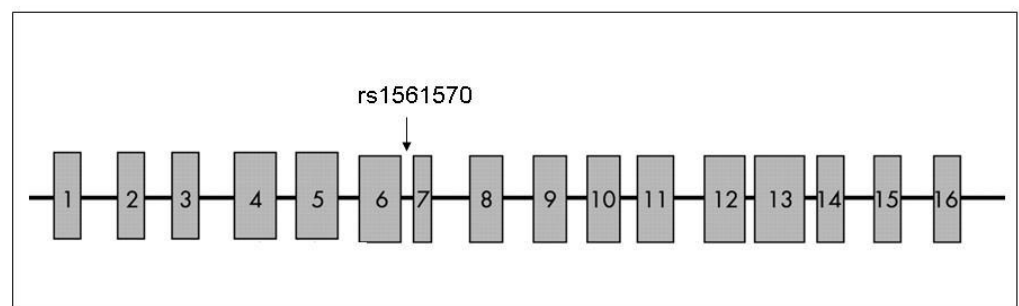
Fig. 7. *OPTN* gene.

The potential interactions with the *TNFRSF11A* polymorphism (rs1805034) previously associated with PDB severity were also explored. In the overall population we observed an increased prevalence of rs1561570 "T" allele in PDB patients than in controls (OR 1.6; p<0.01). This association was higher in sporadic (OR 1.8) than in familial cases (OR 1.5), while became non-significant in familial PDB cases without *SQSTM1* mutation (Fig.8).
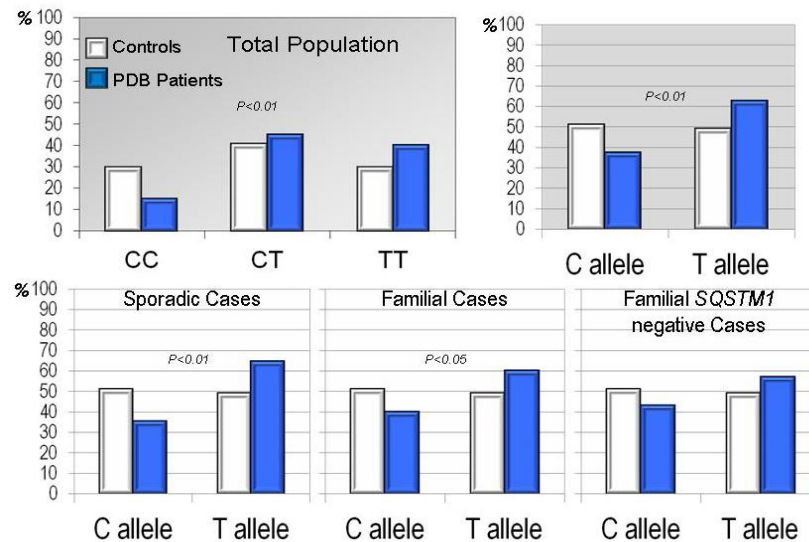
Fig. 8. Prevalence of rs1561570 Alleles of *OPTN* gene.

In contrast to the *TNFRSF11A* "C" variant, which was associated with increased disease severity in both *SQSTM1* negative or positive patients, the *OPTN* variant did not appear to interact with *SQSTM1*. In fact, the presence of the *OPTN* risk allele (T) was significantly associated with an early onset and an increased number of affected sites only in *SQSTM1* negative patients, and particularly in sporadic cases (Table 3).

	Total PDB	WT[SQSTM1 neg]	SQSTM1+
Age of Onset (yrs)			
- CC	56.5±11.7	56.1±12.9	52.6±15.5
- CT	60.4±13.0	61.0±10.3	58.4±9.6
- TT	57.7±10.3	59.9±10.8*	53.2±11.1
Affected skeletal Sites (n)			
- CC	2.9±1.8	2.5±1.4	4.0±2.3
- CT	2.5±1.7	2.3±1.2	3.5±2.7
- TT	2.9±2.1	2.7±0.9*	3.6±2.7

*$P<0.05$ ANOVA

Table 3. Association between clinical characteristics and *OPTN* gene alleles.

Of interest, we observed a particularly higher prevalence of haplotype CC-TT (containing the homozygous risk alleles for both *TNFRSF11A* and *OPTN*, respectively) in sporadic than familial cases or controls (49% vs. 33% vs. 3% in sporadic, familial PDB and controls, respectively; $p<0.01$) (Fig. 9).
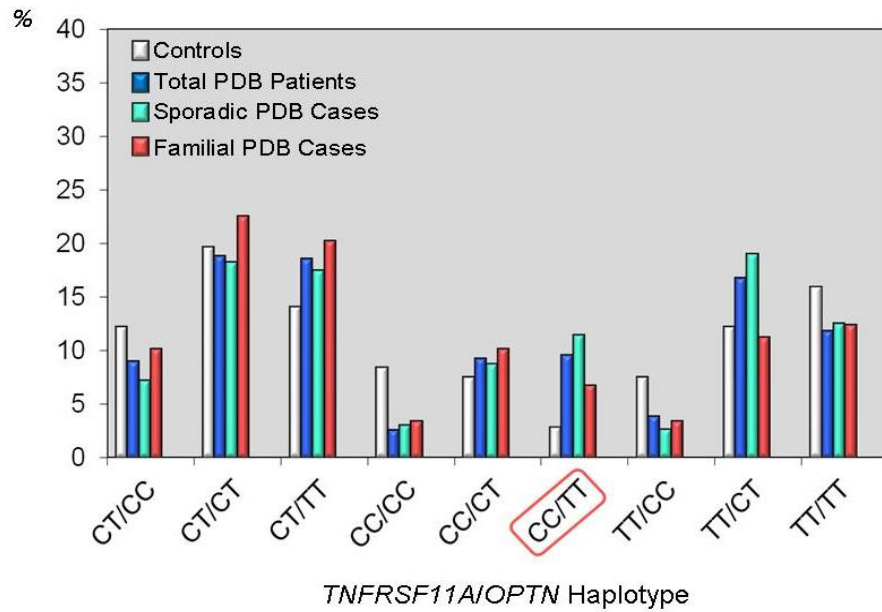
Fig. 9. Haplotype frequencies of TNFRSF11A and *OPTN* SNPs.

Moreover, sporadic *SQSTM1*-negative cases with CC-TT haplotype showed a higher number of affected sites and an earlier age at diagnosis than *SQSTM1*-negative cases with the other haplotypes (Fig. 10-11).
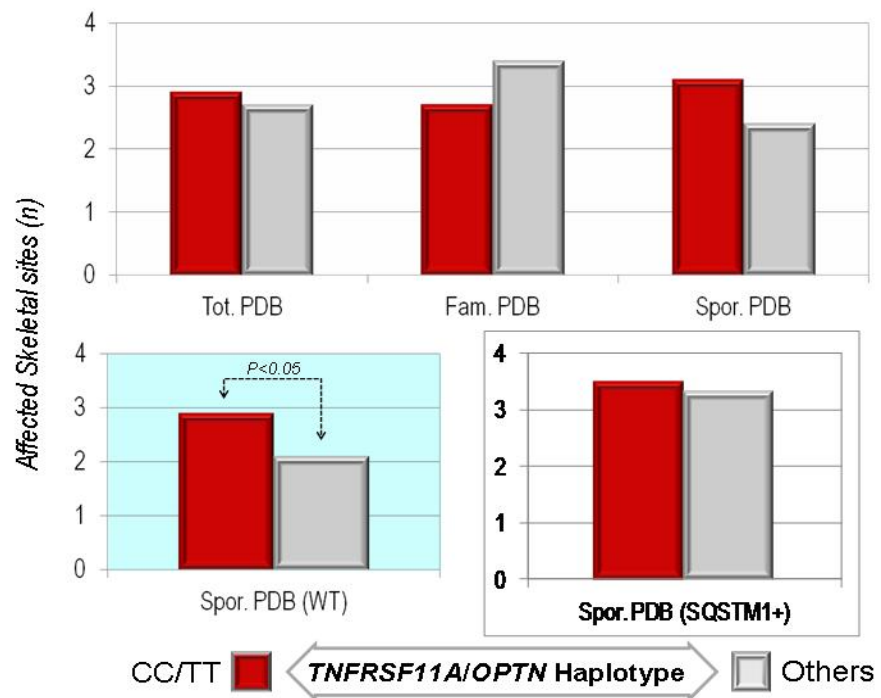
Fig. 10. Association between haplotype frequencies of TNFRSF11A and *OPTN* genes and number of affected sites.
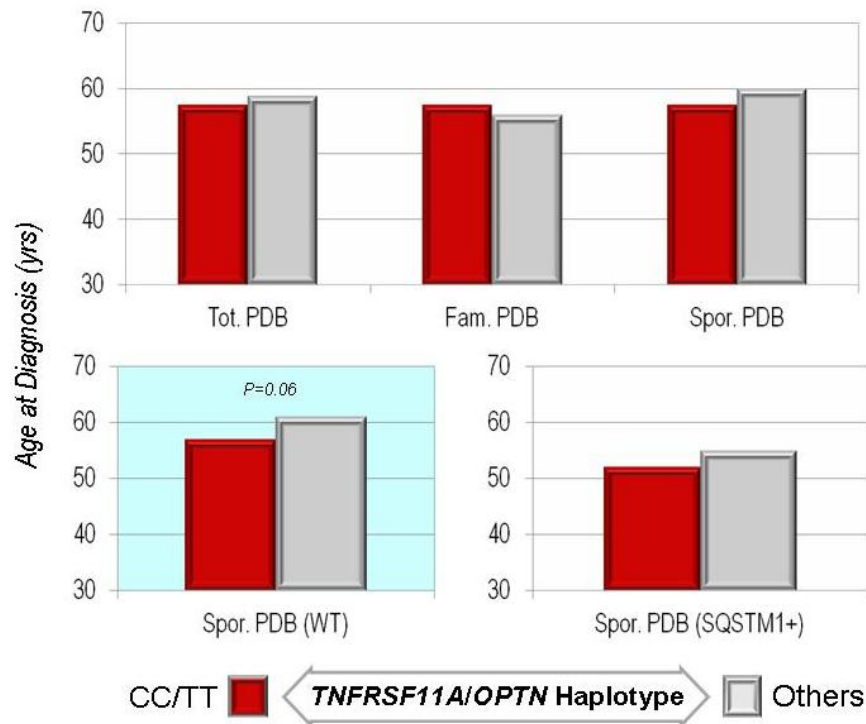
Fig. 11. Association between haplotype frequencies of TNFRSF11A and *OPTN* genes and age at diagnosis.

In summary, this study provides evidence that this *OPTN* variant affects the susceptibility to develop PDB and interacts with rs1805034 polymorphism in *TNFRSF11A* to cause the severity of the disorder in sporadic cases. A different susceptibility gene is probably involved in *SQSTM1* negative families. These results have been recently presented as poster presentation at the ASBMR 2012 Meeting in Minneapolis [Merlotti D, Gianfrancesco F, Gennari L, Rendina D, Di Stefano M, Gallone S, Esposito T, Magliocca S, Formicola D, Mingione A, Muscariello R, Strazzullo P, Isaia G, Nuti R. Interaction Between *OPTN* and *TNFRSF11A* gene variants in sporadic Paget's Disease Of Bone. ASBMR 2012 Annual Meeting, 12-15 October 2012 Minneapolis, Minnesota, USA, Abstract MO0420]

Publication C

A Nonsynonymous *TNFRSF11A* Variation Increases NFκB Activity and the Severity of Paget's Disease

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ABSTRACT

Mutations in the *SQSTM1* gene were identified as a common cause of Paget's disease of bone (PDB) but experimental evidence demonstrated that *SQSTM1* mutation is not sufficient to induce PDB in vivo. Here, we identified two nonsynonymous single nucleotide polymorphisms (SNPs) (C421T, H141Y and T575C, V192A) in the *TNFRSF11A* gene, associated with PDB and with the severity of phenotype in a large population of 654 unrelated patients that were previously screened for *SQSTM1* gene mutations. The largest effect was found for the T575C variant, yielding an odds ratio of 1.29 ($p = 0.003$), with the C allele as the risk allele. Moreover, an even more significant p-value ($p = 0.0002$) was observed in the subgroup of patients with *SQSTM1* mutation, with an odds ratio of 1.71. Interestingly, patients with the C allele also showed an increased prevalence of polyostotic disease (68%, 53%, and 51% in patients with CC, CT, and TT genotypes, respectively; $p = 0.01$), as well as an increased number of affected skeletal sites (2.9, 2.5, and 2.0 in patients with CC, CT, and TT genotypes, respectively, $p = 0.008$). These differences increased when analyses were restricted to cases with *SQSTM1* mutation. In human cell lines, cotrasfection with mutated *SQSTM1* and *TNFRSF11A*[A192] produced a level of activation of NFκB signaling greater than cotrasfection with wild-type *SQSTM1* and *TNFRSF11A*[V192], confirming genetics and clinical evidences. These results provide the first evidence that genetic variation within the OPG/RANK/RANKL system influences the severity of PBD in synergistic action with *SQSTM1* gene mutations. © 2012 American Society for Bone and Mineral Research.

KEY WORDS: SEVERITY OF PAGET'S DISEASE OF BONE; GENE–GENE INTERACTION; *TNFRSF11A* GENE; NFκB ACTIVITY; V192A

Introduction

Paget's Disease of Bone (PDB) (OMIM 602080) is the second chronic metabolic bone disorder after osteoporosis, and typically results in enlarged and deformed bones in one (monostotic form) or more (polyostotic form) regions of the skeleton.[1,2] The osteoclasts in affected bone are increased in size and contain many more nuclei than normal osteoclast.[2]

Moreover, they exhibit enhanced responsiveness to RANKL, tumor necrosis factor (TNF)-α, and 1,25-dihydroxy vitamin D.[1]

PDB lesions are characterized by increased bone resorption, which is accompanied by other abnormalities such as marrow fibrosis, increased vascularity of bone, and increased but disorganized bone formation. These abnormalities disrupt normal bone architecture and lead to various complications such as bone pain, bone deformity, fractures, osteoarthritis,

deafness, nerve compression syndromes, and in rare occasions, osteosarcoma.

The etiology of PDB has remained largely unknown for several decades. Familial clustering has been recognized to occur in up to 40% of cases, and epidemiologic studies indicated that the relative risk of PDB in first-degree relatives of patients is 7 to 10 times greater than the general population.[3–6] Pedigree analysis indicated an autosomal dominant pattern of inheritance with variable penetrance, and initial linkage or genome-wide studies evidenced seven susceptibility loci.[7] In 2002, mutations affecting the ubiquitin-associated domain of SQSTM1 have been identified in up to 10% and 40% of sporadic and familial PDB cases, respectively.[7–9] This gene encodes the p62 protein, which acts as a scaffold protein in the NFκB pathway as well as an intermediate protein in the proteosomal degradation of polyubiquitinated proteins. Although the p62 mutations increased the osteoclastogenic potential of both osteoclast precursors and the marrow microenvironment,[10,11] these changes were not sufficient to induce the complete development of PDB phenotype in some animal models.[1,12–14] Moreover, the prevalence of SQSTM1 mutations is low in sporadic PDB cases, and even in PDB families with SQSTM1 mutations, some affected relatives without the mutation were described, suggesting that additional factors (either genetic or exogenous) may be associated with disease expression.[15,16] Thus, it is possible that mutations or polymorphisms in more than one gene may contribute to the development of PDB, which could also account for the variable penetrance and disease severity found even within PDB families with the same SQSTM1 mutation.[16–18]

Several other candidates have been investigated in relation to PDB, and most attention has focused on genes encoding RANK (TNFRSF11A), RANKL (TNFSF11A), and OPG (TNFRSF11B), because they play a major role in the regulation of osteoclastogenesis via NFκB signaling. In particular, after the discovery of TNFRSF11A gene mutations in PDB-like syndromes (familial expansile osteolysis and expansile skeletal hyperphosphatasia) and in a case of early-onset PDB,[19–22] attention focused on the possibility that TNFRSF11A might contribute to classical PDB, but mutation screening yielded negative results.[19,23,24] More recent studies, including a genome-wide association analysis, suggested that polymorphisms within the TNFRSF11A gene locus may indeed be associated with PDB.[25–27] However, this association has to be replicated in large samples, and the possible underlying molecular mechanisms remains to be investigated. Moreover, in all the previous studies PDB patients with the SQSTM1 mutation were excluded from analysis, leaving us with the question of whether TNFRSF11A variants would have any effect on the phenotype in patients with the SQSTM1 mutation.

In this study we investigated TNFRSF11A variants in a large Italian cohort of sporadic and familiar PDB patients who were previously screened for SQSTM1 gene mutations.[16] This cohort showed a higher heterogeneity of SQSTM1 mutations than observed in other European populations or French–Canadian population. Moreover, even though subjects with SQSTM1 mutations generally showed an increased disease severity, some familial cases with early-onset PDB and a very aggressive phenotype (including the occurrence of giant cell tumors) were negative for the presence of the SQSTM1 mutation.[16] To provide insights on the underlying molecular mechanism and identify the causative single nucleotide polymorphism (SNP) within the TNFRSF11A gene, functional in vitro experiments were also performed.

Materials and Methods

Study population

The participants in this study comprised 654 unrelated and consecutive PDB subjects from different Italian regions, recruited at the Bone Disease Units of Turin, Siena, and Naples. General characteristics of recruited patients and the results of SQSTM1 mutation screening were previously reported.[16] Diagnosis of PDB was based on biochemical evaluation, bone scintigraphy, and subsequent X-ray examination of areas of increased isotope uptake. For all subjects, a detailed medical history was obtained, including family history, skeletal extent, complications, age at onset of PDB symptoms, and age of diagnosis. The study was approved by local ethical committees, and all subjects had given informed consent to being included. All data were collected through common questionnaires shared by all participating centers. The patients having previous report of at least one other family member affected with PDB were defined as familial cases. Patients with negative history were classified as sporadic PDB.

A control population of 500 age and sex-matched individuals of Italian ancestry was also investigated. These individuals were randomly selected from a population-based cohort ($n = 1100$) obtained with the collaboration of the general practitioners who randomly invited subjects aged between 45 and 80 years in their database to undergo a clinical screening for bone and mineral metabolism. All subjects had no indication of PDB-like symptoms, nor a positive family history for PDB. In order to exclude potential asymphomatic PDB cases, the bone resorption markers alkaline phosphatase (Tandem-R Ostase, Beckman Coulter Inc., Fullerton, CA, USA) and serum type I collagen C-telopeptides (CrossLaps RIA, crosslaps, Fan tee) were assessed in all controls. After bone turnover assessment, three individuals with elevated alkaline phosphatase and CTX levels were further investigated, and two of them were excluded from analysis because of asymphomatic PDB. Genetic analysis was completed in 496 controls.

A subset of first-degree family members previously screened for the SQSTM1 mutation were also investigated for the two selected TNFRSF11A polymorphisms.

Sequencing analysis of TNFRSF11A

We conducted mutation screening of all the 10 exons of TNFRSF11A and their intron–exon boundaries using polymerase chain reaction (PCR), followed by automated DNA sequencing. PCR reactions (25 μL) were performed using Taq DNA polymerase (1 U; Fermentas, Glen Burnie, MD, USA), 1× buffer, deoxynucleoside triphosphate (dNTP, 0.2 mM; Amersham, Uppsala, Sweden), primers (0.5 μM), and DNA (50 ng). PCR conditions were as follows: initial denaturation at 94°C for

3 minutes, followed by 35 cycles of 94°C for 30 seconds, 30 seconds at annealing temperature, and extension at 72°C for 45 seconds, and a final extension for 10 minutes at 72°C. Samples were ExoSap-digested (Amersham) and sequenced using the Big Dye Terminator Ready Reaction Kit (Applied Biosystems, Foster City, CA, USA). Sequencing reactions were performed on a 9700 Thermal Cycler (Applied Biosystems) for 25 cycles of 95°C for 10 seconds, 50°C for 5 seconds, and 60°C for 2 minutes. After the sequencing, each reaction was column-purified (Amersham) to remove excess dye terminators. Sequencing of the products was performed on the ABI Prism 3710 Genetic Analyser (Applied Biosystems).

