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Papapoulos, S.E.

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Pamidronate: A model compound of the pharmacology of nitrogen-containing bisphosphonates; A Leiden historical perspective

Socrates E. Papapoulos

Center for Bone Quality, Leiden University Medical Center, Albinusdreef 2, 2333, ZA, Leiden, the Netherlands

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ABSTRACT

Pamidronate [3-amino-1-hydroxypropylidene-1,1-bisphosphonate (APD)] was the first nitrogen-containing bisphosphonate (N-BP) investigated in clinical studies. In contrast to other clinically used bisphosphonates, pamidronate was discovered and its properties were initially studied in an Academic Institution. On the occasion of the 50th Anniversary of the first publications on the biological effects of bisphosphonates, I review in this article the contribution of Leiden investigators to the development of pamidronate that led to the recognition of the significance of the Nitrogen atom in the side chain of bisphosphonates for their action on bone resorption and to the formulation of principles for the use of N-BPs in the management of patients with different skeletal disorders.

1. Introduction

Research in bone and mineral metabolism has been for many years a major activity in the University of Leiden. The early efforts of Pieter Gaillard and his collaborators established the Department of Cell Biology as the centre of basic research in bone biology in the Netherlands with international recognition. Clinical research was initiated in the 1960s at the Department of Endocrinology & Metabolic Diseases but was structured and became a major focus of the Department in the early 1970s by Olav Bijvoet who was appointed Professor of Experimental Endocrinology and Head of the Clinical Investigation Unit, currently the Center for Bone Quality at the Leiden University Medical Center. In this Unit preclinical and clinical research was coupled with optimal care of patients with disorders of bone and mineral metabolism.

Being the first to treat a patient with Paget's disease of bone with calcitonin [1], Bijvoet recognized the potential of antiresorptive agents for treating this and other bone disorders and, following the report of the effects of etidronate on biochemical parameters of bone metabolism in four patients with Paget's disease [2], he was among the first physicians to use this bisphosphonate in clinical studies [2,3].

Early studies of the efficacy and safety of etidronate treatment in patients with Paget's disease in Leiden confirmed the superior efficacy of etidronate compared with calcitonin but revealed also the inability to obtain optimal decrease of the activity of the disease with this bisphosphonate without compromising the normal mineralization of bone tissue [3]. In an attempt to overcome the observed disadvantage of etidronate therapy, a lower oral dose of the bisphosphonate (7.5 mg/kg/day) was administered together with daily subcutaneous injections of calcitonin to patients with Paget's disease [4]. This combination therapy was associated with progressive clinical improvement and induced greater decreases of biochemical parameters of bone turnover than those observed with either monotherapy with no evidence of defective bone mineralization after one year therapy. Although the reduction of disease activity by this combination therapy was superior to that induced by etidronate 7.5 mg/kg/d alone, it was still incomplete and the therapeutic regimen was not convenient for chronic administration. Moreover, the effect of calcitonin, in contrast to that of etidronate, was quickly reversible after its discontinuation. For these reasons Bijvoet sought alternatives to etidronate that might provide a better and safer treatment for Paget's disease. It was then already known from studies with clodronate that impairment of bone mineralization was not a general property of bisphosphonates. Clodronate had lower affinity for hydroxyapatite than etidronate and appeared to impair mineralization only at very high doses suggesting that the effects of a bisphosphonate on bone resorption and mineralization could be dissociated. Although clodronate was reported to be a more potent inhibitor of bone resorption than etidronate, during its evaluation as treatment of Paget's disease a few patients developed haematological malignancy [5] which, although not confirmed in further studies, raised concerns about its safety, particularly in the US.

In an effort to identify a bisphosphonate with the required properties, Bijvoet approached Henkel KGaA in Dusseldorf, Germany. Henkel, a major producer of washing powders, cosmetics, toothpastes and household items, held patents of numerous bisphosphonate compounds that could be used for complexing ions to improve the quality of their

E-mail address: s.e.papapoulos@lumc.nl.

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products [6,7]. He obtained from Henkel four bisphosphonates to test in Leiden; one of these was pamidronate.

2. Preclinical studies

In initial screening studies, high doses of the four Henkel compounds were administered subcutaneously to rats for 6 days and their effects on bone metabolism were compared with those of etidronate and clodronate [8,9]. Three of these bisphosphonates decreased matrix mineralization to a degree similar to that of etidronate whereas clodronate and pamidronate had no or minimal effect on matrix mineralization. Dose-response studies with etidronate, clodronate and pamidronate given subcutaneously for 23 days [8–10], demonstrated further that pamidronate was the most potent inhibitor of bone resorption being about 10 times more potent than clodronate. Inhibition of bone resorption by pamidronate was not associated with a decrease of the number of osteoclasts in metaphyseal tissue and many osteoclasts contained an increased number of nuclei, up to 20 per osteoclast [10]. This finding remained generally unnoticed, due mainly to the difficulty in explaining it, but it was later described in children with osteogenesis imperfecta treated with intravenous pamidronate [11] and attracted considerable attention when the mechanism of action of N-BPs had been elucidated and such osteoclasts were observed and described in detail in bone biopsies from adults treated with alendronate [12]. Treatment increased also calcium retention and weight of bones without detrimental effects on bone matrix. The dose of pamidronate affecting mineralization was about 50 times higher than that required for half-maximal inhibition of bone resorption and the parenteral dose that impaired mineralization was >40 μmol/kg/d (11 mg/kg/d) and the one that completely inhibited bone mineralization and growth of treated animals was 160 μmol/kg/d (45 mg/kg/d). The results of these early studies, demonstrated, thus, a potent inhibitory effect of pamidronate on bone and cartilage resorption with doses much lower than those affecting mineralization.

The long-term skeletal effects of different oral doses of pamidronate given up to 104 weeks to rats were also examined as part of toxicology studies performed by Henkel's scientists [13]. There was a dose-dependent, profound increase in bone mass of treated animals that was not associated with disturbed mineralization or increased incidence of fractures. The increase in bone mass with the highest dose (about 567 mg/kg/d) reduced bone marrow spaces and was associated with extramedullary sites of hematopoiesis in the liver and the spleen as it occurs in osteopetrosis.