RFLP analysis of SNPs within the *TNFRSF11A* region

SNPs within the *TNFRSF11A* region were analyzed by restriction fragment-length polymorphism (RFLP) analysis. C421T polymorphism (rs35211496, H141Y) was amplified by PCR using genomic DNA as templates and a set of primers (EX4F primer: 5′-CT-GGAGCCAGGACTGCGAGT-3′ and primer EX4R 5′-TTGCCCGCA-GTCCCTTTGAA-3′), which amplified a DNA fragment of 293 base pair (bp) in size. Becase the C to T variation creates a BseNI endonuclease recognition site in position 65 of the PCR product, we determined the allele frequencies for this polymorphism by BseNI digestion and 3% agarose gel electrophoresis. The presence of T575C polymorphism (rs1805034, V192A) was performed by amplifying the exon 6 of *TNFRSF11A* by PCR using a set of primers (EX6F: 5′-TGCTGTTCTCCTCCCTGTGG-3′ and EX6R: 5′-AGGGATCTAAACCAGCTGAC-3′), which amplified the 323-bp DNA fragment. Because the T to C variation creates an endonuclease SsiI recognition site in position 216 of the PCR product, we determined the allele frequencies for this polymorphism by reamplification of exon 6, after SsiI digestion and 3% agarose gel electrophoresis. The rs3018362 polymorphism located 29.826 bp from TGA of *TNFRSF11A* gene was amplified by PCR using a set of primers (rs3018362F: 5′-TCTTTTAG-CATCTGGGTCAT-3′ and primer rs3018362REX4R 5′-TTCATT-CATGTCTGGGTGTA-3′), which amplified a DNA fragment of 270 bp in size. Because the A to G variation creates an MaeII endonuclease recognition site in position 183 of the PCR product, we determined the allele frequencies for this polymorphism by MaeII digestion and 3% agarose gel electrophoresis. The rs2957128 polymorphism located 8.467 bp from TGA of *TNFRSF11A* gene was amplified by PCR using a set of primers (rs2957128F: 5′-TGTTGGAGTTTGTAGACGAC-3′ and primer rs2957128R 5′-GGTCGGGTAAGGTTCATAA-3′), which amplified a DNA fragment of 200 bp in size. Because the A to G variation creates a Esp3I endonuclease recognition site in position 149 of the PCR product, we determined the allele frequencies for this polymorphism by Esp3I digestion and 3% agarose gel electrophoresis.

Statistical analysis

Hardy-Weinberg equilibrium (HWE) was calculated with the Finetti program (http://ihg2.helmholtz-muenchen.de/cgi-bin/hw/hwa1.pl). Associations between single SNPs and the disease were analyzed using the Armitage's trend test (ATT). Odds ratios (ORs) and 95% confidence intervals (95% CI) in the ATT were also calculated with respect to the tested allele. A p-value below 0.025 was considered for statistical significance applying the Bonferroni correction for multiple testing. The Haploview software package (http://www.broadinstitute.org/haploview) was used to estimate pairwise linkage disequilibrium. Haplotype analyses were constructed from population genotype data using the Estimate Haplotype (EH) program available to http://linkage.rockefeller.edu/ott/eh.htm. Genotype–phenotype analysis was performed using analysis of variance (ANOVA) for continuous variables and chi-squared test or the Fisher exact test for categorical variables, whichever was appropriate.

Plasmid constructs

The generation of pcDNA3.1/FLAG-*SQSTM1* (wild-type and mutated) plasmids has been reported previously.[28–30] The insert of pcDNA5/FRT/FLAG-*TNFRSF11A* (V192), was excised by BamHI/NotI restriction enzyme digestions and cloned in BamHI/NotI pcDNA3.1 vector to give the expression plasmid pcDNA3.1/FLAG-*TNFRSF11A* (V192). This construct served as a template for mutagenesis reactions. For the preparation of A192 *TNFRSF11A* construct, primers were designed to introduce the following mutations: a T to C at nucleotide +575 from the ATG (V192A). Primer sequences were as follows:

T575C forward 5′-GACAGAGAAATCCGATGcGGTTTGCAGTTC-TTCTC-3′, T575C reverse 5′-GAGAAGAACTGCAAACCgCATCGG-ATTTCTCTGTC-3′. The V192A variant was introduced into the pcDNA3.1/FLAG *TNFRSF11A* (V192) plasmid using the Quik-Change site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA), according to the manufacturer's instructions. The NFκB luciferase reporter (Igκ-Luc) gene used in this study has been reported previously.[31]

Luciferase NFκB reporter assay

HEK293 cell line

For luciferase experiments in HEK293 lines, cells were seeded in 24-well plates before transfection. Transfections were carried out when cells reached ~80% confluence using DOTAP Liposomal Transfection Reagent (Roche, Indianapolis, IN, USA) according to the manufacturer's instructions. Cells were cotransfected with 2 μg pcDNA3.1, pcDNA3.1/FLAG *SQSTM1* (P392, L392) and pcDNA3.1/FLAG *TNFRSF11A* (V192, A192) and 500 ng Ig-Luc luciferase reporter gene, 30 ng PRL-CMV. Four hours after transfection, culture medium was aspirated from cells and replaced with Delbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), without antibiotics. Cells were stimulated, at 24 hours after transfection, with 100 ng/mL of sRANKL (Peprotech, Rocky Hill, NJ, USA) for 48 hours or left untreated before processing. Luciferase activity was measured using Glomax 96 microplate luminometer (Promega, Madison, WI, USA). Individual experiments were performed in triplicate. Measurements of the luciferase reporter assays were statistically examined using the post hoc Bonferroni test for comparison of means (SPSS for windows version 12.0.1, Chicago, IL, USA).

Table 1. Coding Region Polymorphisms of the *TNFRSF11A* Gene

Localization	Variation	dbSNP reference	Codon changes	Amino acids variations
Exon 4	C421T	rs35211496	CAC-TAC	H141Y
Exon 6	T575C	rs1805034	GTG-GCG	V192A
Exon 9	A933G	rs8092336	ACA-ACG	T311T

U2OS cell line

U2OS cells at cell density of 1×10^5 per well were cotransfected with 200 ng NFκB reporter, 60 ng p62, and 240 ng of RANK polymorphic variants (in replicates of four) using the standard protocol for the transfection reagent FUGENE HD. After 30 hours incubation at 37 °C, 5.5% CO_2 cells were washed with phosphate-buffered saline (PBS) and lysed in 100 μL of lysis buffer. They were then frozen at −80 °C and then thawed out for 30 minutes. A total of 5 μL of lysate was then assayed for luciferase activity using the Steady-Glo luciferase assay system (Promega) according to manufacturer's instructions. A GloMax-96 Micro-plate Luminometer (Promega) was used to measure firefly activity.

Results

Molecular analysis of the *TNFRSF11A* gene

The *TNFRSF11A* gene encoding RANK protein, spans a 61-kilobyte (kb) genomic region on chromosome 18 and comprises 10 exons. Each of the 10 exons, including its associated splice junctions, were amplified by PCR of genomic DNA from familial cases negative for *SQSTM1* gene mutation, and evaluated for the presence of mutations and/or polymorphisms. *TNFRSF11A* gene sequencing failed to detect disease specific mutations in these families but we identified several polymorphisms, most of which were already described in previous studies.[19,20,25] In particular, three polymorphisms affecting the coding region of *TNFRSF11A* gene were found in exons 4, 6, and 9, but the last one (rs8092336) was a synonymous previously reported SNP (Table 1). Conversely, in the exon 4, we identified a C421T variation (rs35211496), which results in a H141Y substitution, whereas in the exon 6 we detected a T575C variation (rs1805034) resulting in a V192A.

Analysis of sequence alignments from diverse organisms showed that H141 and V192 are evolutionary conserved amino

acids in mammals suggesting that may be important for RANK function (Fig. 1). Because of potentially functional significance and consistent with a previous association study,[25] we only selected these SNPs located in exons 4 and 6 to do large-scale genotyping.

C421T (H141Y) and T575C (V192A) variants are associated with PDB

Variants C421T (rs35211496) and T575C (rs1805034) were validated to be polymorphic with minor allele frequencies (MAFs) greater than 5% in our population. Both SNPs were genotyped in our well-characterized panel of 654 PDB patients and 496 controls. In controls, SNPs analyzed were in HWE and showed allele frequencies comparable to those previously described in the Caucasian population and reported in the HapMap (http://hapmap.ncbi.nlm.nih.gov/) or SNP database (http://www.ncbi.nlm.nih.gov/snp/). Significant association was observed for T575C (rs1805034) located in the exon 6 of the *TNFRSF11A* gene. Allelic and genotype frequencies significantly differed between cases and controls, showing an increased prevalence of the C575 allele and the CC genotype in PDB patients ($p = 0.003$ and $p = 0.01$, respectively) (Table 2). Stratified analyses were also performed, indicating that the association is particularly evident in the group of PDB patients with mutations in *SQSTM1* gene ($p = 0.0002$ and 0.001, for allele and genotype analysis, respectively) (Table 2). The analysis of the C421T SNP (rs35211496) in cases and controls showed a weaker but statistically significant prevalence of the C421 allele only in PDB patients with mutations in *SQSTM1* ($p = 0.01$ and $p = 0.03$), but not in patients without *SQSTM1* mutation (Table 3). This association is likely because of the linkage disequilibrium between rs35211496 and rs1805034 SNPs, as revealed by Haploview software that showed a coefficient of $D = 0.54$. Moreover, results did not significantly change when gender specific analysis was performed.

	H141			V192
Homo sapiens	ECAPGLGAQ	HPLQLNKDTVCKPCLAGYFSDAFSSTDKCRPWTNCTFLGKRVEHHGTEKSD	V	CSSSLPA
Pan troglodytes	ECAPGLGAQ	HPLQLNKDTVCKPCLAGYFSDAFSSTDKCRPWTNCTFLGKRVEHHGTEKSD	V	CSSSLPA
Pongo abelii	ECAPGLGAQ	HPLQLNKDTVCKPCLAGYFSDAFSSTDKCRPWTNCTFLGKTVEHHGTEKSD	V	CSSSLPA
Nomascus leucogenys	ECAPGLGAQ	HPLQPNKDTVCKPCLAGYFSDAFSSTDKCRPWTNCTFLGKTVEHHGTEKSD	V	CSSSLPA
Callithrix jacchus	ECAPGLGAQ	HPLQLNKDTVCKPCLAGYFSDTFSSADKCRPWTNCTILGKLVEHHGTEKSD	V	CSSSLPS
Oryctolagus cuniculus	ECAPGLGAQ	HPLQLNKDTVCKPCLAGYFSDAFSSTDKCRPWTNCTFLGKRVEHHGTEKSD	V	CSSSLPS
Ailuropoda melanoleuca	ECAPGFGAQ	HPVQLNKDTICEPCLVGYFSDAFSPTEKCKPWTNCSILGETEVHHGTDKSD	V	CSSSLPS
Canis lupus familiaris	ECAPGFGAL	HPVQLNKDTVCKPCLVDVFSSTEKCKPWTNCSILGEKEVRHGTDKSD	V	CSSSLPS
Sus scrofa	ECAPGFGAQ	HPVQLNKDTVCKPCLAGYFSNTSSSTEKCKPWTNCTILGETEARHGTDKSD	V	CSSSLPS
Mus musculus	ECAPGFGAQ	HPLQLNKDTVCTPCLLGFFSDVFSSTDKCKPWTNCTLLGKLEAHQGTTESD	V	CSSSMTL
Rattus norvegicus	ECAPGFGAQ	HPLQLNVDTVCTPCLLGFFSDVFSSTDKCKPWTNCTVLGKMEAHQGTTESD	V	CSSSLTL

Fig. 1. Evolutive conservation of the H141Y and V192A variants.

53

Table 2. Statistically Relationship for the SNP rs1805034 Results in the Amino Acid Substitution V192A Between PDB Patients and Healthy Controls

| | n | Allele frequency | | Armitage's trend test (p) | Odds ratio (OR) | (95% CI) | Genotype frequency | | | Genotypic test (2 df) (p) |
		T	C				TT	CT	CC	
Controls	496	584 (59%)	408 (41%)				170 (34%)	244 (49%)	82 (17%)	
PDB patients (all)	654	689 (53%)	619 (47%)	0.003	1.29	(1.09–1.52)	183 (28%)	323 (49%)	148 (23%)	0.012
PDB patients mutated in SQSTM1	114	104 (46%)	124 (54%)	0.0002	1.71	(1.28–2.28)	24 (21%)	56 (49%)	34 (30%)	0.001
PDB patients not mutated in SQSTM1	540	585 (54%)	495 (46%)	0.031	1.21	(1.02–1.44)	159 (29%)	267 (50%)	114 (21%)	0.09

Given the associations reported above, we also explored the possibility of an additive effect analyzing the distribution of common haplotypes formed by the combination of the two SNPs in the *TNFRSF11A* gene between PDB patients with *SQSTM1* mutations and controls. Haplotype frequencies were significantly different in cases and controls (chi-square = 10.1, df = 3, p-value 0.02). Haplotype-specific testing showed that the putative risk haplotype C421–C575 was more frequent in cases than in controls (48% versus 35%), resulting in an OR of 1.72 (95% CI, p = 0.002). Conversely, the putative protective haplotype T421–T575 was more frequent in controls than in cases (11% versus 5%) resulting in an OR of 0.42 (95% CI, p = 0.01) (Fig. 2). Therefore, our analysis of the combined effects of two variants does not support a synergistic or additive relationship between the two SNPs. These data were supported by in silico analysis with PolyPhen2 software that predicted a deleterious effect of V192A amino acid change on RANK protein function. On the contrary, the H141Y was predicted to be benign.

Recently, a genome-wide association study reported an association between two different SNPs (rs2957128 and rs3018362) near the *TNFRSF11A* gene and PDB.[27] These two SNPs are localized outside of *TNFRSF11A* at the 3′ end (8467 and 29826 bp from TGA). As first, we looked for linkage disequilibrium information between these two SNPs and the rs1805034 (T575C) variant analyzed in this article, using the Hap Map CEU population. We observed that the associated SNPs described in Albagha et al.[27] are moderately correlated with the T575C variant ($D' = 0.45$ and $D' = 0.48$, respectively) in this reference population. Then, we genotyped rs2957128 and rs3018362 SNPs in our PDB cohort to disclose a potential synergistic effect with T575C. We observed for rs3018362 a lower degree of linkage disequilibrium with rs1805034 (T575C) in an Italian population ($D' = 0.163$) than in the Hap Map CEU population. Moreover, these SNPs were not correlated with PDB in our cohort of patients ($p = 0.45$ and $p = 0.98$, respectively, for rs2957128 and rs3018362). Similar negative results were observed when the association between the two SNPs and PDB was restricted to cases with *SQSTM1* mutation ($p = 0.47$ and $p = 0.58$, respectively, for rs2957128 and rs3018362).

Genotype–phenotype correlations

In the overall sample, PDB patients with the C575 (A192) *TNFRSF11A* allelic variant also showed an increased disease severity, with a higher prevalence of polyostotic disease (68%, 53%, and 51% in patients with CC, CT, and TT genotypes, respectively; $p = 0.01$) as well as an increased number of affected skeletal sites (Fig. 3A). Of interest, these differences increased in magnitude when analyses were restricted to cases with *SQSTM1* mutation, both for the prevalence of polyostotic disease (91%, 77%, and 73% in patients with CC, CT, and TT genotypes, respectively; $p < 0.05$) and the number of affected sites (Fig. 3B). Moreover, 100% of patients with truncating *SQSTM1* mutations and the *TNFRSF11A* CC genotype had a polyostotic form with respect to 67% and 50% of patients with CT and TT genotypes, respectively, even though this difference did not reach statistical significance (likely because of the limited number of subjects with truncating *SQSTM1* mutations). A similar but not significant trend for increased disease severity was evidenced when the rs35211496 (C421T) was considered. In contrast, no significant

Table 3. Statistically Relationship for the SNP rs35211496 Results in the Amino Acid Substitution H141Y Between PDB Patients and Healthy Controls

| | n | Allele frequency | | Armitage's trend test (p) | Odds ratio (OR) | (95% CI) | Genotype frequency | | | Genotypic test (2 df) (p) |
		C	T				CC	CT	TT	
Controls	496	808 (81%)	184 (19%)				322 (65%)	162 (33%)	12 (2%)	
PDB patients (all)	654	1092 (84%)	216 (16%)	0.16	1.17	(0.94–1.45)	451 (69%)	190 (29%)	13 (2%)	0.34
PDB patients mutated in SQSTM1	114	201 (88%)	27 (12%)	0.01	1.71	(1.11–2.65)	88 (77%)	25 (22%)	1 (1%)	0.03
PDB patients not mutated in SQSTM1	540	891 (83%)	189 (18%)	0.46	1.09	(0.87–1.36)	363 (67%)	165 (31%)	12 (2%)	0.74

54
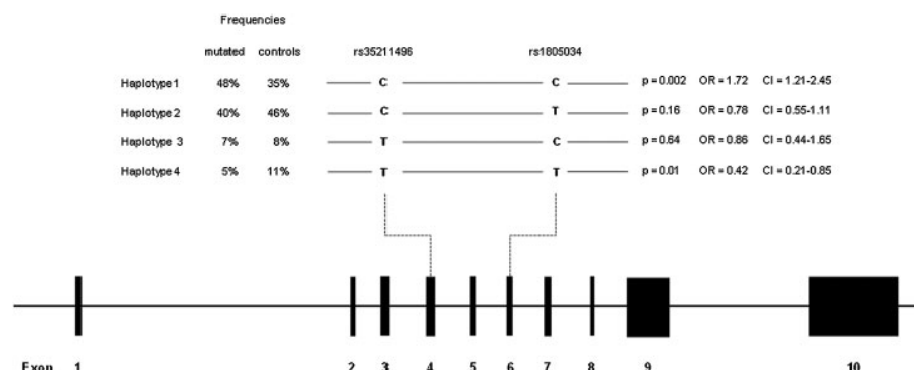
Fig. 2. Haplotype analysis. Frequency and association of four haplotypes identified by C421T (H141Y) and T575C (V192A) variations in PDB patients with mutation in *SQSTM1* and controls. The *p*-value, OR, and 95% CI are indicated for each haplotype.

differences in relation to *TNFRSF11A* genotypes were observed concerning age of diagnosis of PDB.

Interestingly, when we considered affected sib-pairs from six pedigrees with *SQSTM1* mutations, an increased number of affected skeletal sites was observed in carriers of the CC genotype than in those with the CT genotype (Fig. 4A). Conversely, in the three families with giant cell tumor and PDB, there was no evidence for an increased prevalence of the C575 (A192) allelic variant and there were no differences in the number of affected skeletal sites (Fig. 4B) or age at diagnosis in relation to rs1805034 and rs35211496 SNPs.

A192 variant increases NFκB activation in HEK293 and U20S-osteosarcoma cells

As p62 and RANK are implicated in the *NFκB* signaling pathway, we performed luciferase reporter assays in HEK293 and U20S (osteosarcoma-derived) cells to evaluate the effect of the V192A variation alone and coupled with p62 mutations (P392L) on *NFκB* activation.

We initially performed luciferase assays in HEK293 cells to confirm that the *SQSTM1* mutant ($SQSTM1^{L392}$) hyperstimulates activation of *NFκB* relative to wild-type *SQSTM1* ($SQSTM1^{P392}$), as already described elsewhere.[15,30] As expected, P392L mutation

increased *NFκB* activation compared with wild-type *SQSTM1* when HEK293 cells were treated for 24 hours with sRANKL or left untreated (Fig. 5A, D). In a second step, using $TNFRSF11A^{V192}$ and $TNFRSF11A^{A192}$ constructs in sRANKL treated or untreateated HEK293 cells, we did not observe any significant variation in *NFκB* expression between the two *TNFRSF11A* variants, probably because of a not sensitive experimental system (Fig. 5B, E). Importantly, in cotransfection experiments, the *NFκB* activation was significantly attenuated by wild-type *SQSTM1* ($SQSTM1^{P392}$) plus $TNFRSF11A^{V192}$ compared with wild-type *SQSTM1* plus $TNFRSF11A^{A192}$ (Fig. 5C, F: bars 2–3). Moreover, we observed a significant increased transactivation between $TNFRSF11A^{A192}$ plus $SQSTM1^{L392}$ compared with $TNFRSF11A^{A192}$ plus $SQSTM1^{P392}$ when cells were treated ($p < 0.005$) or not ($p < 0.02$) with sRANKL, suggesting that *TNFRSF11A* requires *SQSTM1* to transactivate the NFκB reporter, and this effect is dependent of *SQSTM1* mutational status (Fig. 5C, F: bars 2–4). Finally, the *NFκB* activity was significantly potentiated by mutated *SQSTM1* ($SQSTM1L^{392}$) plus $TNFRSF11A^{A192}$ compared with mutated *SQSTM1* plus $TNFRSF11A^{V192}$ when cells were treated
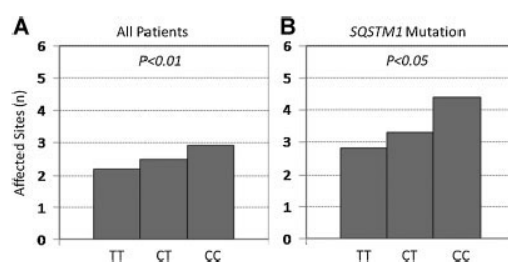
Fig. 3. Genotype–phenotype correlations. Association between *TNFRSF11A* rs1805034 genotype and the number of affected skeletal sites in all patients (*A*) and in patients with *SQSTM1* mutation (*B*).
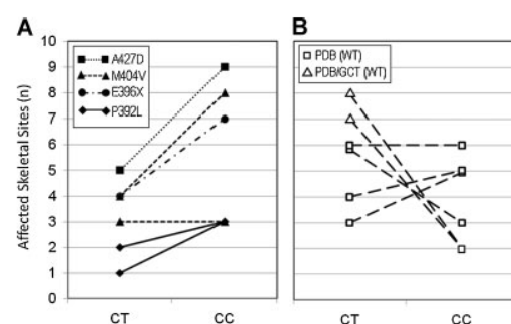
Fig. 4. Genotype–phenotype correlations in six affected pedigrees. Number of affected skeletal sites in (*A*) sib-pairs with *SQSTM1* mutation and (*B*) sib-pairs from PDB/giant cell tumor (GCT) families in relation to CT or TT *TNFRSF11A* rs1805034 genotype.
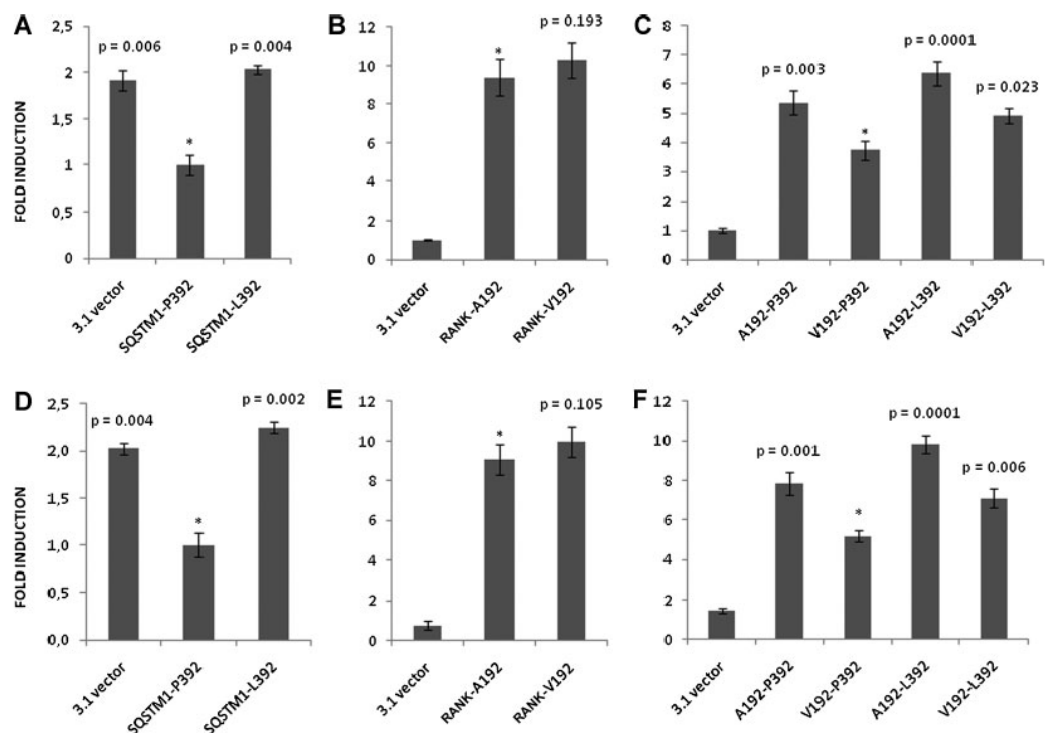
Fig. 5. Luciferase NFκB reporter assay in HEK293 cells. Cells were cotransfected with different vectors combination and Igκ-Luc reporter plasmid. After 4 hours, cells were left untreated (*A, B, C*) or stimulated (*D, E, F*) for 48 hours with sRANKL (100 ng/mL). Luciferase activities were determined. Individual assays were performed in triplicate, and data are presented as the fold induction luciferase activity of at least three independent assays. The *p*-values indicate the difference compared with *.

or not (Fig. 5*C, F*: bars 4–5). Overall, these results suggest an additive effect for $TNFRSF11A^{A192}$ on $NF\kappa B$ activity that can be disclosed only in the presence of *SQSTM1* transfected vector. The highest transactivation capacity is retained by the mutated $SQSTM1^{L392}$ in association with the $TNFRSF11A^{A192}$ variant. Very similar results were observed when these in vitro experiments were replicated in U2OS-osteosarcoma cells (data not shown).

Discussion

The RANKL/RANK/OPG signaling pathway has a critical role in bone remodeling.[32] Based on its dominant function in osteoclastogenesis and its genetic localization in a PDB-candidate region,[33,34] the *TNFRSF11A* gene encoding RANK has long been considered as a serious candidate for PDB, but different studies failed to detect *TNFRSF11A* mutations in patients with PDB, except than in a single family from Japan, with severe and early-onset PDB.[22] Conversely, mutations in the *SQSTM1* gene (encoding p62) were detected in up to 10% and 40% of sporadic and familial PDB cases, respectively.[7–9] Even though the molecular mechanism of *SQSTM1* mutations in the pathogenesis of PDB remains to be clarified in detail, an essential

role of p62 in the osteoclast RANK–NFκB signaling pathway has been demonstrated.[10,11,35] *SQSTM1* mutations, however, show incomplete penetrance, emphasizing the role of environmental factors and/or modifier genes in disease etiology. In a recent large-scale genetic screening of Italian PDB cases we identified some families, mainly originating from the Campania region, that were negative for *SQSTM1* gene mutation.[16] Interestingly, these families were previously characterized for their higher clinical severity and greater frequency of neoplastic degeneration, including giant cell tumors.[36] These peculiar clinical features, together with an early onset, resembled in part the clinical phenotype of the Japanese family with severe PDB because of activating mutations of the *TNFRSF11A* gene.[22] Thus, in the first part of our analysis we specifically performed *TNFRSF11A* mutation screening in these families, but we did not evidence any mutation in the coding region of *TNFRSF11A*. This may suggest the presence of a different predisposition gene responsible of this particular variant of familial PDB, alone or in combination with an environmental trigger.

Interestingly, however, we were able to demonstrate that a particular polymorphic variant of *TNFRSF11A* (rs1805034) is associated with increased NFκB activity in vitro and may affect either the pathogenesis and the clinical severity of PDB.

56

Consistent with the results from a recent analysis in Northern-European populations of sporadic PDB cases without the *SQSTM1* mutation,[25] we observed a different distribution of the two nonsynonymous *TNFRSF11A* polymorphisms (C421T and T575C) in PDB patients compared with the general population. The largest effect was found for the T575C variant (which result in V192A substitution), with a 1.3-fold increased risk in the presence of the C575 (A192) allele. Although the effect of this variant on the overall risk of developing PDB is probably mild, our results also indicate that its importance may be higher in patients with *SQSTM1* mutation. In fact, the prevalence of the C575 allele in affected subjects with *SQSTM1* mutation was higher than in patients without the mutation (54% versus 46%), increasing the relative risk to 1.7. Moreover, a statistically significant increase in the number of affected skeletal sites and in the prevalence of polyostotic disease was observed in CT and CC patients with respect to TT patients. This association was also evident when affected sib-pairs from six families with *SQSTM1* mutation were considered, indicating that the presence of the C575 variant may indeed predispose affected individuals to an increased skeletal involvement and thus to an increased disease severity. As a counterpart, the reduced (even though still significant) association between the C allele and PDB observed in patients negative for the *SQSTM1* mutation might suggest an interaction with additional predisposition genes, and is consistent with the results of two recent genome-wide association studies.[27,37] In fact, in those studies at least seven new candidate genomic regions, including *TNFRSF11A*, were observed in a large sample of PDB cases negative for the *SQSTM1* mutation. The SNPs associated variants near the *TNFRSF11A* gene in these genome-wide analyses (rs2957128 and rs3018362) are moderately correlated with the rs1805034 (T575C) variant (that was not included in Illumina HumanHap300-Duo BeadChip v2 used in the genome-wide analysis) in the Hap Map CEU population but not in our Italian sample. Moreover, neither of these SNPs were associated with PDB in our cohort of patients with and without the *SQSTM1* mutation. Therefore, it is reasonable to speculate that the association between PDB and rs2957128 or rs3018362 in different populations derived from independent signals. This is consistent with the results of the second genome-wide association study,[37] showing the lowest association between rs3018362 and PDB in the two different Italian cohorts (Italian Replication cohorts 1 and 2), with effects estimates for these two individual cohorts well above the overall fixed effect estimates. In fact, concerning the Italian replication cohorts 1 and 2 (which comprises part of our PDB samples) tested in this genome-wide study, the −95% CI for rs3018362 appears below 1.0 and thus nonsignificant (see Supplementary Fig. 2 of ref. 37).

To have conclusive evidence that the T575C variant is the disease-associated variant and to investigate its functional role, a luciferase reporter assay was performed in two different cell lines. The initial assay showed no significant difference in the NFκB activity between the different alleles of this SNP (*TNFRSF11A*^{V192} and *TNFRSF11A*^{A192}) probably because of the restricted sensitivity of the assay (ie, the effect may be too small to be detected). However, in cotransfection experiments, *NFκB* activation was significantly attenuated with *SQSTM1*^{P392} plus *TNFRSF11A*^{V192} compared with *SQSTM1*^{P392} plus *TNFRSF11A*^{A192},

whereas it was significantly potentiated with *SQSTM1*^{L392} plus *TNFRSF11A*^{V192} compared with *SQSTM1*^{L392} plus *TNFRSF11A*^{A192}, suggesting an additive effect for *TNFRSF11A*^{A192} on NFκB activity and thus osteoclast activation. The lack of difference in reporter activation by the *TNFRSF11A*^{V192} and *TNFRSF11A*^{A192} variants alone is consistent with a previous observation from a similar study in Dutch PDB cases.[25] Moreover, it is now well established that *SQSTM1* mutations confer increased sensitivity to RANKL. This has been remarkably demonstrated both in vitro and in vivo by a recent study showing hyperresponsivity to RANKL in mutant SQSTM1/p62 PDB patients, transfected osteoclasts, and transgenic mice.[38] Thus, it is likely that inclusion of *SQSTM1* might increase RANKL sensitivity unmasking the effect of *TNFRSF11A* C575 (A192) variant on reporter assays. This is also in keeping with our clinical data showing an enhanced association between the presence of the A192 allele and the occurrence of PDB or the severity of phenotype in subjects with *SQSTM1* mutations.

The molecular mechanisms through which this *TNFRSF11A* variant may predispose to PDB with increased disease extension remains to be investigated in detail. Mutations in *TNFRSF11A* gene encoding RANK have been found in patients suffering from familial expansile osteolysis (FEO), expansile skeletal hyperphosphatasia (ESH), and early-onset PDB, as well as osteoclast-poor osteopetrosis.[19,21,22,39] This latter condition is a clear loss of function, whereas in the other conditions, small duplications were found in the signal peptide of RANK with an activating effect on NFκB signaling. Then, genetic alterations in *TNFRSF11A* gene may result in hyper- or hypoactivation of NFκB signaling, with an effect on osteoclast formation and activity. Of interest, the V192A variation is localized in the extracellular region of RANK (residues 30–194) and involves the fourth tandem cysteine-rich pseudorepeat domain (CDR), CDR4 (residues 155–197). These CDRs are characteristic of the TNFRSF family members[40] and consists of irregular β-strands linked typically by interstrand disulphides and can be further divided into two structural modules of various types defined by topology and number of disulphides.[41] Although CDR2 and CDR3 are crucial to make close contact with RANKL (thus assuring the high-affinity interaction between the ligand and the receptor), the CDR4 region is important for maintaining the structural integrity of the receptor and mutations within these region have been associated with local conformational changes and functional implications on osteoclastogenesis.[42] The in silico analysis predicted a deleterious effect of this amino acid change on RANK protein function. Thus, one could hypothesize that the presence of an alanine instead of a valine might have some functional effect on CDR4 domain and RANK/RANKL interaction, with potential implications on the efficiency of this signalling pathway. However, further analysis and more specific studies are needed to reveal whether this is indeed the case.

Importantly, our results may also have potential therapeutical and clinical implications for different bone disorders associated with increased NFκB activity and bone resorption (ie, osteoporosis and myeloma bone disease). In fact, our in vitro analysis clearly evidenced a difference in NFκB activity between *TNFRSF11A*^{V192} and *TNFRSF11A*^{A192} allelic variants, even in the absence of *SQSTM1* mutations. This is in keeping with several reports including genome-wide studies that evidenced an

57

association between *TNFRSF11A* gene and the risk of osteoporosis and fractures.[43–45] In this context, the role of this and other *TNFRSF11A* polymorphisms on the response to antiresorptive treatments and particularly to denosumab, a monoclonal antibody against RANKL, which is being marketed for the treatment of osteoporosis,[46,47] remains to be investigated.

Disclosures

All the authors state that they have no conflicts.

Acknowledgments

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59

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Genome-wide association identifies three new susceptibility loci for Paget's disease of bone

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Paget's disease of bone (PDB) is a common disorder characterized by focal abnormalities of bone remodeling. We previously identified variants at the *CSF1*, *OPTN* and *TNFRSF11A* loci as risk factors for PDB by genome-wide association study[1]. Here we extended this study, identified three new loci and confirmed their association with PDB in 2,215 affected individuals (cases) and 4,370 controls from seven independent populations. The new associations were with rs5742915 within *PML* on 15q24 (odds ratio (OR) = 1.34, $P = 1.6 \times 10^{-14}$), rs10498635 within *RIN3* on 14q32 (OR = 1.44, $P = 2.55 \times 10^{-11}$) and rs4294134 within *NUP205* on 7q33 (OR = 1.45, $P = 8.45 \times 10^{-10}$). Our data also confirmed the association of *TM7SF4* (rs2458413, OR = 1.40, $P = 7.38 \times 10^{-17}$) with PDB. These seven loci explained ~13% of the familial risk of PDB. These studies provide new insights into the genetic architecture and pathophysiology of PDB.

PDB is a common skeletal disorder with a strong genetic component that affects up to 2% of individuals of European ancestry aged 55 years and above[2,3]. Mutations of *SQSTM1* are known to cause a high-penetrance form of PDB which is clinically severe[4] and occurs in about 40% of individuals with a family history of the disorder[5,6]. We recently identified additional susceptibility alleles for PDB at the *CSF1*, *OPTN* and *TNFRSF11A* loci by a genome-wide association study (GWAS) involving 692 cases with PDB and 1,001 controls and a replication cohort of 481 cases and 520 controls[1]. In order to identify additional susceptibility loci for the disease, we performed an extended GWAS involving a total of 749 cases with PDB of British descent in whom *SQSTM1* mutations had been excluded and 2,930 British controls derived from the 1958 Birth Cohort[7] with replication in a further 1,474 cases and 1,671 controls from six independent populations.

After applying quality control measures and excluding samples of non-European ancestry, the extended cohort (henceforth referred to as the GWAS stage) comprised 741 cases and 2,699 controls with genotype information for 290,115 SNPs, providing a fourfold increase in power to detect loci of moderate effect size (OR ≥ 1.4) compared with our previous study[1]. To increase SNP coverage, we performed genome-wide SNP imputation for the GWAS stage samples using phased haplotype data from the HapMap project as a reference. The results of the association testing of genotyped and imputed SNPs (a total of 2,487,078 SNPs) from the GWAS stage are shown in **Figure 1**. A locus on chromosome 8q22.3 showed genome-wide evidence of association with PDB ($P < 5.0 \times 10^{-8}$), in addition to the previously identified genome-wide significant loci on 1p13.3, 10p13 and 18q21.33 (ref. 1).

In the second stage of this study, we analyzed the highest ranking SNPs observed in the GWAS stage ($P \leq 5 \times 10^{-5}$) for replication after excluding those in linkage disequilibrium (LD; $r^2 > 0.8$ or

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60
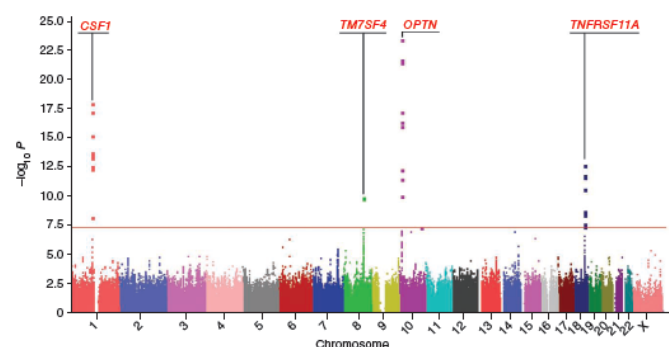
Figure 1 Loci for susceptibility to PDB detected by GWAS. Manhattan plot of association test results of GWAS stage data showing the chromosomal position of 2,487,078 genotyped or imputed SNPs plotted against genomic-control–adjusted $-\log_{10} P$. The red horizontal line represents the threshold for genome-wide significance ($P < 5 \times 10^{-8}$).

$D' > 0.95$) with the highest ranking SNP from each region. We genotyped a total of 27 SNPs in the replication cohorts, which consisted of 1,474 *SQSTM1*-negative cases with PDB from six different geographic regions and 1,671 unaffected controls from the same regions that were matched with the cases by gender, as described in the Online Methods section and **Supplementary Table 1**. We performed a meta-analysis of data from the GWAS stage and the individual replication cohorts, and the results are summarized in **Supplementary Table 2**. This strengthened the association with PDB for the *CSF1*, *OPTN* and *TNFRSF11A* loci that we identified in our previous study[1] and confirmed the association with the 8q22.3 locus that was suggestively associated with PDB in our previous GWAS[1] and which was confirmed to be associated with PDB in a small study of Belgian and Dutch subjects[8]. Furthermore, we identified three additional genome-wide significant loci on 7q33, 14q32.12 and 15q24.1 in the combined dataset ($P < 5 \times 10^{-8}$; **Table 1** and **Fig. 2**).

The strongest signal on 8q22.3 was with rs2458413 (combined $P = 7.38 \times 10^{-17}$, OR = 1.4). There was no significant heterogeneity between the study groups (**Table 1**, **Fig. 3** and **Supplementary Table 3**), and the direction of association was similar in all cohorts. The associated region spans ~220 kb, but the SNPs with the highest association signal appear to cluster within an 18-kb LD block spanning the entirety of *TM7SF4*, the transmembrane 7 superfamily member 4 gene (**Fig. 2** and **Supplementary Fig. 1**). This gene encodes the dendritic-cell–specific transmembrane protein (DC-STAMP)[9], which is a strong functional candidate gene for PDB because it is required for

the fusion of osteoclast precursors to form mature osteoclasts[10]. Previous studies have shown that RANKL-induced DC-STAMP expression is essential for osteoclast formation[11], and a recent study showed that the connective tissue growth factor CCN2 stimulates osteoclast fusion through interaction with DC-STAMP[12]. Because osteoclasts from individuals with PDB are larger in size and contain more nuclei than normal osteoclasts, it seems likely that the genetic variants that predispose to PDB do so by enhancing *TM7SF4* expression or by causing gain of function at the protein level, but further studies will be required to investigate these possibilities.