An important finding of the early studies was the demonstration of the dose-dependent decrease of bone resorption with daily subcutaneous injections of pamidronate. With every dose, a plateau was quickly reached that was also dose-dependent. Thereafter, no further decrease in resorption was observed indicating that the accumulation of pamidronate in the skeleton with time was not associated with a cumulative effect on bone resorption. Furthermore, the response suggested that the dose of the bisphosphonate administrated is more important for effective decrease of bone resorption than the same total dose divided in smaller daily doses. Metaphyseal alkaline phosphatase activity decreased also dose-dependently. However, differently from the reduction of bone resorption, the decrease of alkaline phosphatase was progressive and at least 3 weeks were necessary for the appearance of the maximum effect. These results led Reitsma et al. [10] to challenge the use of fixed periods of treatment in preclinical studies to examine relative potencies of bisphosphonates on different aspects on bone metabolism. They postulated that bone resorption and apposition are cyclical, dynamic inter-related processes, this being the reason for the necessity to perform both sequential and cross-sectional studies.

Treatment was further associated with increases in calcium balance which were, however, not dose-dependent. These results suggested for the first time that it may be possible to modulate bone remodelling mildly and induce significant increases in calcium balance with low bisphosphonate dose and that uninterrupted administration is not accompanied by progressive suppression of bone resorption. These pharmacodynamic observations contributed to the development of N-BPs as treatments of osteoporosis (see Section 3.6). The group proposed a model to explain the action of pamidronate on bone metabolism. They suggested that pamidronate blocks the permissive action of bone mineral on osteoclast activation and function and that reduction of osteoblasts is a secondary effect suggesting, thus, a potential cellular effect of pamidronate on bone resorption.

2.1. Mechanism of action

As expected by the way bisphosphonates were identified, initial studies to explain their action on bone resorption focused on their physicochemical effects. However, the studies with pamidronate raised the question whether bisphosphonates exert their inhibitory effect by rendering bone mineral more resistant to degradation, by diminishing the activity of resorbing cells, or through some combination of both activities. To test this hypothesis the Leiden group collaborated with S. Teitelbaum and A. Kahn in St Louis and tested the effects of pamidronate and clodronate using an in vitro resorption assay consisting of rat peritoneal macrophages co-cultured with particles of ^{45}Calcium labelled devitalized rat bone [14]. They found that "*..the mechanisms of action of APD and Cl_2MDP (clodronate) are markedly different. Cl_2MDP is a potent cytotoxin in the presence of bone and apparently exerts its inhibitory effect in this manner. APD is noncytotoxic at levels adequate to suppress resorption and, therefore, must inhibit macrophage activity by some other mechanism probably dependent upon the direct action of APD on cells*". This was the first demonstration of differences in the mechanism of action of bisphosphonates on bone resorption that emphasized the importance of a potential cellular action of pamidronate on bone resorbing cells. These observations were confirmed using different experimental approaches representative of the in vivo actions of bisphosphonates [15,16] but took >15 years before the molecular mechanism of action of N-BPs was elucidated. Leiden investigators showed later for the first time that farnesyl pyrophosphate synthase (FPPS), an enzyme of the mevalonate metabolic pathway, was the molecular target of N-BPs [17–19].

2.2. Modifications of the pamidronate molecule

2.2.1. Dimethylation of the amino group of the active site

To improve the properties of pamidronate the amino group at the R2 side chain of the bisphosphonate was substituted by two methyl groups (Fig. 1). Dimethylation of the aminogroup, that increases the basic properties of the molecule, increased the in vitro specificity of pamidronate for bone resorption and its potency in vivo and decreased its cellular toxicity [20,21]. The latter was later confirmed in in vitro studies of cultured Caos-2 cells, a cell model of intestinal epithelium, at the Department of Clinical Pharmacy in Leiden [22]. Dimethylation of pamidronate increased its solubility while cellular toxicity was observed at concentrations >10-fold higher than those of pamidronate. The greater potency of this bisphosphonate, (3-dimethylamino-1-hydroxypropy1idene)-1,1-bisphosphonate, dimethyl-APD, olpadronate), combined with its lower toxicity indicated that oral doses that efficaciously decrease bone resorption would be better tolerated as confirmed in later clinical studies. Extensive preclinical and toxicological studies with olpadronate were performed by Gador SA, Argentina.

2.2.2. Aminosubstitution of the hydroxyl group of the binding site

The importance of the cellular actions of bisphosphonates for their clinical use limited temporarily the interest in their physicochemical properties. However, binding and distribution in bone and long-term skeletal retention are essential components of bisphosphonate pharmacology. Studies with hydroxyapatite crystals and later with foetal mouse bone explants showed that the presence of a hydroxyl

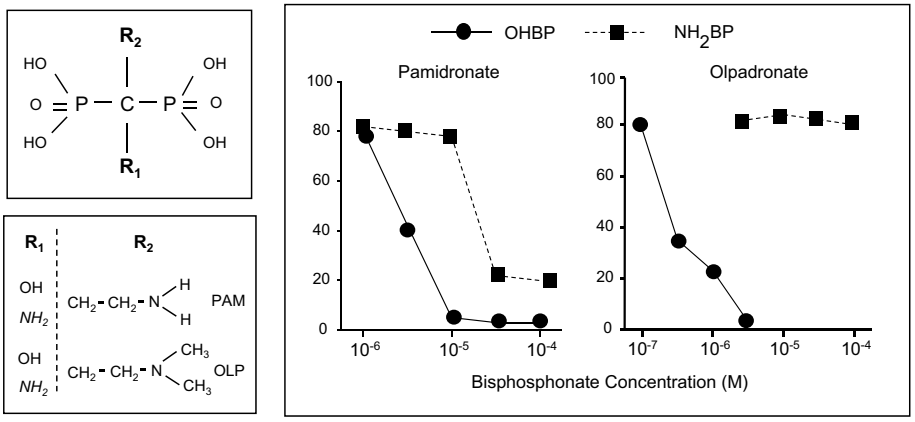

Fig. 1. *Left upper*: Structure of bisphosphonates; *Left lower*: Side chains R1 and R2 of pamidronate (PAM) and olpadronate (OLP), respectively, and their respective analogues with an aminosubstitution at R1. *Right*: Effects of the four bisphosphonates on resorption, measured as ^{45}Ca release from foetal mouse metacarpals in culture. OHBP = bisphosphonate with a hydroxyl group in R1; NH2BP = bisphosphonate with an amino group at R2. Note: acid forms of bisphosphonates are depicted.
Modified from ref [25].

substitution in R1 increases the binding of bisphosphonates to bone mineral, due probably to the tridentate binding of hydroxyl substituted bisphosphonates to calcium, and that this action was independent of the structure of the R2 side chain [23]. In all experiments that led to these conclusions, R1 was usually kept unaltered, and R2 was varied but the opposite, namely varying substitutions at R1 while keeping R2 unaltered was rarely tested. Only in early crystal growth experiments it was shown that substitution of the hydroxyl group with an amino group, that provides also a tridentate binding, in pentane bisphosphonates had the same effect on the growth of hydroxyapatite crystals [24]. The properties of analogues of etidronate, pamidronate, and olpadronate generated by substituting the OH group at R1 with an NH2 group were tested in cultures of bone explants [25]. The pamidronate and olpadronate analogues with a NH2 substitution were shown to bind equally well to bone explants as their hydroxyl counterparts and to share the same physicochemical properties, such as inhibition of the growth of calcium crystals. Surprisingly, however, the NH2 substitution at R1 while retaining the affinity of these analogues for bone led to profound differences in the ability of the two N-BPs to decrease bone resorption (Fig. 1). These findings were confirmed in vivo in mice and in a pure cellular experimental model, the slime mould *Dictyostelium discoideum*, in which binding to mineral is not required for bisphosphonate action [26]. These observations suggested for the first time that the whole bisphosphonate molecule is important for the action of N-BPs on bone resorption. Notably, the R1 aminosubstituted analogue of olpadronate was later shown by another group [27] to prevent osteoblast and osteocyte apoptosis as well as loss of bone strength in glucocorticoid-treated mice, without affecting bone turnover, but this bisphosphonate was not tested in clinical studies.

3. Clinical investigations

Following the recognition of the properties of pamidronate and toxicological studies performed at the laboratories of Henkel and after approval by Medical Ethical Committees, the efficacy and safety of the bisphosphonate was examined in a series of clinical studies published in The Lancet [28–33]. These studies confirmed the superior potency of pamidronate and identified the main indications for its clinical use namely, Paget's disease of bone, malignancy-associated hypercalcemia, skeletal complications of cancer and bone disease of multiple myeloma. In addition, the use of pamidronate in the treatment of rare bone disorders was reported [34,35]. In the following paragraphs I discuss selected observations of early clinical studies performed in Leiden that led to a better understanding of the action of N-BPs and provided the background for optimal therapeutic use of such bisphosphonates. Important for the successful completion of these studies was the support of the staff of the Department of Clinical Pharmacy of the Leiden University Hospital who prepared formulations of pamidronate, including enteric-coated tablets, suitable for human use.

3.1. Paget's disease

The pathophysiology of Paget's disease characterized by high rates of bone resorption tightly coupled with high rates of bone formation, commonly measured by biochemical markers of bone turnover or, infrequently, by bone histomorphometry [36], provided an appropriate human model to study the kinetics of forced changes in bone cellular dynamics by a potent inhibitor of bone resorption such as pamidronate.

During the early phase of treatment of patients with Paget's disease with pamidronate there was a rapid decrease of bone resorption whereas bone formation was not affected but decreased later at a slower rate due to the coupling of bone resorption and formation. The induced uncoupling of bone resorption and bone formation during this period was associated with an increase in calcium balance, the magnitude of which was probably limited only by the capacity of the intestine to absorb calcium (Fig. 2). As the equilibrium between bone resorption and bone formation was slowly restored, a new steady state of lower rate of bone turnover was obtained 3 to 6 months after the start of treatment. This marked, transient dissociation of bone resorption and bone formation decreased serum calcium concentrations but adaptive changes of calciotropic hormones prevented the development of symptomatic hypocalcaemia in vitamin D-replete subjects. Secretion of PTH and, consequently, of 1,25-dihydroxycholecalciferol production increased, leading to stimulation of renal tubular reabsorption and of intestinal absorption of calcium, respectively [37,38]. The studies also suggested that continuation of therapy of patients with Paget's disease with pamidronate might not be necessary after a maximum decrease of bone resorption is achieved because this will be predictably followed by reduction of bone formation. This therapeutic principle was documented in a study of 142 patients with Paget's disease with raised serum alkaline phosphatase (AP) activity with primary objective the normalization of serum AP, defined as biochemical remission [39]. Patients were treated with 3 different regimens of pamidronate: 1. Oral pamidronate 600 mg/d continued for 6 months after normalization of serum AP activity, 2. Oral pamidronate 600 mg/d until urinary hydroxyproline excretion returned to normal and 3. Intravenous pamidronate 20 mg/d for 10 days. Biochemical remission was obtained in 91% of patients with no differences between the 3 groups and was associated with clinical improvement and in some patients with remarkable radiological changes of affected bones [40]. The median duration of remission, assessed from the time of the firstly observed normal serum AP value to the time when AP increased above the upper limit of normal, was 2.7 years without significant differences among the 3 treatment modalities. The most important determinant of the duration of remission was the value of AP obtained with treatment. Patients who had post-treatment serum AP values below the median value had also significantly longer remissions. These observations were later confirmed in a study with olpadronate showing that the lower the decrease of serum AP activity within the normal range the longer the period of