The first new locus for PDB susceptibility was on 7q33, which is tagged by rs4294134 (combined $P = 8.45 \times 10^{-10}$, OR = 1.45). The direction of association was similar in all study cohorts, and analysis of the combined dataset showed no evidence for heterogeneity between study groups (**Table 1**, **Fig. 3** and **Supplementary Table 3**). The associated region spans ~350 kb (**Fig. 2**), but the strongest signal was with rs4294134, located within the twenty-second intron of *NUP205*. This gene encodes nucleoporin 205 kDa, which is one of the main components of the nuclear pore complex involved in the regulation of transport between the cytoplasm and nucleus[13]. All SNPs with $P < 1 \times 10^{-5}$ in the 350-kb associated region were in moderate to strong LD with rs4294134 ($r^2 \geq 0.5$, $D' \geq 0.95$), with the exception of two SNPs (rs3110788 and rs3110794) that were poorly correlated with rs4294134 ($r^2 \leq 0.21$, $D' \geq 0.95$; **Fig. 2**). Conditional analysis in the GWAS stage indicated that the association signal appeared to be driven by rs4294134 ($P = 8.8 \times 10^{-3}$) after adjusting for rs3110788 ($P = 0.31$) and rs3110794 ($P = 0.10$). None of the genes located in this region are known to affect bone metabolism, and further studies will be required to identify the functional variant(s) responsible for association with PDB.

The second new susceptibility locus was located on 14q32.12 and was tagged by rs10498635. This SNP showed borderline evidence of association with PDB in our previous study ($P = 9.69 \times 10^{-8}$)[1] but reached genome-wide significance in the present study (combined $P = 2.55 \times 10^{-11}$, OR = 1.44). Association testing showed no evidence for heterogeneity between the study groups (**Table 1**, **Fig. 3** and **Supplementary Table 3**). The 62-kb associated region is bounded by two recombination hotspots and contains *RIN3* (**Fig. 2**), which encodes the Ras and Rab interactor 3, a protein that plays a role in vesicular trafficking through

Table 1 Summary of the seven loci showing genome-wide significant association with Paget's disease of bone

Chr.	SNP	RA	GWAS Stage		Replication Fixed effect		Combined overall effect Fixed effect		Heterogeneity		Closest gene
			P	OR (95% CI)	P	OR (95% CI)	P	OR (95% CI)	P_{het}	I^2	
1	rs10494112	G	5.83×10^{-17}	1.75 (1.54–1.99)	4.93×10^{-19}	1.69 (1.50–1.89)	7.06×10^{-35}	1.72 (1.57–1.87)	0.97	00.0	*CSF1, EPS8LS*
7	rs4294134	G	1.20×10^{-5}	1.50 (1.25–1.79)	2.29×10^{-5}	1.42 (1.20–1.66)	8.45×10^{-10}	1.45 (1.29–1.63)	0.83	00.0	**NUP205**
8	rs2458413	A	7.85×10^{-11}	1.51 (1.34–1.71)	1.09×10^{-7}	1.32 (1.19–1.46)	7.38×10^{-17}	1.40 (1.29–1.51)	0.10	44.3	*TM7SF4*
10	rs1561570[a]	T	9.56×10^{-18}	1.71 (1.51–1.93)	2.09×10^{-21}	1.64 (1.48–1.81)	4.37×10^{-38}	1.67 (1.54–1.80)	0.01	65.7	*OPTN*
14	rs10498635	C	1.51×10^{-5}	1.45 (1.23–1.71)	5.64×10^{-7}	1.42 (1.29–1.63)	2.55×10^{-11}	1.44 (1.29–1.60)	0.62	00.0	**RIN3**
15	rs5742915	C	1.40×10^{-7}	1.38 (1.22–1.54)	3.99×10^{-8}	1.32 (1.20–1.46)	1.60×10^{-14}	1.34 (1.25–1.45)	0.56	00.0	**PML**
18	rs3018362	A	1.87×10^{-11}	1.50 (1.34–1.69)	1.27×10^{-10}	1.40 (1.26–1.55)	7.98×10^{-21}	1.45 (1.34–1.56)	0.46	00.0	*TNFRSF11A*

RA, risk allele; OR, odds ratio for the risk allele; CI, confidence interval; I^2, heterogeneity statistics; P_{het}, P value for heterogeneity. Newly identified loci are shown in bold. [a]rs1561570 showed significant heterogeneity but random-effect results were genome-wide significant ($P = 4.34 \times 10^{-12}$, OR = 1.68).

61

Figure 2 Regional association plots of loci showing genome-wide significant association with PDB. (**a–d**) Details of loci on chromosome 7q33 (**a**), 15q24.1 (**b**), 8q22.3 (**c**) and 14q32.12 (**d**) showing the chromosomal position (based on NCBI human genome build 36) of SNPs in each region plotted against $-\log_{10} P$ values. Genotyped (squares) and imputed (circles) SNPs are color coded according to the extent of LD with the SNP showing the highest association signal (represented as purple diamonds) from each region in the combined analysis. The estimated recombination rates (cM/Mb) from HapMap CEU release 22 are shown as light blue lines, and the blue arrows represent known genes in each region. We defined the associated regions based on LD with the highest association signal ($r^2 > 0.2$) within a window of 500 kb.
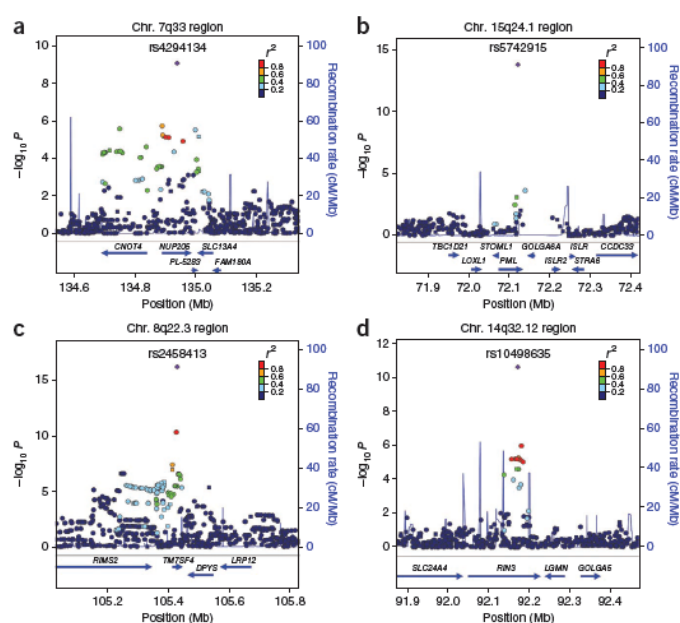
interaction with small GTPases such as Ras and Rab[14],[15]. The function of *RIN3* in bone metabolism is currently unknown, but it could play a role in bone resorption in view of the importance that small GTPases play in vesicular trafficking and in osteoclast function[16],[17]. It is of interest to note that mutations affecting VCP, a protein also involved in vesicular trafficking, cause the syndrome of inclusion body myopathy with early onset Paget's disease and frontotemporal dementia[18].

The third new susceptibility locus was located on 15q24.1, and the strongest association was with rs5742915 (combined $P = 1.60 \times 10^{-14}$; OR = 1.34; **Table 1**, **Fig. 3** and **Supplementary Table 3**). The associated region is bounded by two recombination hotspots and spans ~200 kb, but we observed a gap spanning ~40 kb in this region with no SNP coverage in the Illumina arrays or the HapMap European CEU population. The associated SNPs were clustered within *PML*,

the promyelocytic leukemia gene (**Fig. 2**), and we observed the strongest signal for rs5742915, which results in a phenylalanine to leucine amino acid change at codon 645 (p.Phe645Leu) of PML. The function of *PML* in bone metabolism is unclear, but it is known to be involved in TGF-β signaling[19]. Accordingly, researchers from a previous study showed that cells from *Pml* knockout mice were resistant to TGF-β–dependent growth arrest and apoptosis and had impaired induction of TGF-β target genes[19]. Because TGF-β is known to play a role in the regulation of bone remodeling, it is possible that the association between PDB and *PML* could be mediated by an effect on TGF-β signaling, but further research will be required to investigate this possibility. *GOLGA6A* is also located in the associated region and encodes a protein that belongs to golgin, a family of coiled-coil proteins associated with the Golgi apparatus and which play a role in membrane fusion and as structural supports for the Golgi cisternae. This gene is located in the 40-kb gap region that contains a large low-copy repeat sequence. Although *GOLGA6* has no known role in bone metabolism, mutations in other members of the golgin family have been shown to cause lethal skeletal dysplasia[20] and a severe form of osteoporosis[21].
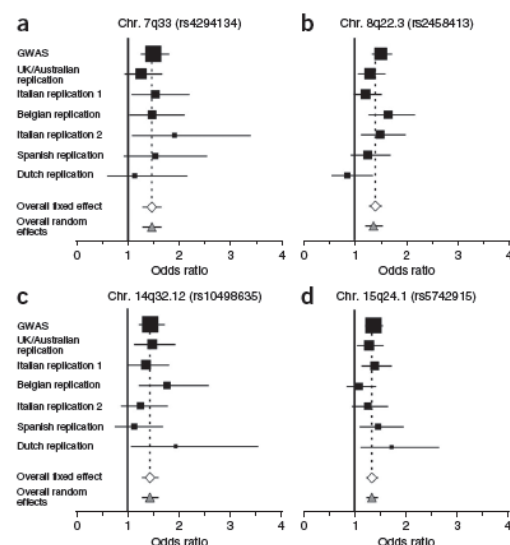
Figure 3 Forest plots showing association in the different datasets for SNPs at 7q33, 8q22.3, 14q32.12 and 15q24.1. (**a–d**) Forest plots of overall effect size for SNPs associated with PDB risk from the identified loci on 7q33 (rs4294134) (**a**), 8q22.3 (rs2458413) (**b**), 14q32.12 (rs10498635) (**c**) and 15q24.1 (rs5742915) (**d**). We estimated the overall effect size using meta-analysis of the GWAS sample and the six replication samples. The black squares represent the effect estimates for the individual cohorts, and the horizontal lines represent the 95% CIs of the estimates. The sizes of the squares are proportionate to the weights of the estimates. The diamonds and triangles represent the overall estimate under fixed-effect and random-effect models, respectively. The dotted vertical lines represent the overall fixed effect estimates.

62
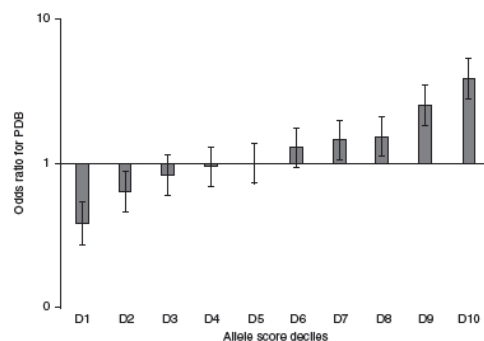
Figure 4 Cumulative contribution of genome-wide significant loci to the risk of PDB. Risk allele scores defined by the seven loci associated with PDB risk are plotted against the OR for PDB. We weighted risk alleles according to their estimated effect size and divided weighted risk allele scores into ten equal parts (deciles) using data from the replication cohorts. We calculated the OR for PDB risk for each decile in reference to the fifth decile (D5). Vertical bars represent 95% CIs.

We were also able to replicate our previously reported association between variants at the *CSF1*, *OPTN* and *TNFRSF11A* loci and PDB in the present study[1]. The results of our meta-analysis of the combined dataset for these loci are shown in **Table 1** and **Supplementary Figure 2**, which provide conclusive evidence for association of variants at *CSF1* ($P = 7.06 \times 10^{-35}$), *OPTN* ($P = 4.37 \times 10^{-38}$) and *TNFRSF11A* ($P = 7.98 \times 10^{-21}$) with PDB. We observed evidence of heterogeneity between the study groups for rs1561570 ($I^2 = 65.7\%$, $P_{het} = 0.01$) at *OPTN*, but this was because of differences in effect size rather than the direction of effect, and the association remained genome-wide significant after accounting for heterogeneity ($P = 4.34 \times 10^{-12}$, OR = 1.68). The heterogeneity was caused by the larger effect size observed in the Dutch cohort (**Supplementary Fig. 2**) and possibly because of the small sample size of this cohort. These observations provide highly robust evidence for association between these loci and PDB and extend those recently reported[8] in the Dutch and Belgian populations, which were also included in the present study.

We next wanted to determine if the identified loci on 15q24.1, 7q33 and 14q32.12 interacted with each other or with the previously identified loci on 1p13.3, 8q22.3, 10p13 and 18q21.33 to affect the risk of PDB. Pairwise interaction analysis showed weak evidence for interaction of 7q33 (rs4294134) with 8q22.3 (rs2458413, $P = 0.03$) and 10p13 (rs1561570, $P = 0.02$). However, these interactions were not significant after adjusting for multiple testing, and none of the other loci showed evidence for interaction ($P > 0.05$), suggesting a multiplicative model of association with PDB risk. In order to estimate the effect size of the identified loci on the development of PDB, we calculated the proportion of familial risk explained by the genome-wide significant loci in the replication sample assuming a sibling relative risk for PDB of 7.0 (ref. 22). This showed that the proportion of familial risk explained was ~13%, which is much greater than that observed for other common bone diseases, such as osteoporosis[23]. We also estimated the cumulative population attributable risk of these loci in the replication cohort and found it to be 86%, and we found that the risk of PDB increased with the increasing number of risk allele scores defined by the seven loci (per risk allele OR = 1.44, 95% CI 1.38–1.51, $P = 5.4 \times 10^{-57}$). When we weighted allele scores according to their estimated effect size, we found that subjects in the top

10% of the allele score distribution (D10; $n = 315$) had a 10.1-fold (95% CI 7.0–14.6, $P = 2.4 \times 10^{-39}$) increase in risk of developing PDB compared to those in the bottom 10% of the distribution (D1; $n = 315$) from the replication dataset (**Fig. 4**). Although these data suggest that a large part of the genetic risk of PDB in individuals without *SQSTM1* mutations is accounted for by these loci, we acknowledge that the functional variants need to be identified before we can precisely estimate the contribution that these loci make to the risk of developing PDB. To assess the functional effect of the identified SNPs on gene expression, we tested the association between the top PDB-associated SNPs (or those in LD with these SNPs; $D' \geq 0.8$) from each of the seven loci and *cis*-allelic expression of genes located in the associated regions using publicly available expression quantitative trait loci (eQTL) data. This showed highly significant associations for transcripts of *TM7SF4* (rs2458415, expression $P = 1.22 \times 10^{-18}$) and *OPTN* (rs1561570, expression $P = 6.61 \times 10^{-62}$) in peripheral blood monocytes[24], suggesting that the association with PDB risk for these loci could be mediated by influencing gene expression levels.

In addition to the loci mentioned above, we identified additional variants that showed suggestive evidence for association with PDB. For example, a locus on chromosome Xq24 showed borderline evidence for association with PDB (rs5910578 within *SLC25A43*, combined $P = 1.26 \times 10^{-7}$, OR = 1.34), as did another locus on chromosome 6p22.3 (rs1341239 near *PRL*, combined $P = 3.83 \times 10^{-6}$, OR = 1.20; **Supplementary Table 2**). Given that we observed six genotyped variants with $P < 1 \times 10^{-5}$ in the GWAS stage after removal of confirmed SNPs and associated variants when only three are expected by chance (**Supplementary Fig. 3**), it is likely that some of the associations observed are true, but our study was not sufficiently powered to detect them at a genome-wide significant level.

This study has been successful in identifying seven loci that contribute substantially to the risk of developing PDB. The identified loci have relatively large effect sizes compared to other common diseases of the musculoskeletal system such as osteoporosis and rheumatoid arthritis. This indicates that susceptibility to PDB is probably mediated by inheritance of a relatively small number of genes with large effect sizes as opposed to a large number of genes with small effect sizes, as seen in other complex diseases. Many of the susceptibility variants are within or close to genes that are known to play important roles in regulating osteoclast differentiation and function, whereas other variants are within genes not previously implicated in the regulation of bone metabolism. Although further work will be required to identify functional variants, the present study has provided new insights into the genetic architecture of PDB and has identified several genes that were not previously suspected to play a role in bone metabolism. Finally, the large effect size of the variants identified means that it may be possible in the future to identify people at risk of developing PDB by genetic profiling.

METHODS
Methods and any associated references are available in the online version of the paper at http://www.nature.com/naturegenetics/.

63

AUTHOR CONTRIBUTIONS

COMPETING FINANCIAL INTERESTS

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64

ONLINE METHODS

GWAS stage study subjects. This study describes an extension to our previously reported GWAS of PDB, in which we used genotype data from 692 cases with PDB from our previously described study[1] and extended the case group by genotyping an additional 57 cases with PDB. The additional cases were selected from recently recruited subjects in the PRISM study[25], which was a randomized trial of two different treatment strategies for cases with PDB from the UK. We also increased the size of the control group by using genotype data from 2,930 subjects from the British 1958 Birth Cohort genotyped by the Wellcome Trust Case Control Consortium[7]. This control group is a better match to our cases with PDB than the previous controls, which were recruited from Scotland[1] because, like the PRISM participants, they were recruited from all over the UK. The extended samples size used in this study provided 90% power to detect disease-associated alleles with minor allele frequency of 0.2 and genotype relative risk of 1.4, assuming a multiplicative model and a disease with population prevalence of 2%. This represents a substantial increase in power compared to our previous study[1], in which we had 20% power to detect alleles with genotyped relative risk of 1.4.

GWAS-stage genotyping and quality control. Genotyping and quality control for the 692 cases with PDB were performed using Illumina HumanHap300-Duo arrays as described previously[1]. The additional 57 cases with PDB were genotyped using Illumina Human660W Quad version 1 arrays, and quality control measures were applied as previously described[1]. Briefly, SNPs with call rate <95% were excluded, and samples with call rate <90% ($n = 1$), excess heterozygosity ($n = 1$), and non-European ancestry ($n = 6$; **Supplementary Fig. 4**) were removed before analysis. The genotyping of the British 1958 Birth Cohort was previously performed by the Wellcome Trust Case Control Consortium using the Illumina Human 1.2M Duo custom array (see URLs)[7]. For the control group, SNPs with call rate <95% were excluded, and we removed 231 samples because they failed at least one of the following quality control criteria: low call rate, non-European ancestry, gender mismatch or cryptic relatedness. Population ancestry was determined using multidimensional scaling analysis of identity-by-state distances matrix as previously described[1]. After quality control, we analyzed 741 cases with PDB and 2,699 controls with genotype data for 290,115 SNPs, which were common to the three different genotyping arrays. To ensure consistent genotyping between different platforms, a subset of samples were genotyped using at least two different platforms, and the cross-platform genotype concordance rate was >99.7% (**Supplementary Table 4**). Additionally, the genotype cluster plots for all SNPs showing association with PDB at $P < 1.0 \times 10^{-4}$ were visually inspected in cases and controls, and only high quality genotype data were included in the analysis. Furthermore, genotype call rate for the top associated SNPs was consistent between cases and controls (**Supplementary Table 5**).

Replication samples. The replication study groups were derived from clinic-based individuals with PDB and gender-matched controls selected from the same region. Individuals with *SQSTM1* mutations were excluded, and all study participants provided informed consent. The first replication cohort comprised 175 individuals with PDB from the UK, 8 cases with PDB from Sydney, Australia and 215 cases with PDB from Western Australia. These individuals were of British descent and were matched with 485 unaffected British controls. The second replication cohort (Italian replication cohort 1) comprised 354 cases with PDB and 390 unaffected controls enrolled from various referral centers in Italy who took part in the GenPage project[26]. The third replication cohort (Italian replication cohort 2) comprised 205 Italian cases with PDB and 238 unaffected controls enrolled from referral centers in northern, central and southern Italy as previously described[27]. The fourth replication cohort comprised 246 individuals with sporadic PDB recruited from various referrals centers in Belgium, and these individuals were matched with 263 controls with no clinical evidence of PDB as previously described[8]. The fifth replication cohort comprised 85 individuals with PDB and 93 controls recruited from various centers in The Netherlands, as previously described[8,28]. The sixth replication comprised 186 cases with sporadic PDB recruited from the Salamanca region in the Castilla-Leon region of Spain and 202 unaffected controls from the same region.