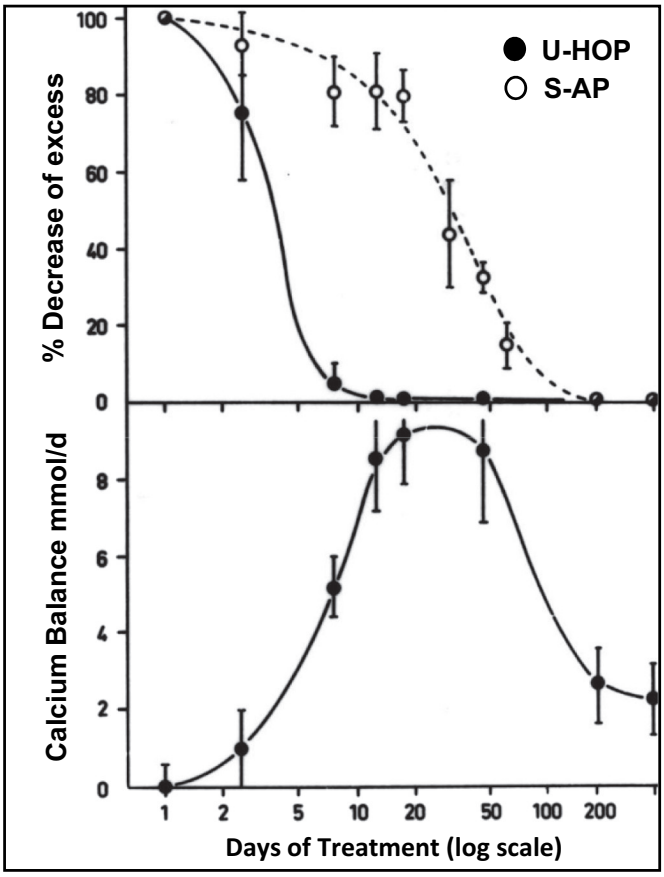

Fig. 2. Early changes of biochemical markers of bone turnover (*upper panel*) and calcium balance (*lower panel*) of patients with Paget's disease treated with oral pamidronate. U-HOP = urinary hydroxyproline excretion, S-AP = serum alkaline phosphatase activity. Adapted from references [28, 43].

biochemical remission of the disease [41]. These findings, supported by shorter term studies using biochemical markers of bone resorption more specific than urinary hydroxyproline [42], established the currently accepted way of treating the disease.

A problem in bisphosphonate research at the time of pamidronate development, was the difficulty to accurately assess the relative antiresorptive properties of bisphosphonates with existing in vitro assays that assessed different stages of the resorption sequence and provided results that could differ widely from in vivo findings. Consequently, relevant therapeutic doses of bisphosphonates could not be predictably defined. During short-term administration of pamidronate to patients with Paget's disease, Harinck et al. [43] observed that the decrease of urinary hydroxyproline excretion was exponential and independent of initial values allowing the expression of the response to the bisphosphonate in a uniform way and to study dose-response relationships. This way, information of the antiresorptive potency of the bisphosphonate could be obtained from small groups of patients treated for short periods (e.g. up to 10 days). The approach was prospectively tested with newer bisphosphonates and efficacious doses relative to those of pamidronate were defined with investigation of small groups of patients with Paget's disease particularly when combined with an *vitro* resorption assay representative of the action of bisphosphonates in vivo [44]. Thus, doses of bisphosphonates effectively decreasing bone resorption could be quickly selected for further clinical testing shortening considerably the time of phase 2 dose-finding studies.

The dose of oral pamidronate used in the treatment of Paget's disease (600 mg/d) caused gastric irritation and about half of the patients developed epigastric complaints that necessitated temporary withdrawal of the drug in 19 patients. This side-effect of oral pamidronate was also observed in a study of patients with bone metastases from breast cancer in whom the dose of 600 mg/d had to be reduced to 300 mg/d for long-term treatment [32,45]. Gastric intolerance of oral pamidronate was dose-dependent and indicated that in disorders characterized by increased rates of bone resorption (e.g Paget's disease, malignancy-associated hypercalcaemia) in which higher bisphosphonate doses are needed for optimal efficacy, intravenous administration should be the preferred mode of treatment. Epigastric complaints were attributed to the amino group of the molecule as also later confirmed with other aminobisphosphonates.

In summary, the use of pamidronate in the treatment of patients with Paget's disease suggested: a. The optimal way to treat the disease with a potent N-BP; b. The assessment of the potency of bisphosphonates by their ability to normalize biochemical markers of bone turnover than by the relative decrease of these markers, as was then the practice; c. The use of clinical and biochemical remissions as long-term treatment targets. These concepts were further applied to the development of other N-BPs that led to the current efficacious treatment of the disease with a single intravenous infusion of zoledronate [46].

3.1.1. Acquired resistance to pamidronate

In the early studies of Paget's disease there was no evidence of diminished response to consecutive pamidronate therapy in patients treated for a relapse of the disease [43]. However, with longer term observations progressive reduction in responsiveness was observed in some patients. These impaired responses to pamidronate were related to the extent of skeletal involvement but not to the dose of pamidronate or to biochemical activity of the disease and only patients with three or more affected bones were more likely to develop true resistance to pamidronate [47]. To assess whether this acquired resistance to treatment was specific for pamidronate, data obtained in a large group of patients treated with olpadronate were also analysed [47,48]. There was no evidence of impaired responsiveness to olpadronate even when patients with extensive skeletal disease were separately analysed. Thus, acquired resistance to treatment was not a general property of bisphosphonates and appeared to be specific for pamidronate.

Theoretically, for Pagetic osteoclasts to become unresponsive to bisphosphonate treatment with time, either the intracellular uptake of bisphosphonate should be reduced or its interaction with its molecular target should be altered. There were no known mechanisms that could impair the uptake of the bisphosphonate by the osteoclasts with time and studies with statins that target the same intracellular biochemical route upstream FPPS, had shown no evidence of development of resistance to their action [49,50]. Thus, resistance to the action of pamidronate in Paget's disease was more likely due to disease-related factors rather than to decreased responsiveness at the molecular level. This was supported by the findings that the response to pamidronate could be impaired only in patients with extensive Paget's disease. Alternatively, it was observed that a significant part of the antiresorptive activity of pamidronate in vitro involves targets additional to FPPS [51] and it may be that altered interaction of pamidronate with these targets might contribute to the impaired response with time.

3.2. Acute phase response

During the initial clinical studies with pamidronate some patients with Paget's disease showed a increase in body temperature within the first 5 days of treatment associated with flu-like symptoms such as malaise, headache and muscle pain (summarized in [39,52]). Such reaction had not been previously observed in studies with either etidronate or clodronate and appeared to be specific for pamidronate. The reaction was transient, reversed with no specific treatment and did not occur in patients previously exposed to pamidronate but was observed in patients previously treated with etidronate. In a detailed analysis of 63 patients with Paget's disease treated with pamidronate during admission to a metabolic ward, an increase in body temperature >0.5 °C

was observed in 54% patients treated with oral pamidronate 600 mg/d and in 63% of those treated with daily intravenous infusions of pamidronate. The peak increase in temperature occurred within the first 72 h after the start of treatment and was not related to the mode of administration of pamidronate, or to the severity or activity of the disease. It was, however, associated with transient reductions in blood lymphocytes and increases in granulocytes and in serum C-reactive protein (CRP). Clinical and laboratory findings resembled, thus, the acute phase reaction induced by infectious or inflammatory factors but the magnitude of serum CRP changes was much lower. The question raised then was whether this was a side-effect of treatment or whether it was related to the mechanism of action of pamidronate on bone resorption [52]. The lack of such response in patients previously treated with pamidronate made the first explanation unlikely and the authors hypothesized that pamidronate may modulate monocyte-lymphocyte interaction at sites that they have been accumulated [52]. The acute phase response (APR) following intravenous pamidronate was confirmed by Adami et al. [53] in patients with different bone disorders who showed, in addition, that the response could be also induced by two other N-BPs (alendronate and neridronate) but not by clodronate and it was absent in patients previously treated with a N-BP. The Leiden group showed later in studies with olpadronate that the APR was associated with transient increases in circulating IL-6 that preceded the increase in body temperature [54] (Fig. 3) and were significantly related with serum CRP levels, a finding confirmed by other investigators in patients with bone metastases treated with intravenous pamidronate [55]. These showed, in addition, concurrent increases in circulating TNF-α that were also significantly related with serum CRP but not as strongly as IL-6 levels. Thus, APR occurred exclusively in patients treated with N-BPs, appeared to be dose-dependent and was associated with increased production of proinflammatory cytokines, particularly IL-6; all studies suggested a link of APR to the mechanism of action of N-BPs, a hypothesis confirmed later following the elucidation of the molecular mechanism of action of N-BPs [56].

3.3. Pharmacokinetics/dynamics

Information of pharmacokinetic/pharmacodynamic (PK/PD) relationships of bisphosphonates could only be obtained at later stages of their development [57]. This was due to the specific pharmacological properties of bisphosphonates and to difficulties in determining their

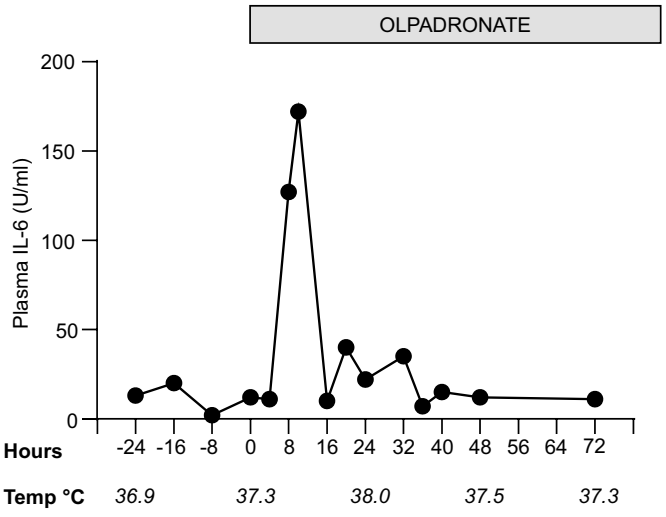

Fig. 3. Sequential measurements of plasma IL-6 activity in a patient with Paget's disease and Acute Phase Response before and after treatment with intravenous olpadronate 4 mg/d. The increase in plasma IL-6 values preceded the rise of body temperature (Temp) and was captured by the frequent sampling (every 4 h). Modified from reference [54].

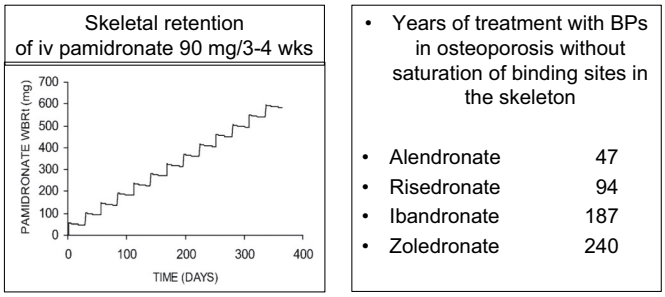

Fig. 4. *Left panel*: Mean Whole Body Retention (WBR) of pamidronate in patients with breast cancer with bone metastases treated with intravenous pamidronate 90 mg every 3 or 4 weeks for 1 year. *Reproduced from reference* [67]. *Right panel*: Years of continuous treatment with different bisphosphonates used in the treatment of osteoporosis without decrease in their WBR; see text for explanation of calculations.

concentrations accurately and reproducibly in serum and urine. Even with improved methodology such relationships have not yet been accurately defined [58,59]. The Department of Clinical Pharmacy in Leiden succeeded in developing methods for measuring pamidronate (and olpadronate) in serum and urine [60–63] that were applied to PK/PD studies in patients with different skeletal disorders [64–68]; these studies provided information about the effect of renal function and rate of bone turnover as well as of characteristics specific of studied diseases on bisphosphonate retention in the skeleton. Two studies helped to better understand not only pamidronate pharmacology but also that of N-BPs in general.