Replication sample genotyping and quality control. Genotyping of replication samples was performed by Sequenom using the MassARRAY iPLEX platform. To minimize genotyping bias caused by variations between runs, DNA from cases and controls from the six different replication cohorts were distributed into 384-well plates so that each plate had the same number of cases and controls. We included 4,000 known genotypes as a quality control measure, and the concordance rate between the genotype calls was >99.8%. We removed 64 samples because of low call rate (<90%), and the call rate for all genotyped SNPs was >95%.

Imputation. Genome-wide genotype imputation for autosomal SNPs was performed using MACH[29], and the HapMap European (CEU) phased haplotype data from release 22 were used as a reference. We excluded SNPs with poor imputation quality based on the estimated correlation between imputed and true genotypes ($r^2 < 0.3$). Additionally, a subset (2%) of known genotypes were masked during imputation, and then imputed genotypes were compared with true genotypes, and the average per allele imputation error rate was 2.9%. Imputed SNPs were tested for association using ProbABEL software[30] implementing a logistic regression model in which the allelic dosage of the imputed SNP was used to adjust for uncertainty in imputed genotypes.

Statistical analysis. Statistical analyses were performed using PLINK (Version 1.07)[31] and R (v2.11.1). In the GWAS stage, genotyped SNPs were tested for association with PDB using a standard allelic (1-degree-of-freedom) χ^2 statistic. We also performed association testing using regression models in which we adjusted for gender and population clusters (as determined by multidimensional scaling analysis), but the results were essentially identical to those obtained from the standard allelic test reported here (data not shown). The genomic inflation factor λ_{GC} was calculated based on the 90% least significant SNPs as described previously[32]. The observed test statistic values were corrected using the genomic control method ($\lambda_{GC} = 1.05$; **Supplementary Fig. 3**). Logistic regression was used to test for the independent effects of SNPs where the allelic dosage of the conditioning SNP was entered as a covariate in the regression model. To assess if the reported associations were confounded by age, age of onset or recruitment center, we performed a regression analysis using case-only data from the GWAS stage to test if any of these factors were associated with the top hits using linear regression models. The results of this analysis showed no evidence to suggest that the reported association is confounded by age, age of onset or recruitment center ($P > 0.10$). The cut-off point for genome-wide significance was set as $P < 5 \times 10^{-8}$, as recently proposed[33]. Association testing of replication data was performed in each replication cohort using a standard (1-degree-of-freedom) χ^2 statistic. To assess combined genetic effects, we performed a meta-analysis of all studies using the inverse-variance method assuming a fixed-effect model. We also tested the random-effects model using the DerSimonian-Laird method[34], and between-study heterogeneity was assessed using the Cochran's Q and I^2 metrics. Heterogeneity was considered significant if $P_{het} < 0.05$. The population attributable risk (PAR) for markers showing association with PDB was calculated according to the following formula:

$$PAR = p(OR - 1)/(p(OR - 1) + 1)$$

where p is the frequency of the risk allele in controls, and OR is the risk allele odds ratio. The cumulative PAR was calculated as follows:

$$\text{cumulative PAR} = 1 - (\Pi_{1 \to n} (1 - PAR_i))$$

where n is the number of variants, and PAR_i is the individual PAR for the ith SNP. The proportion of familial risk attributable to the identified loci was calculated as previously described[35] assuming a multiplicative model of association and a sibling relative risk $\lambda_s = 7.0$, as estimated from previous epidemiological studies[22]. Regional association plots were generated using the locuszoom tool[36].

eQTL analysis. SNPs showing genome-wide significant association with PDB (or those in strong LD; $D' \geq 0.8$) were tested for association with *cis*-allelic expression of gene transcripts located in the associated regions using publicly available eQTL data[24,37–40]. Only *cis*-acting allelic associations located within 250 kb of either 5' or 3' end of the associated gene with expression $P < 1 \times 10^{-5}$ were considered. To avoid false detection, we excluded expression data if the gene probe contained a polymorphic SNP or was located in a highly repetitive sequence.

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STUDY AIM #3

New susceptibility gene causing giant cell tumour in PDB patients

A large pedigree with 14 affected members of whom 4 developed giant cell tumors at pagetic sites was further characterized from both the clinical and the genetic point of view. Of interest all the affected members had polyostotic PDB, but subjects developing giant cell tumors showed an increased disease severity with a reduced clinical response to bisphosphonate treatment and an increased prevalence of bone pain, deformities, and fractures. Together with an increased occurrence of common pagetic complications, affected patients of this pedigree also evidenced a 5-fold higher prevalence of coronary artery disease with respect to either the unaffected family members or a comparative cohort of 150 unrelated PDB cases from the same geographical area. This association was further enhanced in the 4 cases with PDB and giant cell tumors, all of them developing coronary artery disease before 60 years of age. Despite the early onset and the severe phenotype, PDB patients from this pedigree were negative for the presence of *SQSTM1* or *TNFRSF11A* mutations, previously associated with enhanced disease severity. Genome-wide linkage analysis identified 6 possible candidate regions on chromosomes 1, 5, 6, 8, 10, and 20. Since the chromosome 8 and 10 loci were next to the *TNFRSF11B* and *OPTN* genes we extended the genetic screening to these 2 genes but we failed to identify any causative mutation at both genomic and transcription level, suggesting that a different genetic defect is associated with PDB and potentially giant cell tumor of bone in this pedigree. Thus we have planned to apply a next generation sequencing approach to this pedigree in order to identify a new susceptibility gene (Publication E).

These results have been presented also as poster presentation at the ASBMR 2011 Meeting in San Diego [D Merlotti, F Gianfrancesco, D Rendina, T Esposito, A Mingione, D Formicola, R Muscariello, P Strazzullo, R Nuti, L Gennari. Identification of Susceptibility Loci to Giant Cell Tumor and Paget's Disease of

Bone. 2011 ASBMR Annual Meeting, San Diego 16-20 September 2011, abstract

MO0163].

Publication E

ORIGINAL ARTICLE

JBMR

Giant Cell Tumor Occurring in Familial Paget's Disease of Bone: Report of Clinical Characteristics and Linkage Analysis of a Large Pedigree

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ABSTRACT

Neoplastic degeneration represents a rare but serious complication of Paget's disease of bone (PDB). Although osteosarcomas have been described in up to 1% of PDB cases, giant cell tumors are less frequent and mainly occur in patients with polyostotic disease. We recently characterized a large pedigree with 14 affected members of whom four developed giant cell tumors at pagetic sites. The high number of affected subjects across multiple generations allowed us to better characterize the clinical phenotype and look for possible susceptibility loci. Of interest, all the affected members had polyostotic PDB, but subjects developing giant cell tumors showed an increased disease severity with a reduced clinical response to bisphosphonate treatment and an increased prevalence of bone pain, deformities, and fractures. Together with an increased occurrence of common pagetic complications, affected patients of this pedigree also evidenced a fivefold higher prevalence of coronary artery disease with respect to either the unaffected family members or a comparative cohort of 150 unrelated PDB cases from the same geographical area. This association was further enhanced in the four cases with PDB and giant cell tumors, all of them developing coronary artery disease before 60 years of age. Despite the early onset and the severe phenotype, PDB patients from this pedigree were negative for the presence of *SQSTM1* or *TNFRSF11A* mutations, previously associated with enhanced disease severity. Genome-wide linkage analysis identified six possible candidate regions on chromosomes 1, 5, 6, 8, 10, and 20. Because the chromosome 8 and 10 loci were next to the *TNFRSF11B* and *OPTN* genes, we extended the genetic screening to these two genes, but we failed to identify any causative mutation at both the genomic and transcription level, suggesting that a different genetic defect is associated with PDB and potentially giant cell tumor of bone in this pedigree. © 2012 American Society for Bone and Mineral Research.

KEY WORDS: PAGET'S DISEASE OF BONE; GIANT CELL TUMOR; CARDIOVASCULAR DISEASE; GENETICS OF PAGET'S DISEASE OF BONE

Introduction

Paget's disease of bone (PDB, OMIM 602080) is a common skeletal disorder of the elderly characterized by focal abnormalities of bone remodeling.[1] The abnormal bone turnover disrupts normal architecture and structure of single or multiple bones, leading to bone pain, deformity, and the development of various complications including pathological fractures, deafness, nerve entrapment syndromes, and secondary osteoarthritis. Studies have also indicated that patients with PDB have an increased incidence of osteosarcoma in the affected bone.

The etiology of PDB has remained largely unknown for several decades and still has to be conclusively determined. Both a genetic and a viral etiology have been suggested for this disorder.[1–3] Initial studies demonstrated the presence of paramyxovirus material in pagetic osteoclasts, suggesting a viral etiology for the disease.[1–3] However, familial clustering has been clearly recognized in up to 40% of cases, and at least seven potential susceptibility loci for the disease have been identified

1

by genome-wide searches and candidate locus linkage studies.[2,4] Within most families, the disease is inherited as an autosomal dominant trait with genetic heterogeneity and incomplete penetrance.

In 2000, Hughes and colleagues identified a mutation on the *TNFRSF11A* gene, encoding receptor activator of NF-κB (RANK) in a single family with severe, early onset, polyostotic PDB.[5] This mutation was similar to those reported in familial expansile osteolysis, a rare autosomal dominant skeletal disorder related to PDB.[5,6] However, further screening in different populations excluded mutations in *TNFRSF11A* as a common cause of PDB.[7] Of interest, since 2002 mutations in a different gene, *SQSTM1*, have been identified in up to 10% and 40% of sporadic and familial PDB cases, respectively.[8] This gene encodes the p62/sequestosome 1 protein, which acts as a scaffold protein in the NF-κB pathway as well as an intermediate protein in the proteosomal degradation of polyubiquitined proteins. Even though patients with *SQSTM1* mutations generally show an increased disease severity than *SQSTM1*-negative patients,[9,10] we recently identified *SQSTM1*-negative patients with a severe phenotype and the presence of peculiar phenotypic characteristics, including the occurrence of giant cell tumors (GCT) originating from affected skeletal sites.[9,11,12] This complication represents a very uncommon clinical feature of PDB (described in less than 100 cases worldwide), and mainly occurs in patients with severe polyostotic PDB.[11–15] GCTs may be multifocal and aggressive, leading to increased morbidity and mortality of patients. Patients with extensive, recurrent, and/or biologically more aggressive tumors may require wide excision, and often do not respond to antiresorptive compounds commonly in use to treat PDB such as calcitonin or bisphosphonates.

Importantly, both familial clustering and the evidence that GCT occurs with higher prevalence in PDB patients from Campania (accounting for up to 50% of all GCT cases), even if they have lived for several years in other countries, strongly suggest the hypothesis of a genetic factor in the etiology of this complication.[13–15] However, some studies demonstrated the presence of the typical nuclear inclusions seen in PDB in the ultrastructure of the giant cells, suggesting a viral etiology.[16]

We recently characterized a large Italian pedigree with 14 affected members of whom four developed GCT, which despite the early onset and the severe phenotype were negative for the presence of *SQSTM1* and *TNFRSF11A* mutations.[9,17] The high number of affected subjects across multiple generations (clinically followed from 1977 to date) allowed us to better characterize the clinical phenotype and look for possible susceptibility loci. A mutation screening of other candidate genes encoding for components of the RANKL/OPG/RANK/NF-kB signaling pathway (namely *TNFSF11A* and *TNFRSF11B* genes) or previously associated with PDB-related syndromes (namely *VCP*, encoding for valosin containing protein) or classical PDB in *SQSTM*-negative patients (*OPTN* gene, encoding for optineurin, and *CSF1*, encoding for MCSF-1) was also performed.

Materials and Methods

Pagetic pedigree

The pedigree of the examined family is reported in Fig. 1. All family members were born in Campania, and the majority of them were born in a rural area in the surroundings of Avellino, approximately 40 miles from Naples, within the region at the highest prevalence rate for the occurrence of GCT in PDB.[11,13,15] Only subjects IV-2, IV-3, IV-4, and their children were born in Naples, outside that region. The diagnosis of PDB was confirmed in all patients by clinical examination, [99m]techenetium methylene diphosphonate ([99m]TC-MDP) bone scan, and subsequent X-ray examination of areas of increased isotope uptake. Moreover, all family members underwent a biochemical screening of total alkaline phosphatase (ALP) by standard techniques. In patients III-1, III-3, and IV-3, the PDB diagnosis was performed after the admission of their affected first-degree relatives in the Department of Clinical and Experimental Medicine of the "Federico II" University. An initial clinical screening of patients V-8 and V-9 patients, performed in July 2005 using a [99m]Technetium methylene diphosphonate bone scan and the measurement of total and bone-specific alkaline phosphatase serum levels, was not indicative for PDB.
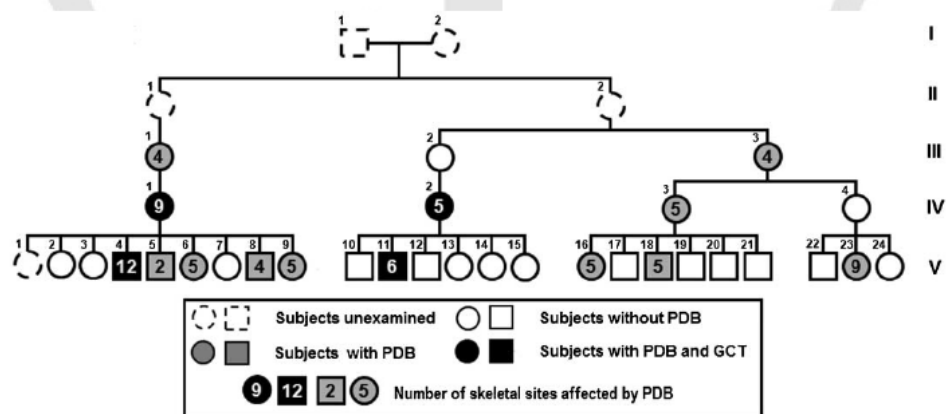

Fig. 1. Pedigree of the PDB/GCT family.

However, a subsequent screening performed in 2010 for the occurrence of bone pain demonstrated the presence of polyostotic PDB in both family members. All nonaffected subjects from generations III to V underwent a ^{99m}TC-MDP bone scan to exclude the presence of asymptomatic PDB. As shown in Fig. 1, four of the 14 PDB patients developed GCT in one or more affected bones. Clinical characteristics of these patients developing GCT were previously described[15] and are summarized in Table 1 and Fig. 2. Briefly, patient IV-02 developed a single GCT lesion of the jaw at the age of 59 years and did not respond to calcitonin or intravenous clodronate treatment. Then she underwent surgical curettage and subsequent radiation (external beam cobalt-60 with a total dose of 40 Gy during 6 weeks), with marked clinical improvement and resolution of the facial swelling. Conversely, the other cases (IV-1, V-4, and V-11) developed multifocal GCT with unsatisfactory clinical response to either calcitonin or bisphosphonate treatment (intravenous clodronate or pamidronate) and variable response to radiotherapy. In particular, patient V-4 (Fig. 2A–E) developed three GCT lesions of the skull (of the parietal, temporal, and occipital regions, respectively) and did not respond to multiple radiotherapy cycles (50 Gy). He died at the age of 61 years (after 9 years from the diagnosis of GCT) resulting from cerebral ischemia. All these patients had received previous bisphosphonate treatment (clodronate intravenously) at the time of GCT diagnosis, with partial biochemical response and total ALP levels well above the normal range (Table 1).

A specific questionnaire exploring the place of birth and residence, place of residence during childhood and adolescence, housing, animal contacts, dietary habits, occupation, and pharmacological history was performed in all patients. A detailed medical history including the record of common complications of PDB and other comorbidities was also performed in affected family members. When available, information from echocardiography and carotid Doppler ultrasound was recorded, particularly concerning the occurrence of signs of high cardiac output and the presence of valvular or carotid artery calcifications. When more than one analysis (performed at different ages) was available from a single patient, the closest one to the date of PDB diagnosis was selected. Cardiac index (a vasodynamic parameter that relates the cardiac output to body surface area) was calculated as a major index for high cardiac output.[18] The study was approved by the local ethical committee, and all subjects had given informed consent to being included.

The general and clinical characteristics of patients from this pedigree were compared with those observed in 150 consecutive and unrelated PDB patients living in the same geographical region who were referred to the Department of Clinical and Experimental Medicine of "Federico II" Medical School of Naples.

Candidate gene analysis

After excluding SQSTM1(p62) and TNFRSF11A (RANK) mutations, we conducted mutation screening of all exons of other candidate genes (TNFRSF11B [OPG], TNFSF11A [RANKL], and VCP [p97]). Genomic DNA of four affected patients was specifically amplified using several sets of primers designed to amplify each of the coding exons of all five genes and was analyzed by direct sequencing. Given the results from linkage analysis in SQSTM1-negative PDB familes[19] and of two genome-wide association studies[20,21] suggesting a possible susceptibility locus in 10p13 within a region encoding for optineurin (OPTN) and an additional locus next to the CSF1 gene, we also searched for possible OPTN or CSF1 mutations in affected members of our pedigree.

Linkage analysis

Genomic DNA from seven affected and three unaffected members were hybridized on the Affymetrix (Santa Clara, CA, USA) Genome-Wide Human SNP Array 6.0 according to the manufacturer's protocol. This array contains more than 1.8 million genetic markers, including more than 906,600 single nucleotide polymorphisms (SNPs) and more than 946,000 probes for the detection of copy number variation (CNV). Genotype of each SNP was generated with Birdseed v2. Quantile normalization was performed at probe level on the whole data set (sample + 240 references). For each single marker (SNP or CNV), the ratio in log 2 scale between the sample and reference set was then calculated.

Results

General and clinical characteristics of affected members with and without GCT

All family members reported animal contacts for at least 10 years before the clinical onset of PDB (without any difference between PDB cases with or without GCT). These included pet ownership (cats and dogs) and contacts with pigs and rabbits. All examined subjects also referred the recurrent use of unpasteurized milk and of fresh, homemade meat products without sanitary controls, without any difference between affected and nonaffected family members. All cases affected by PDB showed a polyostotic disease (sites affected mean 5.7 ± 2.6, median 5, range 2 to 12) with a preferential localization in the skeletal axial bones (skull 12/14, 85.7%; pelvis 12/14, 85.7%; and vertebrae 13/14, 92.8%). As is evident in Table 2, there was no decrease in disease severity (as expressed by total ALP levels and the number of affected skeletal sites) across generations. On the contrary, there was an apparent decrease in age at diagnosis from generation III to generation V, likely owing to the extension of detailed clinical analysis in all family members of generation V, at a younger age than in generations III and IV.

Pagetic patients who developed GCT had an increased number of affected skeletal sites than patients without GCT (8.7 ± 2.9 versus 4.8 ± 1.7, $p < 0.0001$), without significant differences in age at diagnosis (44.2 ± 9.3 versus 52.9 ± 17.1, $p = 0.36$). ALP levels were also significantly higher in patients with GCT than in patients without GCT (1592.7 ± 646.8 versus 500.5 ± 296.1 IU/L, $p < 0.001$). Moreover, as shown in Table 2, there was an increased prevalence of bone pain (100% versus 60%), bone deformity (100% versus 30%), and fractures at pagetic sites (50% versus 10%) in PDB patients with GCT than in those without GCT. These differences reached statistical significance concerning bone deformities ($p < 0.05$ Fisher's exact test). Of interest, the prevalence of coronary heart disease

70

Table 1. Clinical Characteristics and History of Paget's Disease of Bone (PDB) Cases With Giant Cell Tumor (GCT)

	Patient IV-01	Patient V-02	Patient V-04	Patient V-11
Age at PDB diagnosis (years)	57	44	35	41
Age at first GCT diagnosis (years)	67	58	51	51
ALP at GCT diagnosis (IU/L)	6940	4224	3200	3259
Sites affected by GCT	Mandible (67 years) Skull (77 years) Maxilla (78 years)	Mandible (58 years)	Parietal skull (51 years) Temporal skull (51 years) Occipital skull (51 years)	Right clavicle (51 years) Mandible (52 years) Pelvis (55 years) Lumbar spine (56 years) Maxilla (58 years)
Medical therapy	Calcitonin iv (for 3 years) CLN iv (for 6 years) PAM iv (for 1 years)	Calcitonin iv (for 3 years) CLN iv (for 16 years)	Calcitonin iv (for 3 years) CLN iv (for 4 years) PAM iv (for 5 years)	CLN iv (for 10 years) PAM iv (for 1 years) NER iv (for 4 years) ZOL iv (for 2 years)
Radiotherapy	1 cycle (45 Gy, mandible) 1 cycle (41 Gy, skull) 1 cycle (45 Gy, maxilla)	1 cycle (40 Gy)	3 cycles (50 Gy)	No radiotherapy
Surgery	Surgical curettage (mandible)	Surgical curettage	No surgery	Surgical curettage (clavicle) Surgical curettage (mandible) Laminectomy (lumbar spine)
Age at death (years)	80	76	61	58
Cause of death	Myocardial infarction	Myocardial infarction	Cerebral ischemia	Hearth failure (2 years after a myocardial infarction)

ALP = alkaline phosphatase; CLO = clodronate; PAM = pamidronate; .