In the first study, patients with bone metastases from breast cancer received intravenous pamidronate 90 mg every 3 to 4 weeks up to 1.5 years [67]. Skeletal retention of pamidronate was constant, about 50% of the administered dose, for the whole duration of the study and was related to the rate of bone resorption only at the start of treatment; this relationship was lost with treatment prolongation and pamidronate was progressively accumulated in the skeleton (Fig. 4) indicating that available sites for pamidronate binding were not saturated. While the measurement method did not allow the definition of separate binding compartments, it demonstrated the great capacity of the skeleton to retain the bisphosphonate. If we extrapolate these findings to N-BPs approved for the treatment of osteoporosis (oral alendronate 10 mg/d, risedronate 5 mg/d, ibandronate 2.5 mg/d and intravenous zoledronate 5 mg once-yearly) and we assume an intestinal absorption of oral bisphosphonates of 0.7% and renal excretion of 50% of the dose in the circulation, patients could be treated continuously with these bisphosphonates for 47 to 240 years without any evidence for reduced retention of the bisphosphonate in the skeleton (Fig. 4). Thus, saturation of the skeleton with bisphosphonate in clinical practice, an early concern in bisphosphonate development, is impossible. The second study reported observations in young patients treated for long periods with pamidronate in whom urinary excretion of the bisphosphonate was measured after cessation of treatment [68]. It was found that pamidronate was measurable in urine up to 8 years following treatment arrest. This was the first direct demonstration of the persistence of bisphosphonate release long after treatment discontinuation.

3.4. Malignancy-associated hypercalcaemia (MAH)

Following the initial observations of successful treatment of osteolytic bone disease and MAH with pamidronate [30], studies in Leiden focused mainly on the effect of treatment on bone and kidney in the pathogenesis and correction of hypercalcaemia [31,69] and the overall experience of the treatment of 132 patients with MAH was reported by Harinck et al. [70]. Intravenous pamidronate, 15 mg/d was administered until normalization of serum calcium concentrations up to a maximum of 10 days. Three groups of patients were analysed according

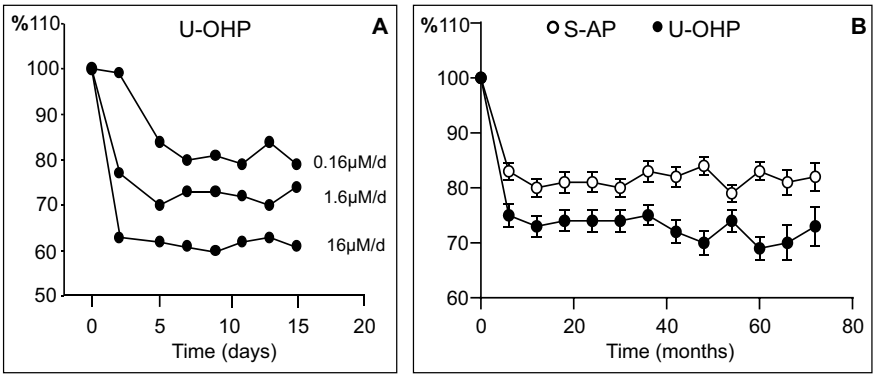

Fig. 5. *Left panel* (A): Percentage decrease of urinary hydroxyproline excretion (U-OHP) in rats treated with daily subcutaneous injections of different doses pamidronate. *Modified from reference* [10]. *Right panel* (B): Percentage decreases of serum alkaline phosphatase activity (S-AP), open circles, and U-OHP, closed circles, during long-term treatment of patients with osteoporosis with oral pamidronate 150 mg/d. Adapted from reference [71].

to administration of physiological saline. 1. Patients who received ≥1lt normal saline at least 48 h before and during pamidronate treatment; 2. Patients who started saline infusion together with pamidronate and 3. Patients in whom use of normal saline was left to the discretion of treating physicians. Serum calcium concentrations normalized in 91% of patients confirming the efficacy of pamidronate therapy. The number of patients studied, the variety of malignant tumours with and without bone metastases as well as the treatment regimens used, allowed additional, detailed evaluation of the relationships between serum calcium concentrations and urinary calcium and sodium excretion (787 measurements of clearances were available). Results provided evidence of inappropriately high, sodium-independent, calcium reabsorption in patients with certain types of tumours that supported the assumption that some tumours can produce substances with a PTH-like effect on renal handling of calcium. These were predominantly patients with renal and lung cancers which were also those with the lowest prevalence of bone metastases. Importantly, the investigators concluded that the ability of the kidney to handle an increased load of calcium, through augmentation of fractional calcium excretion, is reduced in MAH. Tumour-derived humoral factors contribute to this reduction but the major limitation in calcium excretion is the augmentation of calcium-associated sodium reabsorption capacity that compensates the sodium diuretic effect of a high calcium concentration in the glomerular filtrate. Effective treatment of MAH should, therefore, not only aim at reversing the increased bone-generated calcium load but requires also administration of sodium chloride for as long as hypercalcaemia persists, the accepted approach to treat this condition.

3.5. Metastatic bone disease

van Breukelen et al. [30] showed in a pilot study inhibition of tumour-induced osteolysis in a few normocalcaemic patients with malignant diseases treated with oral pamidronate. Consequently, the first prospective, randomized study of patients with breast cancer and bone metastases treated or not with oral pamidronate 300 mg/d was initiated in 1983. The end point of the study was the prevention of skeletal morbidity due to progression of metastatic disease [32]. Events related to skeletal morbidity included hypercalcaemia, bone pain requiring radiotherapy or surgery and pathological or imminent fractures. In addition, the changes in antitumour therapy required due to progression of osteolytic metastases were evaluated. In pamidronate-treated patients hypercalcaemia did not occur and bone pain and pathological or imminent fractures were significantly reduced compared to the control group after a median follow-up of 13 months. In addition, supportive pamidronate treatment more than halved the requirement for specific therapies due to metastatic disease. The study demonstrated for the first time the efficacy of a N-BP in the treatment of patients with bone metastases from breast cancer that led to the successful application of intravenous pamidronate and later of zoledronate in the management of patients with metastatic disease.

3.6. Osteoporosis

At the time of pamidronate discovery the rationale for the use of bisphosphonates in the treatment of osteoporosis was not immediately obvious due to the complexity and heterogeneity of the disease as well as concerns about the chronic use of bisphosphonates [71,72]. While it was generally believed that osteoporosis results from an imbalance between bone resorption and formation that worsens when accompanied by activation of new remodelling units, bone turnover was not generally increased and there was no evidence of accelerated bone loss in many elderly patients with osteoporosis. The early clinical studies of patients with diseases characterized by increased bone resorption and turnover demonstrated that decrease of bone resorption by pamidronate will be invariably followed by decrease of bone formation leading to a state of lower bone turnover. It was feared that such effect might reduce the ability of bone to remodel in individuals with normal or low bone turnover and might increase the risk of skeletal damage, an undesirable effect in patients with osteoporosis. Furthermore, pamidronate given at doses that effectively reduced bone resorption had prolonged effects on bone metabolism that appeared to depend on the dose of the bisphosphonate as well as on the prevalent rate of bone turnover. In addition, about 50% of the administered bisphosphonate was retained for long in the skeleton and there was uncertainty about its long-term fate and its effects on bone metabolism. Questions, therefore, relevant to treatment of osteoporosis included the dose and the duration of the response to pamidronate as well as of the activity of the bisphosphonate removed from the bone surface and embedded in the skeleton on bone remodelling with time. To address these questions extensive pilot studies, preferably of long duration, were necessary.

Two observations, described above, provided the rationale and helped in the design of initial studies in Leiden. The first was the rat study that illustrated the feasibility to modulate bone remodelling mildly and induce significant increases in calcium balance with low dose pamidronate and that uninterrupted daily administration was not accompanied by progressive decrease of bone turnover (Fig. 5A) [10]. The second was the observation that the equilibrium calcium balance after 6–9 months treatment of Paget's disease with pamidronate was about 1 mmol more positive than the baseline balance [28]. This observation suggested persistence of the effect of treatment on calcium retention. These studies were, however, performed in models characterized by increased bone turnover and their reproducibility in osteoporotic patients needed to be confirmed.

In an initial study 7 patients with osteoporosis and normal bone turnover were treated with oral pamidronate 600 mg/d. As expected, bone resorption decreased with treatment and calcium balance increased significantly within 2 weeks. The dose was, however, high for long-term administration to patients without increased bone turnover and a lower dose (150 mg/d) was given to 14 patients for one year [73]; this dose induced a lower decrease of biochemical parameters of bone turnover but was associated with a positive calcium balance after one

year (from −0.72 to 1.33 mmol/, $p < 0.005$). This increase in calcium balance corresponds to a gain in BMD of about 3% in a patient with osteoporosis, confirmed in a further group of patients treated with oral pamidronate in whom BMC of the spine was measured by DPA. After a mean period of 2.2 years, BMC increased by 6.8% while in a parallel studied group with osteoporosis and similar clinical and densitometric characteristics no significant change in BMC was observed. The observed effect at the spine did not occur at the expense of cortical bone shown by SPA measurements. The study was extended and included more patients and showed that the BMC increase was not confined to the first two years and continued to increase at a an overall rate of 2.4% per year for at least 4 years of treatment [71]. Notably, values of urinary hydroxyproline excretion and serum AP, measured 6-monthly, after an initial reduction, remained stable for the whole period of treatment up to 72 months (Fig. 5B). This result indicated that there is no cumulative effect of long-term administration of oral pamidronate on bone turnover. Additional support was obtained from a limited number of bone biopsies taken between 2 and 4 years of treatment that showed bone of normal structure without a mineralization defect. It was further shown that acute calcium mobilization following a strong hypocalcaemic stimulus was not impaired and did not differ between patients treated with oral pamidronate for 5 years compared with a group of treatment-naïve patients with osteoporosis [74]. Combined the results of these studies suggested that long-term efficacious treatment of osteoporosis with pamidronate was feasible and safe justifying the design of controlled studies with daily, low dose, oral pamidronate or other N-BPs.

The next question addressed was whether the long-term (>5 years) sustained reduction of bone turnover by pamidronate was reversible after cessation of treatment. Women and men with osteoporosis and prevalent vertebral fractures treated for a mean period of 6.5 years (range 5 to 9 years) with oral pamidronate 150 mg/d were followed for 2 years after discontinuation of treatment [75]. Urinary hydroxyproline levels increased, but not to pre-treatment levels while BMD at the spine and the hip did not change (Fig. 6). The incidence of vertebral fractures after cessation of treatment in this high risk group was similar to that observed during treatment. The study demonstrated, thus, lack of rebound of bone remodelling and indicated persistent protection of the skeleton following arrest of long-term treatment with pamidronate. It was hypothesized that the small quantities of the drug released locally by desorption although insufficient to sustain a reduction of bone turnover may have been adequate to correct the imbalance between bone resorption and formation at remodelling sites.

These pilot studies with daily oral pamidronate in patients with severe osteoporosis described the complete sequence of expected responses to N-BP therapy in osteoporosis shown later in large clinical trials with other N-BPs.

3.7. Effects on the growing skeleton

In 1985 Hoekman et al. [35] reported clinical, biochemical and radiological observations of a 13.5 year-old boy with severe juvenile osteoporosis with multiple metaphyseal and vertebral fractures treated with pamidronate. Results were striking with normalization of increased indices of bone resorption and of a negative calcium balance within one week of treatment associated with clinical improvement and healing of metaphyseal and one diaphyseal fracture.

Osteoporosis in children is rare and is usually secondary to factors responsible also for the disease in adults. Differently from adults, in the growing skeleton bone is deposited not only by bone remodelling but also by bone modeling at surfaces that have not been previously resorbed by osteoclasts. Evaluation of therapeutic interventions, should, therefore, consider the growth potential of the skeleton as well the continuous changes of parameters used for the evaluation of bone metabolism during growth, especially during puberty (e.g. biochemical parameters of bone turnover, BMD). In children, as shown earlier by bone scintigraphy with ^{99} Tc bound to bisphosphonate, the uptake of bisphosphonate in the skeleton is characteristic of the growth process as they concentrate primarily in areas where active growth takes place, such as the metaphysis of long bones close to the growth plate. The pattern of bisphosphonate uptake may have practical, as well as theoretical, consequences not encountered in the treatment of adults. In addition, the growing skeleton is particularly sensitive to factors that adversely affect bone metabolism and any potential deleterious effect of long-term bisphosphonate administration might be, thus, best identified in children.