Show panels A and B in black and white instead of color.
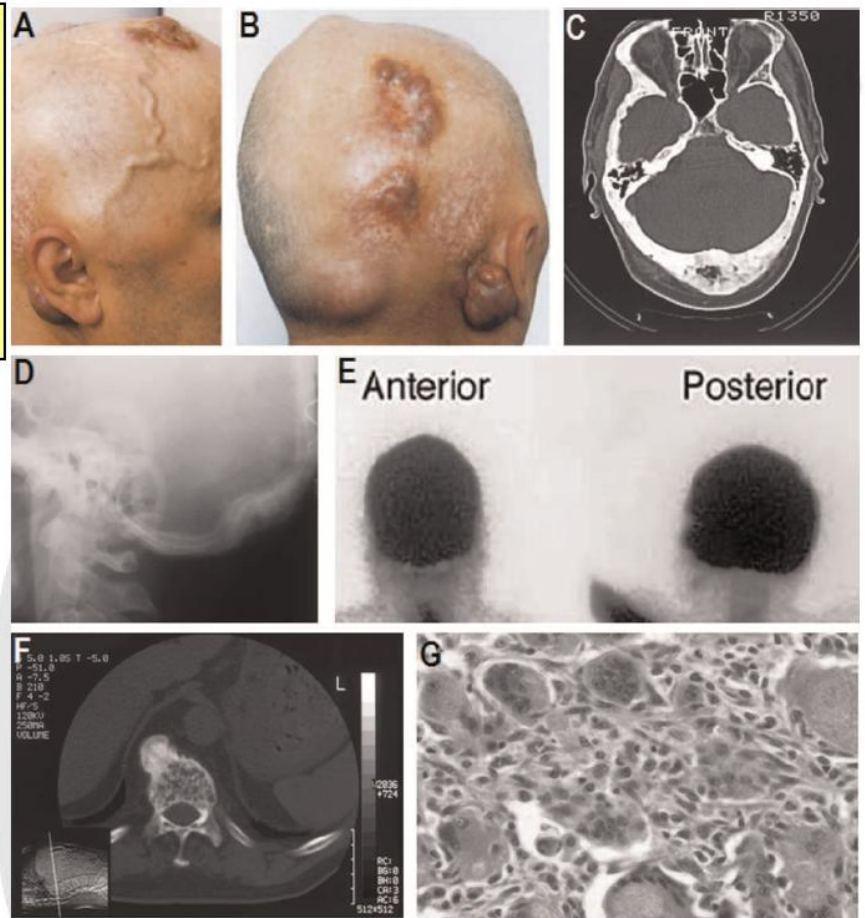
Fig. 2. Phenotype characteristics of two patients with GCT complicating PDB. Patient V-04: (*A, B*) pictures showing dilated scalp veins and multiple giant cell tumors of the skull; (*C*) CT scan and (*D*) radiograph of the skull showing an occipital giant cell lesion originating from pagetic bone; (E) anterior and posterior bone scan images showing marked and diffuse radioisotope captation of the entire skull. Patient V-11: (*F*) CT scan showing the giant cell lesion originating from the vertebral body of L_4; (*G*) Bone biopsy showing the typical aspect of the giant cell tumor with numerous osteoclast-like giant cells.

was also higher in patients with GCT than in PDB patients without GCT or nonaffected family members (100%, 40%, and 6%, $p < 0.001$ in PDB/GCT, PDB, and nonaffected members, respectively). This difference was also evident in generation V (100%, 57%, and 0%, $p < 0.001$ in PDB/GCT, PDB, and nonaffected members, respectively), despite the relatively younger age of subjects. The onset of coronary heart disease was lower in patients with PDB complicated by GCT than in patients with PDB without GCT (55.0 ± 8.4 versus 61.2 ± 11.1 years, respectively, $p = 0.38$) with an overall age of onset of 58.4 ± 9.9 years. Moreover, all four PDB cases with GCT had myocardial infarction or died from cardiovascular complications. Subjects affected by PDB (with or without GCT) died at a younger

age than nonaffected family members, with a difference approaching statistical significance (73.0 ± 10.1 versus 86.5 ± 4.8, respectively, $p = 0.09$).

Fig. 3 summarizes the variation of ALP levels in PDB cases with or without GCT from the diagnosis to the last follow-up analysis. Importantly, GCT occurred in those patients who did not respond to repeated treatment courses with calcitonin and/or bisphosphonates and in a condition of persistent active disease. Conversely, patients who responded to treatment with a marked reduction in ALP activity did not develop GCT. Moreover, the occurrence of GCT was associated with a consistent increase in ALP levels, as previously described in the case of osteosarcomas complicating PDB.[22]

72

Table 2. Clinical Characteristics and Complications of Family Members Affected by Paget's Disease of Bone (PDB) and Giant Cell Tumor (GCT) or PDB Alone
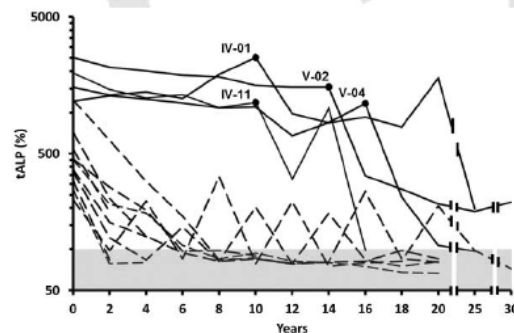
	PDB with GCT				PDB									
	IV-1	IV-2	V-4	V-11	III-1	III-3	IV-3	V-5	V-6	V-8	V-9	V-16	V-18	V-23
Age at diagnosis (years)	57	44	35	41	70	74	75	38	46	60	59	41	41	25
Total alkaline phosphatase (%)	1536	2523	1207	1950	457	386	541	231	371	276	321	724	1241	457
Affected skeletal sites (n)	9	5	12	6	4	4	5	2	5	4	5	5	5	9
Bone pain	+	+	+	+	−	−	+	+	+	−	+	+	−	+
Bone deformity	+	+	+	+	+	+	−	−	−	−	−	−	+	−
Fractures	+	−	−	+	−	−	−	−	−	−	−	−	+	−
Osteoarthritis	+	−	−	−	−	−	+	+	+	+	+	−	−	−
Hearing loss	−	−	+	−	+	+	−	−	−	−	−	−	+	+
Nephrolitiasis	−	−	−	+	−	−	+	−	−	−	−	−	−	−
Neurological complications	+	−	−	−	−	+	−	−	−	−	−	−	+	−
Diabetes	−	−	−	−	−	+	−	−	−	−	−	−	−	−
Hypertension	−	−	−	−	−	−	+	+	+	+	+	−	+	−
Coronary artery disease	+	+	+	+	−	−	−	+	−	−	+	+	+	−
Myocardial infarction	+	+	−	+	−	−	−	−	−	−	−	−	−	−

Total alkaline phosphatase was expressed as percent increase from the upper normal limit.

Clinical characteristics of PDB patients from this pedigree were compared with those observed in a cohort of 150 unrelated familial and sporadic PDB cases from the same geographic region (Table 3). Overall, the affected members of the pedigree showed an increased number of affected skeletal sites and an increased prevalence of polyostotic disease than either sporadic or familiar PDB cases from the same region. Moreover, the mean number of PDB complications per patient was significantly elevated in affected members of this pedigree than in the unrelated group of PDB patients (2.09 ± 0.9 versus 1.96 ± 0.8 versus 2.64 ± 1.3, in sporadic PDB versus familial PDB versus PDB/GCT pedigree, respectively, $p < 0.05$ ANOVA). Even though some PDB complications such as bone pain, fractures, nephrolitiasis, and neurological syndromes were more represented in the PDB/

Fig. 3. Temporal occurrence of GCT in relation to variation in ALP (expressed as percent increase from the upper normal limit). Time 0 indicates the time of PDB diagnosis. The black circles indicate the temporal occurrence of GCT in the four members of the pedigree (IV-01, IV-11, V-02, and V-04). Dotted lines indicate the variation in ALP in PDB members from the pedigree who did not develop GCT.

GCT pedigree than in the group of sporadic or familial unrelated patients, these differences did not reach the threshold for statistical significance. Conversely, a statistically significant increased prevalence of coronary artery disease was observed in patients from the PDB GCT pedigree (57.1%) than in the reference group of sporadic (11.7%) or familial (13.3%) PDB cases (p for trend <0.01).

To provide further insight into the relationship between PDB and the occurrence of cardiovascular complications, information concerning the presence of carotid artery calcifications, valvular calcifications, and echocardiography parameters was obtained from clinical records of patients from the pedigree. Echocardiography data were available in 12/14 cases from the PDB/GCT pedigree (12/12 in generations IV and V) and were compared with those obtained from 20 unrelated polyostotic PDB patients from Campania. All four cases with GCT underwent the echocardiography screening 3 to 5 years before the occurrence of cardiovascular complications. Of interest, the cardiac index was higher in PDB cases from the pedigree than in the other PDB patients (3.77 ± 0.72 l/min/m^2 versus 2.92 ± 0.82 l/min/m^2, $p < 0.01$), and particularly in those family members who developed GCT (4.44 l/min/m^2). Cases from the pedigree also presented an increased prevalence of left ventricular hypertrophy (75% versus 25%, $p < 0.01$) and valvular calcifications (42% versus 20%, $p = 0.09$) than the group of unrelated PDB patients. Moreover, a significant and positive correlation between cardiac index and ALP levels at the time of echocardiography ($r = 0.43$, $p < 0.05$) or the number of affected skeletal sites ($r = 0.51$, $p < 0.01$) was observed in the overall population of the 12 PDB/GCT and 20 unrelated PDB cases. The correlation between cardiac index and ALP was increased when analysis was restricted to cases from the PDB/GCT pedigree ($r = 0.58$, $p < 0.05$), whereas it decreased and became not statistically significant in the 20 unrelated PDB patients ($r = 0.23$, $p = 0.3$). Results of carotid Doppler ultrasounds were available from all 14

73

Table 3. Comparison of Clinical Characteristics of Cases From the Paget's Disease of Bone (PDB)/Giant Cell Tumor (GCT) Pedigree and PDB Patients From the Same Geographical Area

	PDB from Campania		
	Sporadic	Familial	PDB/GCT family
No. of cases	120	30	14
Males/females (n)	67/53	16/14	5/9
Age at diagnosis (years)	57.6 ± 10.6	54.7 ± 9.7	50.4 ± 15.4
Affected family members (n)	—	2.93 ± 1.2***	14
Monostotic/poliostotic	34/86*	5/25	0/14
Affected sites (n)	2.80 ± 1.9***	4.00 ± 2.4*	5.93 ± 2.7
Bone pain	54 (45.0%)*	20 (66.7%)	11 (78.6%)
Bone deformity	74 (61.7%)	19 (63.3%)	7 (50.0%)
Fractures	9 (7.5%)	3 (10.0%)	3 (21.4%)
Osteoarthritis	49 (40.8%)	12 (40.0%)	6 (42.8%)
Hearing loss	34 (28.3%)	6 (20.0%)	4 (28.6%)
Nephrolitiasis	10 (8.3%)	4 (13.3%)	3 (21.4%)
Neurological complications	21 (17.5%)	5 (16.7%)	3 (21.4%)
Diabetes	14 (11.7%)	4 (13.3%)	2 (14.3%)
Hypertension	39 (32.5%)	9 (30.0%)	6 (42.8%)
Coronary artery disease	14 (11.7%)***	4 (13.3%)**	8 (57.1%)
Myocardial infarction	3 (2.5%)*	2 (6.7%)	3 (21.4%)

*$p < 0.05$; **$p < 0.01$; and ***$p < 0.001$ vs. PDB/GCT family.

affected members of the pedigree and from the group of 20 unrelated PDB patients. Consistent with the echocardiography findings, affected family members of the pedigree had an increased prevalence of atherosclerotic plaques than the other PDB cases (78% versus 35%, $p < 0.05$). A trend for an increase in vascular calcifications was also observed (50% versus 25%, $p < 0.10$).

Genetic analysis

As is evident in Fig. 1, both PDB and GCT were inherited as autosomal dominant traits. In previous analysis, all the affected members of the pedigree were screened for mutations in the entire coding regions of *SQSTM1* and *TNFRSF11A* genes, failing to detect any genetic alteration.[9,17] In the current candidate gene analysis, we now excluded the presence of mutations in five additional genes: *OPTN* encoding for optineurin, *TNFSF11A* encoding for RANKL (the ligand of RANK), *CSF1* encoding for M-CSF, *TNFRSF11B* encoding for OPG (the decoy receptor of RANKL), and *VCP* encoding for valosin-containing protein. Mutations in the latter two genes were previously associated with juvenile PDB (also known as idiopathic hyperphosphatasia) and the syndrome of hereditary inclusion body myopathy-PDB-frontotemporal dementia (IBMPFD), respectively.[23–25]

As shown in Table 4, genome-wide screening allowed us to identify five possible candidate regions containing putative genes predisposing to PDB on chromosomes 8 (39 Mb between rs278559 and rs2280871), 5 (50 Mb between rs12514992 and rs17597145; 15 Mb between rs353287 and rs10462946), 6 (56 Mb from 6pter), 20 (2 Mb near the SNP rs11905231), and 1 (47 Mb between rs12142090 and rs6702754). Assuming a dominant model and a 100% penetrance, parametric analysis resulted in

Table 4. Summary Results From Parametric (P) and Nonparametric (NP) Linkage Analysis of the Pedigree

Locus	Region (cM)	Size	Linkage	Interval
chr 8	94324446-133791851	39 Mb	P	rs278559-2280871
chr 5	75554502-125888955	50 Mb	P	rs12514992-rs17597145
	148770478-164095625	15 Mb	P	rs353287-rs10462946
chr 6	110391-56774326	57 Mb	P	rs4959515-rs9382665
chr 20	5127693-7128056	2 Mb	P	rs1292244-rs6085920
chr 1	104184672-151117049	47 Mb	P	rs12142090-rs6702754
chr 10	24006978-24063687	57 Kb	NP	rs4491127-rs11013680
	30784089-30870635	86 Kb	NP	rs650788-rs3124176
	100584411-100919720	335 Kb	NP	rs10883215-rs2796755
chr 8	76976762-77100149	124 Kb	NP	rs17321328-rs830437
	10424956-120586912	16 Kb	NP	rs2447179-rs17794271

74

LOD score of 2.06 on chromosome 8 (94 to 133 Mb). Conversely, nonparametric analyses revealed most suggestive linkage on chromosomes 10 (Zmax = 6,2 at 20 to 30 Mbp from 10pter) and 8 (Zmax = 6,2 at 120 Mb). Given the consistency of the former association with some previous studies in *SQSTM1*-negative patients,[19–21] we performed a genetic screening of the *OPTN* and *CSF1* gene loci (see above), but we failed to identify any causative mutation at both the genomic and transcription levels.

Discussion

Epidemiological and clinical evidence clearly indicated a higher prevalence of neoplastic degeneration in PDB, with up to 1% increased risk of developing osteosarcomas at pagetic skeletal sites.[22,26] GCT is also a very rare but well-recognized neoplastic complication of PDB, accounting for only a small proportion of all neoplasms arising from pagetic bone.[16] All GCTs complicating PDB occur exclusively in bone affected by the disease and generally differ in the age of onset and the skeletal localization with respect to GCTs occurring in nonpagetic subjects.[16,27,28] In fact, GCT usually occurs in PDB patients older than 50 years and involves the craniofacial bone, the humerus, the femur, the pelvis, or the vertebrae. Conversely, nonpagetic GCT generally affects patients from 20 to 40 years of age and involves predominantly the distal femur, the proximal tibia, or the distal radius.[16,27,28] Of interest, the skeletal sites more frequently affected by GCT in PDB patients were those preferentially affected by PDB in subjects with familial disease from Campania.[12] Moreover, multicentric giant cell tumors arising in PDB patients have been described in 11 cases, including those presented in this study.[15,16,27–30] In the international literature, up to 50% of cases of CGT complicating PDB have been reported in patients from Campania or with ancestry in this geographical area.[11–16,27]

Despite the description of different GCT cases occurring in PDB, the clinical phenotype of patients with this complication has not been fully investigated. Moreover, at this stage, the evidence for a familial clustering on the occurrence of neoplastic PDB complications is virtually limited to a few patients from Campania. In fact, other than our patients and those described by Jacobs and colleagues,[13] only Wu and colleagues described neoplastic degeneration in 2 of 3 family members of a PDB family from Castellammare di Stabia (about 30 miles from Avellino), with long-standing polyostotic disease. However, in this case, the two subjects developed osteogenic sarcoma, not GCT.[31]

In this study, we extend the knowledge about this rare complication. In fact, we clearly evidenced an increased disease severity in our pedigree of PDB/GCT patients and particularly in the four patients who developed GCT. Moreover, in contrast to the recent evidences from other PDB cohorts, we did not find a decrease in both clinical extension and severity of the disorder in the last generation with respect to the previous generations. In a previous study, we demonstrated that the region of Campania is associated with an enhanced clinical severity of PDB with respect to the other regions,[8] and thus we preferred to compare the clinical characteristics of patients from this PDB/GCT pedigree to a cohort of unrelated patients from the same region. This also reduced the potential bias related to a different genetic background or lifestyle between the PDB/GCT pedigree and the reference cohort of PDB cases. Of interest, in this pedigree, in addition to an increased occurrence of common pagetic complications, we also evidenced a fivefold higher prevalence of coronary artery disease with respect to the cohort of unrelated PDB cases. This association has not been previously reported in the literature and might be owing to either disease activity (because of enhanced skeletal extension) or to a mutation in a new gene simultaneously affecting the skeleton and the cardiovascular system. Indeed, total ALP levels at diagnosis were significantly higher in subjects developing coronary artery disease than in the other PDB cases from the same pedigree (1111 ± 631 versus 414 ± 91; $p < 0.05$, respectively), partly supporting the former hypothesis. Moreover, an increase in the cardiac index was observed in cases from the pedigree rather than in the group of unrelated PDB patients, with levels above the threshold for high output cardiac state (>3.9 l/min/m^2) in most members. Notably, cardiac index was also positively correlated with the extension of the disorder (as assessed by the number of affected skeletal sites) and disease activity (as reflected by ALP levels at the time of echocardiography). A similar behavior was observed concerning GCT because at the time of the occurrence of this complication, all four cases showed a limited response to treatment and a persistent active disease, with ALP levels well above the normal range. Taken together, these data suggest that the persistence of a state of active PDB might increase the risk of GCT and cardiovascular complications, at least in genetically predisposed subjects such as those from this GCT/PDB pedigree.

Of interest, current and previous[9,17] candidate gene analysis in this pedigree excluded the presence of mutations in all the major genes associated with PDB or PDB-related syndromes, suggesting that a different genetic defect is associated with PDB and potentially GCT. In keeping with our observation, a recent study in a North-American cohort of PDB patients excluded the presence of *SQSTM1* mutations in three cases of Italian origin who developed GCT.[35] Genome-wide linkage analysis identified different genomic regions potentially associated with the occurrence of the disorder in our pedigree. In particular, two regions on chromosomes 10 and 8 were significantly linked to PDB occurrence in this family. It is interesting to note that the region of chromosome 10p is very close to a recently identified locus in either familial and sporadic PDB cases negative for *SQSTM1* mutation. In a first study of families of British ancestry without *SQSTM1* mutations, multipoint parametric linkage analysis showed strong evidence of linkage to a single locus on chromosome 10p13 close to the marker D10S1653.[19] More recently, a genome-wide association study in 750 sporadic PDB cases without *SQSTM1* mutations and 1002 controls identified three candidate disease loci, replicated in an independent set of 500 cases and 535 controls.[20] One of these loci was located on chromosome 10p13. Three SNPs (rs1561570, rs825411, and rs2095388), all located within a 30-kb region, were analyzed in both stages of the study, and the strongest signal was observed for SNP rs1561570.[20] These findings were replicated in a larger cohort of PDB patients from different countries.[21] *OPTN*, a candidate gene located in this region, negatively regulates TNFα-

induced NF-κB activation, and a putative NF-κB binding site has been identified in the *OPTN* promoter.[20] We identified a second interesting locus on chromosome 8, near *TNFRSF11B* gene (encoding OPG), the gene associated with juvenile PDB (JPD; OMIM 239000).[23,24] This association was suggested by both parametric and nonparametric analysis. However, we did not identify any mutation in both *TNFRSF11B* and *OPTN* genes, indicating that genetic variation in other genes located in these regions might confer susceptibility to PDB and possibly to GCT. Alternatively, mutations in a yet unidentified gene within the other genomic regions of chromosomes 1, 5, 6, or 20 might be responsible for the susceptibility to PDB in this pedigree.

Given the peculiar familial and geographic clustering of GCT in patients with PDB, it is also likely that only particular variations within this region may be associated with the occurrence of GCT, alone or in combination with other triggers (either environmental or genetics). Although further work will be required to identify the functional variant as well as the pathogenetic mechanism, the current study has provided new insights into the clinical phenotype and the genetic cause of GCT in patients PDB.

Disclosures

Acknowledgments

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STUDY AIM #4

**Long term efficacy of bisphosphonate treatment in PDB and possible**

pharmacogenetic implications.

To fulfil this aim, we performed a pilot study, analyzing *SQSTM1* mutations in 90

patients with active PDB involved in a comparative trial with intravenous amino-

bisphosphonates [111]. At baseline, patients were randomly assigned to receive

pamidronate (30 mg, iv, for 2 consecutive days every 3 months; n=60) or

zoledronate (4 mg, iv; n=30). After 6 months, 33/60 patients in pamidronate group

did not respond to treatment and were crossed over to zoledronate 4 mg (n=18) or

neridronate (100 mg, iv, for 2 consecutive days, n=15). Follow-up analysis has

been extended to 36 months in all treatment groups. No bisphosphonate was given

during the extension study (12 to 36 months) except in case of relapse. *SQSTM1*

gene analysis revealed the presence of 4 different mutations (Y383X, P392L,

E396X, M404V) in 18/90 patients. At baseline, patients with *SQSTM1* mutation

showed an increased severity of disease with a higher number of affected skeletal

sites and higher alkaline phosphatase levels than patients without mutation.

Interestingly, an increased proportion of patients with *SQSTM1* mutation showed

resistance to pamidronate at 6 months (11/13, 85% vs. 22/47, 47% in patients

without mutation, p=0.02) (Fig.12).
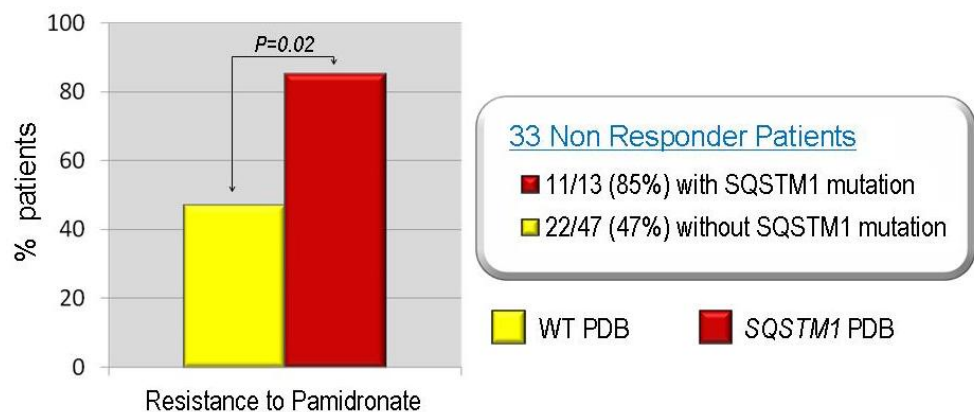
Fig. 12. Resistance to intravenous pamidronate treatment at 6 months in wild type PDB patients or
SQSTM1 mutated PDB patients *(Merlotti D et al ASBMR 2009, #1031)*

Conversely there was no significant difference in the response to zoledronate between patients with or without mutation at all time points from 6 to 36 months. Overall, therapeutic response to zoledronate was achieved in 97%, 83% and 69% of patients at 12, 24, and 36 months from infusion, respectively (Fig.13).
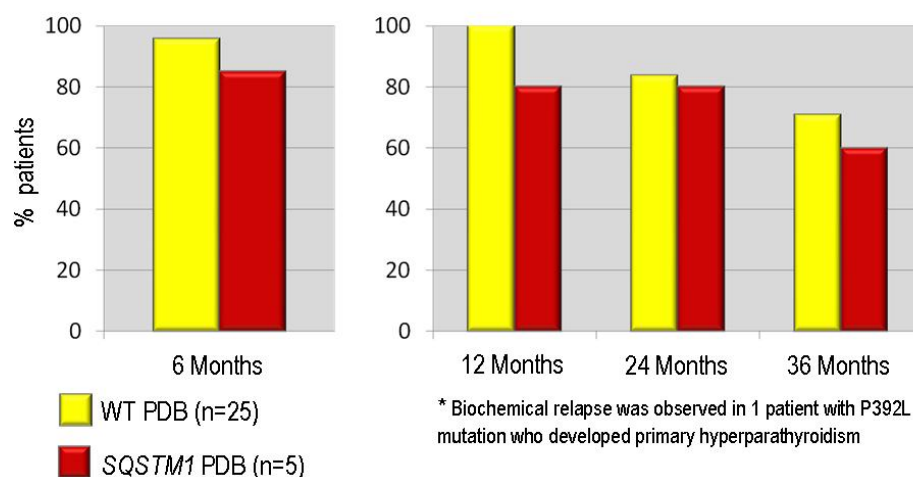
Fig. 13. Long term therapeutic response to intravenous zoledronate treatment in wild type PDB patients or *SQSTM1* mutated PDB patients *(Merlotti D et al ASBMR 2009, #1031)*

Patients with recurrence of disease were treated with a new zoledronate 4 mg infusion, and all achieved therapeutic response. Among non-responders patients to pamidronate, 93% in the neridronate group and 94% in the zoledronate group achieved therapeutic response after 6 months from cross-over. Response was maintained in 82%, 53% and 41% of patients with neridronate and in 94%, 83% and 67% of patients with zoledronate at 12, 24 and 30 months from cross-over, respectively. All the 3 patients with *SQSTM1* mutation sustained clinical relapse between 24 and 30 months from cross-over to neridronate. These results suggest that PDB patients with *SQSTM1* mutation may require a more aggressive treatment regimen for disease remission. We are actually collecting the available retrospective clinical information from all cases of the Italian PDB Registry in order to confirm these preliminary and interesting observations.The results from the pilot study were presented as an oral communication at the ASBMR 31st Annual Meeting in 2009 [D Merlotti, L Gennari, F Gianfrancesco, G Mossetti, D Rendina, T Esposito, G Martini, P Strazzullo, R Nuti. Long term effects of

intravenous bisphosphonates in Paget's Disease of bone and interaction with

SQSTM1 mutations. ASBMR 31st Annual Meeting, Denver 11-15 September 2009,

abstract 1031].

Following the positive indications of the comparative study on different

intravenous regimens we decided to perform a small trial aimed to evaluate the

long term efficacy of an intramuscular neridronate regimen, given with an identical

cumulative dosage (200 mg) of the intravenous dosage, but in 8 weekly

administrations. Briefly, 56 patients with active PDB were randomized to receive

neridronate as intravenous (100-mg infusion for 2 consecutive days) or

intramuscular (25-mg injection weekly for 2 months) regimen, and followed for 36

months. All patients were advised to receive calcium plus vitamin D

supplementation throughout the study period. At 6 months, 92.6% and 96.5% of

patients receiving intravenous and intramuscular neridronate, respectively,

achieved a therapeutic response [defined as normalization of alkaline phosphatase

(ALP) levels or a reduction of at least 75% in total ALP excess]. The response to

treatment was significantly correlated with baseline ALP and 25-hydroxyvitamin D

[25(OH)D] levels at 6 months. The decrease in ALP levels was highest in patients

with higher baseline total or bone specific ALP levels and with higher 25(OH)D

levels at 6 months. Response rates were maintained at 12 months but decreased

progressively at 24 and 36 months without significant differences between the two

neridronate regimens. Both regimens were well tolerated. The only relevant side

effect was an acute-phase response occurring in 14% of the patients. (Publication

F).

JBMR

Comparison of Intravenous and Intramuscular Neridronate Regimens for the Treatment of Paget Disease of Bone

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ABSTRACT

Aminobisphosphonates actually represent the most common treatment for Paget disease of bone (PDB). In a previous study we demonstrated that either zoledronic acid (4 mg) or neridronate (200 mg) given as a single intravenous infusion showed a similar short-term efficacy in achieving biochemical remission in up to 90% of patient nonresponders to pamidronate. In this study we compared the long-term (36 months) effects of a same neridronate dose (200 mg) given as an intravenous (100-mg infusion for 2 consecutive days) or intramuscular (25-mg injection weekly for 2 months) regimen in 56 patients with active PDB. All patients were advised to receive calcium plus vitamin D supplementation throughout the study period. At 6 months, 92.6% and 96.5% of patients receiving intravenous and intramuscular neridronate, respectively, achieved a therapeutic response [defined as normalization of alkaline phosphatase (ALP) levels or a reduction of at least 75% in total ALP excess]. The response to treatment was significantly correlated with baseline ALP and 25-hydroxyvitamin D [25(OH)D] levels at 6 months. The decrease in ALP levels was highest in patients with higher baseline total or bone-specific ALP levels and with higher 25(OH)D levels at 6 months. Response rates were maintained at 12 months but decreased progressively at 24 and 36 months without significant differences between the two neridronate regimens. Both regimens were well tolerated. The only relevant side effect was an acute-phase response occurring in 14% of the patients. In conclusion, these results indicate that a 200-mg intramuscular neridronate course has a similar efficacy as an intravenous infusion of the same dose for the treatment of PDB and might be of particular value for patients intolerant to oral bisphosphonates and unwilling or unable to undergo intravenous infusions. © 2011 American Society for Bone and Mineral Research.

KEY WORDS: PAGET DISEASE OF BONE; NERIDRONATE; INTRAMUSCULAR REGIMEN; INTRAVENOUS REGIMEN; BISPHOSPHONATES

Introduction

Paget disease of bone (PDB) is a chronic disorder of bone remodeling affecting up to 1% to 5% of the elderly population, which typically results in enlarged and deformed bones in one or more regions of the skeleton.[1–4] Excessive bone breakdown and formation can cause the bone to weaken. As a result, bone pain, arthritis, bone deformities, fractures, and other complications (eg, deafness) can occur, contributing to substantial morbidity and reduced quality of life.[5] Neoplastic degeneration of pagetic bone is a relatively rare event, occurring with an incidence of less than 1%, but it has a grave prognosis.

Specific therapy for PDB is aimed at decreasing the abnormal bone remodeling, and different bisphosphonates are currently considered the treatment of choice.[6,7] These treatments are associated with a reduction in bone turnover markers and an improvement in radiologic and scintigraphic appearance, as well as a reduction in bone pain and bone deformity, whereas the effects of treatment on the development or progression of other PDB complications are poorly understood.[6–8] Different bisphosphonates have been used successfully for the treatment of PDB. Compounds such as etidronate and clodronate were used initially and were associated with about 50% reduction in levels of serum alkaline phosphatase (ALP) or other bone turnover markers.

However, because of their modest antiresorptive potency, treatment effects were transient, and failure was not uncommon.[7,8] Moreover, a consistent portion of patients tended to become

resistant.[9] Recently, the availability of newer, more potent nitrogen-containing bisphosphonates has improved treatment outcomes. These compounds have a high binding affinity to hydroxyapatite as well as increased potency in terms of inhibition of bone resorption.[10–12]

While some aminobisphosphonates such as alendronate and risedronate are given as oral formulations and may affect long-term compliance,[13,14] others, owing to their ability to achieve optimal control of bone turnover at lower doses than the previous compounds, can be delivered as intravenous or intramuscular administration. Moreover, their greater binding affinity offers the potential for sustained remission. In a previous study we demonstrated that either zoledronic acid (4 mg) or neridronate (200 mg) given as a single intravenous regimen showed a similar short-term efficacy in achieving biochemical remission at 6 and 12 months in up to 90% of patient nonresponders to pamidronate.[15] The aim of this study was to evaluate the long-term efficacy of a same neridronate dose (200 mg) given as an intravenous or intramuscular regimen in 57 patients with active PDB followed for 3 years.

Subjects and Methods

Patients and study design

This was a 36-month open-label, randomized survey comparing intravenous neridronate or intramuscular neridronate in 57 subjects with active PDB. Eligibility was defined by the presence of serum total ALP above the upper limit of the normal range (120 IU/L) on two consecutive measurements and no treatment with bisphosphonates or other drugs affecting bone metabolism for at least 6 months before the study. Exclusion criteria included major comorbidity, metabolic bone disease other than uncomplicated osteoporosis, recent fracture at pagetic bone, clinically significant liver disease, and renal impairment. The diagnosis of PDB was confirmed in all the recruited patients by bone scintigraphy and X-ray examination of areas of increased isotope uptake, unless recently performed. The study was approved by the local ethics committee, and written informed consent was obtained from all participants.

At baseline, patients were randomly assigned to receive either a 100-mg infusion of neridronic acid over a 2-hour period ($n = 27$) for 2 consecutive days or an intramuscular dose of 25 mg once a week for 2 months ($n = 29$). Randomization was stratified according to baseline ALP levels and previous bisphosphonate treatment (as a binary variable, yes or no). All patients were advised to receive calcium plus vitamin D supplementation throughout the 36-month length of the study (1 g of calcium and 800 IU of colecalciferol per day).

The presence of bone pain owing to PDB (ie, never pain, disappearance, decrease, or no change) was recorded at baseline and during follow-up, as described previously.[15] It was the patient's and investigator's decision whether the pain was or was not related to PDB. Moreover, a pain score was registered for each patient every 6 months based on Tong and colleagues,[16] as described previously.[17] Briefly, the score was obtained by multiplying the severity of pain (ie, none, mild, moderate, or severe) by the frequency of pain (ie, no pain, occasional,

intermittent, or constant), with each of these measures graded from 0 to 3. A score of 0 denotes the absence of pain, whereas a score of 9 denotes severe and constant pain.

Adverse events were recorded at all postbaseline visits, and their severity and relationship to treatment were evaluated.

Biochemical evaluation and efficacy endpoints

Subjects were evaluated at baseline and after 3, 6, 12, 18, 24, 30, and 36 months. At each visit, venous blood was collected in the fasting state for serum analysis. Serum ALP, aspartate and alanine aminotransferases, complete blood cell count, and ionized and total calcium, phosphate, and creatinine levels were determined by standard methods. Bone ALP (bALP; Alkphase-B, Metra Biosystem, Mountain View, CA, USA), serum C-terminal telopeptides of type I collagen (sCTX, serum CrossLaps; Osteometer, Herlev, Denmark), 25-hydroxyvitamin D [25(OH)D; 25-Hydroxyvitamin D ^{125}I RIA Kit, Diasorin Diagnostics Saluggia (Vercelli), Italy], and intact parathyroid hormone (PTH; N-tact PTH, IRMA Kit, DiaSorin, Stillwater, MN, USA) also were evaluated. The observed intra- and interassay coefficients of variation for each marker were, respectively, as follows: below 1.6% for ALP, 2.0% and 4.1% for bALP, 6.1% and 5.4% for sCTX, 10.7% and 15.0% for 25(OH)D, and 2.8% and 4.0% for PTH.

The primary efficacy endpoint was the rate of therapeutic response assessed at 6, 12, 24, and 36 months and defined as the normalization of ALP levels or a reduction of at least 75% in total ALP excess (the difference from the midpoint of the reference range). Secondary endpoints were the reduction of bone pain, time of nadir of ALP (defined as the number of days from the beginning of the treatment to the time when the lowest value was observed), time to a therapeutic response (defined as the number of days between the onset of treatment and the visit at which a response was observed), and the average percent decrease in ALP or the other bone turnover markers at each time point compared with baseline levels.

Statistical analysis

The proportion of patients who demonstrated a therapeutic response was compared between treatment groups using a standard chi-square or Fisher exact test. Biochemical data for each treatment group and interactions between treatment and time were evaluated by analysis of variance (ANOVA) and covariance (ANCOVA) using baseline values as covariates. Linear regression analysis with Pearson's correlation coefficient was used to determine the relationships between the percent decrease in ALP and different biochemical parameters. Analysis was performed using Statistica 5.1 (Statsoft, Inc., Tulsa, OK, USA) and SPSS (Release 6.1; SPSS, Inc., Chicago, IL, USA).

Results

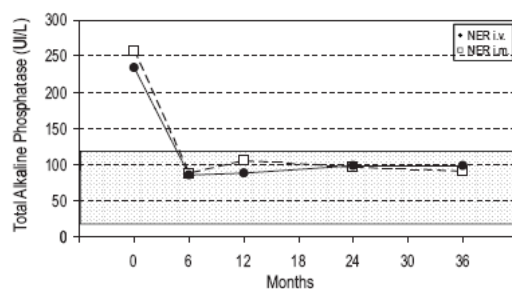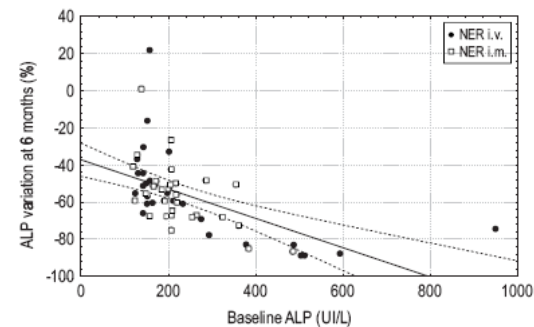### Short- and long-term response to intravenous and intramuscular neridronate

A total of 28 patients were enrolled in the intravenous neridronate group and 29 in the intramuscular neridronate group. Patient's characteristics at baseline are shown in Table 1. The two groups did not differ significantly in terms of age, age at

82

Table 1. Characteristic of Patients at Baseline

	Neridronate i.v. (n = 28)	Neridronate i.m. (n = 29)
No. of men/no. of women	16/12	16/13
Age (years)	69.6 ± 9	65.9 ± 8
Skeletal sites (n)	2.9 ± 2	3.5 ± 2
Polyostotic cases (n)	21	25
Previously treated patients (n)	19/28	21/29
Alkaline phosphatase (IU/L)	224.2 ± 98	257.2 ± 91
Pain score	5.23 ± 2.5	5.62 ± 2.1
HAQ functional disability index	0.92 ± 0.4	0.81 ± 0.4

Fig. 2. Relationship between baseline serum ALP and ALP changes at 6 months after the initiation of treatment.

diagnosis, gender, weight, previous therapy for PDB, and baseline ALP, bALP, PTH, or 25(OH)D levels. All patients previously treated for PDB (n = 40, 70.0%) had received pamidronate (n = 13), clodronate (n = 19), or risedronate (n = 8) in the past, whereas none had received neridronate or zoledronate.
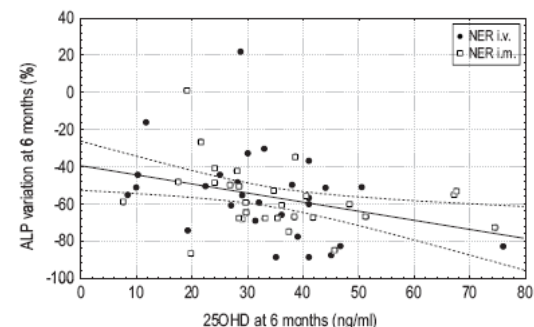
A significant 50% to 60% decrease in ALP levels was observed with both regimens after 3 months. Overall, 27 of 28 patients receiving intravenous neridronate and 29 of 29 patients receiving intramuscular neridronate completed the follow-up at 6 months and continued the study. Mean ALP levels during the 3 years of the study in subjects with intramuscular and intravenous regimens are shown in Fig. 1. At 6 months, 25 of 27 (92.6%) and 28 of 29 (96.5%) of patients receiving intravenous and intramuscular neridronate, respectively, achieved a therapeutic response. Normalization of ALP levels at 6 months was achieved in 24 of 27 patients (88.9%) in the intravenous group and in 26 of 29 patients (89.6%) in the intramuscular group. Similar results were obtained when bALP or CTX level instead of ALP levels was considered. Interestingly, the response to treatment was significantly correlated with baseline ALP ($r = -0.56$, $p < .0001$) and 25(OH)D levels at 6 months ($r = -0.34$, $p < .01$). In fact, the percent decrease in ALP after 6 months was highest in patients with higher ALP activity at baseline or with higher 25(OH)D levels at 6 months (Figs. 2 and 3). Moreover, in both groups, the response to neridronate was significantly affected by previous bisphosphonate treatment. In fact, ALP

decreased at a greater extent in previously untreated patients than in those with previous bisphosphonate treatment ($-63.7\% \pm 22.6\%$ versus $-52.8\% \pm 16.7\%$ at 6 months, respectively, $p < .05$). In particular, all 17 previously untreated patients achieved therapeutic response at 6 months compared with 36 of 39 patients (92.3%) with previous bisphosphonate treatment.

Therapeutic response was maintained at 12 months in 24 of 27 (88.9%) and 25 of 29 patients (86.2%) in the intravenous and intramuscular neridronate groups, respectively, without significant differences between the two regimens. Response rates at 6, 12, 24, and 36 months from the treatment course with intramuscular or intravenous neridronate are shown in Fig. 4. A progressive decrease in normalization and therapeutic response was observed for both regimens without significant differences. At the 36-month follow up, therapeutic response was maintained in 13 of 27 (48.1%) and 13 of 29 patients (44.8%) in the intravenous and intramuscular neridronate groups, respectively. Overall, therapeutic response at 36 months was observed in 12 of 17 (70.6%) of the previously untreated patients compared with 14 of 39 patients (35.9%) reporting a previous treatment with other bisphosphonates ($p < .05$).

Analysis of other secondary endpoints did not evidence significant differences between the intravenous and intramuscular regimens in terms of time of nadir of ALP (211.2 ± 136 versus 226.6 ± 174 UI/L, respectively), average ALP decrease at

Fig. 1. Mean serum ALP levels during the 36-month study for the intramuscular and intravenous neridronate regimens. The shaded area represents the normal range of serum ALP.

Fig. 3. Relationship between serum ALP variation and serum 25(OH)D levels at 6 months after the initiation of treatment.
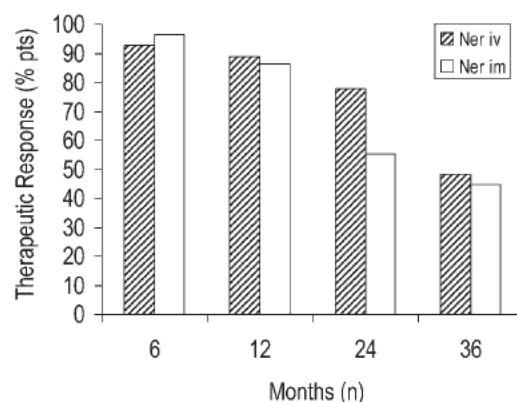
Fig. 4. Percentage of patients with a therapeutic response (defined as normalization of ALP levels or a reduction of at least 75% in total ALP excess) after 6, 12, 24, and 36 months of intramuscular or intravenous neridronate treatment.

nadir ($-60.4\% \pm 16\%$ versus $-66.3\% \pm 14\%$, respectively), time to a therapeutic response (within 3 months in all patients), and the average percent decrease in ALP from 3 to 36 months. The average ALP decrease at nadir was significantly higher in patients without previous bisphosphonate treatment than in patients previously treated with one or more bisphosphonate courses ($-71.8\% \pm 15\%$ versus $-59.6\% \pm 13\%$, respectively, $p < .005$), irrespective of baseline ALP levels.

Response to a second neridronate course

Overall, from month 12 to 36, 11 of 27 (40.7%) and 16 of 29 patients (55.2%) in intravenous or intramuscular neridronate group received a new treatment course owing to relapse ($n = 21$) or an increase in bone pain at pagetic sites ($n = 6$). The lag time between the first and second neridronate course did not differ significantly between intravenous and intramuscular groups (621.8 ± 246 days versus 573.7 ± 164 days, respectively). Among patients with relapse after the first neridronate course, 6 of 9 (66.7%) and 9 of 12 (75.0%) in the intravenous and intramuscular groups, respectively, achieved therapeutic response after 6 months from the second treatment course. All the 6 nonresponder patients to the second neridronate regimen had been treated previously with other bisphosphonates before recruitment in this study. The percent decrease in ALP at 6 months from retreatment was similar in the intramuscular and intravenous groups. Three of the 6 patients who did not achieve therapeutic response after the second neridronate regimen were treated with zoledronic acid 5 mg intravenously, and all achieved therapeutic response after 6 months from crossover.

Treatment effects on pain and quality of life

The effects of neridronate on pain and quality of life were evaluated at 6 months in each treatment arm. As shown in Table 2, both neridronate regimens were effective in decreasing and/or abolishing bone pain at pagetic sites in a consistent group of patients (77.8% versus 86.2% in the intravenous versus

the intramuscular groups, respectively). Moreover, significant reductions in pain score at 6 months were observed with both the intravenous (5.23 ± 2.5 versus 1.69 ± 1.8, $p < .001$) and the intramuscular (5.62 ± 2.1 versus 1.20 ± 1.2, $p < .001$) regimens.

At 12 months from treatment, 2 of 27 (7.4%) and 4 of 29 patients (13.8%) in the intravenous and intramuscular groups, respectively, reported the recurrence or worsening of bone pain with respect to month 6 and were treated with a second treatment course. All but one patient reported a decrease in bone pain after 6 months of retreatment.

Analysis of baseline Health Assessment Questionnaire (HAQ) scores indicated a relatively preserved health function because most patients were in categories 0 to 2. A mild but not significant improvement in quality of life was observed after 6 months of treatment, as indicated by a reduction in HAQ score (cumulative HAQ score at 6 months 0.74 ± 0.39 versus 0.86 ± 0.40 at baseline, $p = .26$) without any significant difference between treatment groups.

Safety and adverse events

All treatment regimens were well tolerated. Significant adverse effects resulting in withdrawal from the study occurred in one patient in the intravenous neridronate group, who withdrew after 3 months of treatment because of the diagnosis of colon cancer. This was considered unrelated to the neridronate treatment. Major adverse events for each treatment group are summarised in Table 3. These were mainly influenza-like symptoms that are known to occur mostly with the use of intravenous amino-bisphosphonates. In this study, these

Table 2. Modification of Pain and Pain Score at 6 Months With Neridronate

	Neridronate i.v. ($n = 27$)	Neridronate i.m. ($n = 29$)
Never pain	2 (7.4%)	1 (3.5%)
Disappearance of pain	7 (25.9%)	9 (31%)
Decrease of pain	14 (51.8%)	16 (55.2%)
No change in pain	4 (14.8%)	3 (10.3%)
Pain score variation	$-68.7\% \pm 34\%$	$-81.1\% \pm 20\%$

Table 3. Reported Adverse Events With Different Neridronate Regimens

	Neridronate i.v. ($n = 27$)	Neridronate i.m. ($n = 29$)
Influenza-like illness	4 (14.8%)	4 (13.8%)
Myalgia	4 (14.8%)	2 (6.9%)
Pyrexia	4 (14.8%)	4 (13.8%)
Fatigue	5 (18.5%)	4 (13.8%)
Headache	3 (11.1%)	2 (6.9%)
Diarrhea	1 (3.7%)	2 (6.9%)
Osteoarticular pain	3 (11.1%)	5 (17.2%)
Hypocalcemia	2 (7.4%)	1 (3.4%)
Dermatitis	0	1 (3.4%)

84

symptoms also were observed in patients with the intramuscular neridronate regimen, generally after the first or the second 25-mg intramuscular administration. Moreover, in both treatment groups, symptoms of acute-phase reaction occurred mostly in previously untreated patients, were mild to moderate in severity, and generally resolved within a few days. Of interest, patients experiencing an acute-phase reaction showed lower vitamin D levels than those without the acute-phase reaction, either before (18.3 ± 13 versus 29.7 ± 15, $p = .05$) or after (18.3 ± 13 versus 32.2 ± 16, $p = .05$) the exclusion of subjects with previous bisphosphonate treatment. No significant differences in other adverse events were observed between the two treatment groups. There were no clinically relevant changes over time in routine hematologic and biochemical tests.

Discussion

Since the discovery of the profound effects of bisphosphonates on calcium metabolism, the treatment of PDB has evolved remarkably over the last decades, from using drugs simply to reduce bone pain to using other drugs designed to induce remission or prevent deformity and possibly other long-term complications. Despite the fact that nitrogen-containing bisphosphonates are currently considered the treatment of choice in PDB,[6–8] a recent survey in PDB patients evidenced inappropriate dosing regimens and short duration of treatment, particularly with oral regimens.[14] In this context, the development of potent intravenous compounds may present advantages with regard to outpatient management and patient adherence to treatment, with the potential to improve the control of bone turnover as well as to maintain PDB remission over long-term follow-up. As a counterpart, acquired resistance to the intravenous infusion of some amino-bisphosphonates such as pamidronate has been reported in different studies.[9,15,18,19] In one of these studies, we demonstrated that intravenous infusions with either zoledronate or neridronate are effective in achieving therapeutic response at 6 months in up to 90% of patient nonresponders to multiple courses of pamidronate.[15] Results from this study confirm and extend these observations and indicate that a single course with neridronate 200 mg is effective and normalizes bone turnover in up to 85% of patients with PDB within the first year of treatment and in up to 55% after the second year. The estimated period of remission was about 590 days, but more than 45% of patients showed normal ALP levels even at 3 years after treatment. A second course generally was sufficient to achieve disease remission in most patients who relapsed with the first administration. Interestingly, both intravenous and intramuscular regimens demonstrated a similar efficacy and safety profile, showing that the selected cumulative dose is effective in most patients irrespective of the route of administration.

Neridronate also has been used successfully in PDB patients with acquired resistance to non–amino-bisphosphonates such as etidronate and clodronate.[20–22] While etidronate and clodronate are less potent non–amino-bisphosphonates, pamidronate is a nitrogen-containing bisphosphonate with the same or a slightly lower in vitro antiresorptive potency as neridronate.[23] However, neridronate demonstrated a higher skeletal uptake

and hydroxyapatite binding than pamidronate,[23] which might explain its increased efficacy in PDB, as observed in this and other studies.[15]

Overall, the long-term tolerability of both neridronate regimens was excellent and comparable with that of the previous short-term studies with intravenous infusion.[15,22,25] No serious adverse events related to treatment were reported. In keeping with previous reports on postmenopausal women with osteoporosis,[26] a certain percentage of patients with acute-phase reaction also was observed with the intramuscular regimen, with a prevalence that did not differ significantly from that observed in the intravenous regimen. The overall number of patients experiencing acute-phase reaction with both regimens (8 of 57, 14.0%) was lower than in other studies. However, acute-phase reaction generally is more common in previously untreated patients than in subjects with previous amino-bisphosphonate treatment. Indeed, when we excluded patients with previous treatments with amino-bisphosphonates, the prevalence of acute-phase reaction was higher (8 of 35, 22.9%). Moreover, patients with the intramuscular regimen mainly reported fever as the major symptom of the acute-phase reaction, whereas the association between fever and muscular pain was observed more frequently in patients undergoing intravenous neridronate treatment. Interestingly, we also observed lower vitamin D levels in patients reporting acute-phase reaction, which is consistent with findings from a recent study in osteoporotic women undergoing zoledronate treatment.[27] This suggest that acute-phase response following amino-bisphophonate treatment may be related to vitamin D status, possibly through direct effects on $\gamma\delta$ T cells, the subpopulations of T cells mainly involved in acute-phase reaction.[28] Moreover, in our population of PDB patients, 25(OH)D levels also were associated with the response to neridronate treatment. In fact, the percent decrease in ALP levels achieved at 6 months was highest in patients with higher 25(OH)D levels at 6 months. The reason for this observation remains unclear but could be related to a parallel increase in PTH levels negatively influencing bone turnover in subjects with lower vitamin D status. Indeed, a positive association between ALP decrease and PTH levels at 6 month also was observed, even though slightly above the threshold for statistical significance, likely owing to the higher daily variability of serum PTH levels with respect to 25(OH)D. Interestingly, when we compared PTH levels at 6 months in responders versus nonresponders, we found statistically significant differences (PTH 62.0 ± 29.3 versus 36.8 ± 11.9 pg/mL, $p < .005$, respectively). Previous observations in other PDB samples suggested that the development of secondary hyperparathyroidism is associated with reduced response to bisphosphonate treatment.[9] These data are also consistent with similar observations derived from studies of amino-bisphosphonates for osteoporosis, generally showing increased bone mineral density (BMD) gain and higher antifracture efficacy in vitamin D–depleted compared with vitamin D–deficient subjects.[29–32] Taken together, these observations further underline the necessity to recommend adequate vitamin D intake in PDB patients undergoing bisphosphonate treatment. Possibly, achievement of adequate 25(OH)D levels should be reached before high-dose bisphosphonate

administration in order to maximize treatment response and minimize the risk of acute-phase reaction.

In conclusion, the results of this study indicate that a 200-mg intramuscular neridronate course (administered as a single 25-mg injection every week for 2 months) has a similar efficacy to an intravenous infusion of the same dose for the treatment of PDB and might be of particular value for patients intolerant of oral bisphosphonates (ie, with serious gastroesophageal diseases or unable to fast) and unwilling or unable to undergo intravenous infusions. This regimen avoids all the limitations of oral bisphosphonates and may be offered as a home treatment without major contraindications. Moreover, both intramuscular and intravenous neridronate regimens may be relevant in terms of cost-effectiveness, as appears from the comparative analysis of the costs of recommended regimens for PDB with the other amino-bisphosphonates that have been licensed for this indication in our country (approximately 90 and 115 euros for an intravenous or intramuscular neridronate 200-mg course, respectively, versus 425, 695, and 530 euros for intravenous pamidronate, oral risedronate, and intravenous zoledronate courses, respectively). In the case of relapse, additional neridronate courses are able to achieve a therapeutic response in most patients, even though impaired response to a second treatment course was described in a few patients in this study, particularly those with acquired resistance to previous regimens with other bisphosphonates. In the latter circumstance, treatment with a more potent compound (eg, zoledronic acid) may be indicated.

Disclosures

All the authors state that they have no conflicts of interest.

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86

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87

5. SUMMARY AND CONCLUSIONS

In summary, our prospective analysis of a large and well characterized sample of patients with PDB has provided novel and interesting insights on the pathogenesis and management of this invalidating disorder.

As first we evidenced the presence of different *SQSTM1* mutations in 37% and 10% of familial and sporadic PDB patients, respectively. Together with an additional study in a smaller Italian cohort [50], 15 different *SQSTM1* mutations have been described in Italian PDB cases. This heterogeneity is higher than what observed in populations of British descent or in other European populations and might reflect the complex history of Italy as well as the several foreign invasions and dominations that occurred between sixth and nineteenth centuries. To this regard, a single *SQSTM1* mutation has been demonstrated in the French-Canadian population [47]. Interestingly, the highest prevalence and heterogeneity of mutations was observed in southern Italy than in central and northern Italy, explaining at least in part the increased disease severity and the early onset of PDB frequently described in patients from this area [9, 23, 42]. Consistent with other reports we also demonstrated that subjects with *SQSTM1* mutation have an increased disease extension. Moreover, we also demonstrated the presence of gene-environment and gene-gene interactions on the severity of PDB phenotype. In fact PDB was more severe and occurred earlier in those cases reporting animal contacts (suggesting the exposure to viral infections) and in those bearing a particular polymorphic variant of the gene encoding RANK, the receptor of RANKL (the major factor stimulating osteoclastogenesis and osteoclast activity). Following our results from in vitro analyses and the data reported from other groups [71, 74, 77] we can hypothesize that either the presence of the predisposing RANK variant or the contact with viral infections (i.e. paramixoviruses) may facilitate the occurrence and the skeletal diffusion of PDB, particularly in subjects with mutations in *SQSTM1* gene. Conversely, a particular polymorphic variant of *OPTN* gene,

encoding for optineurin seems to act as a predisposing factor in sporadic PDB cases, while it is likely that the effect of this gene is overcome in *SQSTM1* positive cases as well as in familial cases without *SQSTM1* mutation. This latter evidence also suggests that at least one additional gene is involved in the pathogenesis of familial PDB in those pedigrees negative for *SQSTM1* mutation. In order to uncover new genes predisposing to PDB we performed a collaborative genome-wide association study in *SQSTM1* negative cases and we extended clinical and genetic analyses in a particular pedigree with 14 affected members (associated with the occurrence of giant cell tumours at the affected skeletal sites).

In the first collaborative analysis we confirmed the association of PDB with variations in *TNFSRF11A* (encoding RANK) or *OPTN* genes, but we also demonstrated that at least 5 additional genes (*CSF1, TM7SF4, PML, RIN3, NUP205*) are associated with PDB in *SQSTM1* negative patients. In the second study we further characterized the clinical phenotype of the PDB-giant cell tumour pedigree demonstrating that affected subjects also show and increased prevalence of cardiovascular complications with respect to unaffected family members and to a comparative cohort of 150 unrelated PDB cases from the same geographical area [Publication E]. An initial linkage analysis in this pedigree evidenced some interesting loci that are actually under investigation in order to uncover the causative gene. According to all the above evidences a likely pathogenetic model for PDB is summarized in Fig.14.
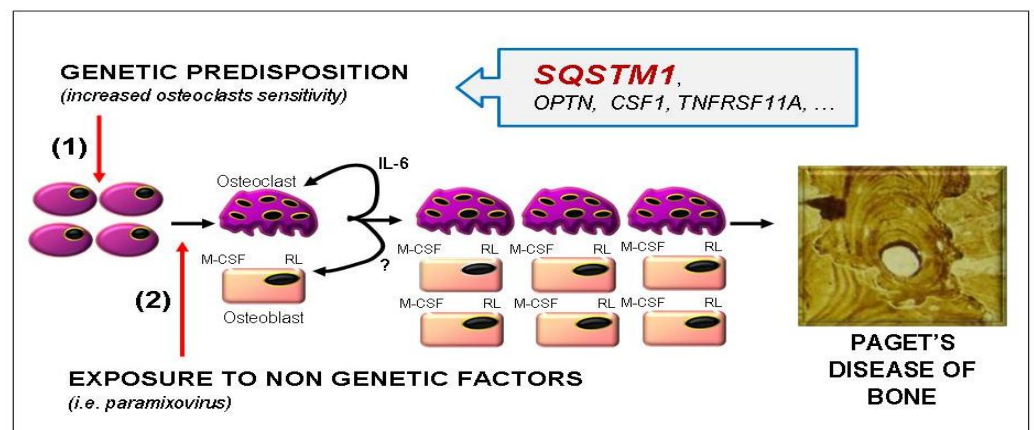
Fig. 14. Proposed physiopathological model for PDB
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Finally, we provided interesting insight on the long term treatment of PDB. In a first study we demonstrated that that in addition to the oral and intravenous regimens with different bisphosphonates (commonly used in different countries), the suppression of disease activity either in monostotic or polyostotic cases can be also achieved by the use of intravenous neridronate administration, given at the same cumulative dosage than the intravenous regimen [Publication F]. This might be of particular value for patients intolerant to oral bisphosphonates (i.e. due to gastro-esophageal irritation) and unwilling or unable to undergo intravenous infusions (that in our Country can be administered only in hospital services). In a second preliminary analysis we evidenced important pharmacogenomic implications demonstrating that patients bearing the *SQSTM1* mutation may require a more aggressive and intensive treatment regimen for disease remission. This analysis will be extended to all cases of the Italian PDB Registry and integrated with the genetic information concerning polymorphic variation in the other PDB predisposing genes in order to extend the knowledge about the pharmacogenetic of bisphosphonate treatment in PDB.

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