Long-term effects of pamidronate on the growing skeleton were studied in young patients treated with daily oral pamidronate for a mean period of 6.7 years and were followed after discontinuation of treatment for a mean total period of 12.9 years (range 7 to 19 years) [76]. Thus, although number of studied patients was small, periods of observation were long enough to detect adverse effect of treatment on the growing skeleton. The most clinically relevant results of all studies of young patients in Leiden were: 1. No impairment of linear growth and fracture healing, 2. Improvement of calcium balance within a week of starting treatment to a level maintained for at least 3 years (Fig. 7A), 3. Increase in BMD at both the spine and hip in young patients with a slope different from that of their peers if treatment started before puberty, 4. Striking radiological changes, consisting of band-like metaphyseal sclerosis [76,77]; the extent of sclerosis depended on the duration of treatment and growth activity (Fig. 7), 5. Reversal of spinal deformities in prepubertal patients reminiscent to those of young patients with Cushing's disease after successful surgical treatment [78]. 6. No abnormal histological findings on bone biopsies taken between 2 months and 6 years treatment [76]. Notably, the presence of

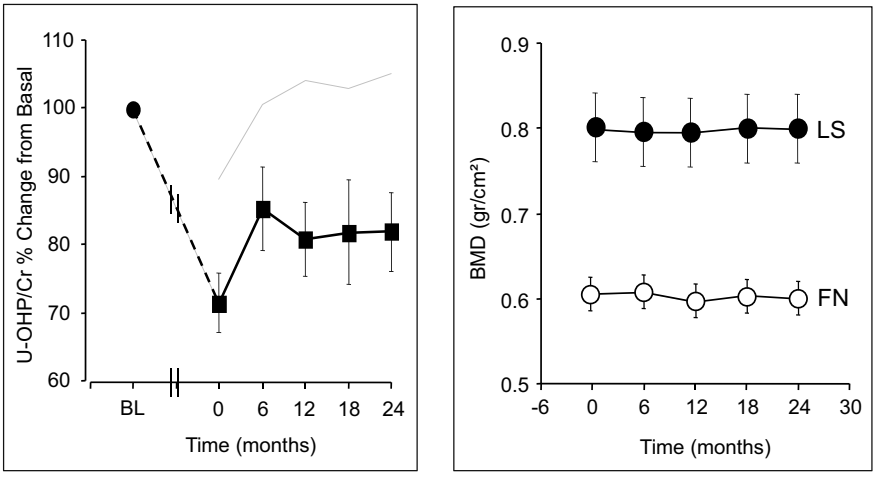

Fig. 6. *Left panel*: Percent change (mean ± SE) of urinary hydroxyproline excretion (U-OHP) of patients with osteoporosis after a mean treatment period of 6.5 years with oral pamidronate 150 mg/d (from baseline-BL- to 0) and two years after discontinuation of treatment (0 to 24 months). *Right panel*: BMD of the lumbar spine (LS) and the femoral neck (FN) after discontinuation of treatment. Modified from reference [75].

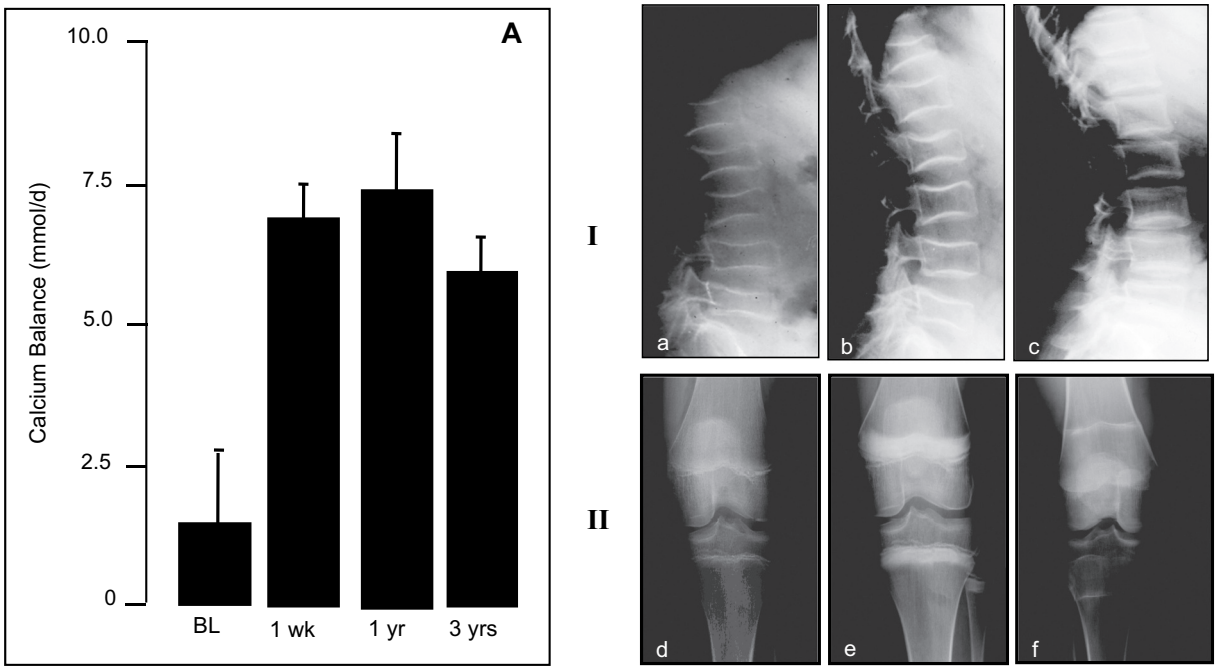

Fig. 7. *Left panel* (A): External calcium balance (mean ± SEM) measured during admission to a metabolic ward before, after one week, one year and three years of 8 young patients with osteoporosis treated with pamidronate. *Adapted from reference* [76]. *Right panel*: I. Sequential spine radiographs (a-c) of a child treated with oral pamidronate for 6 years. Note, radiograph a. was made after the start of treatment because vertebrae were hardly seen in the pre-treatment radiograph. II Sequential radiographs of the knee of a child treated with pamidronate; d. before treatment, e. after treatment for one year, f. after treatment for 6 years; note the undisturbed linear growth.

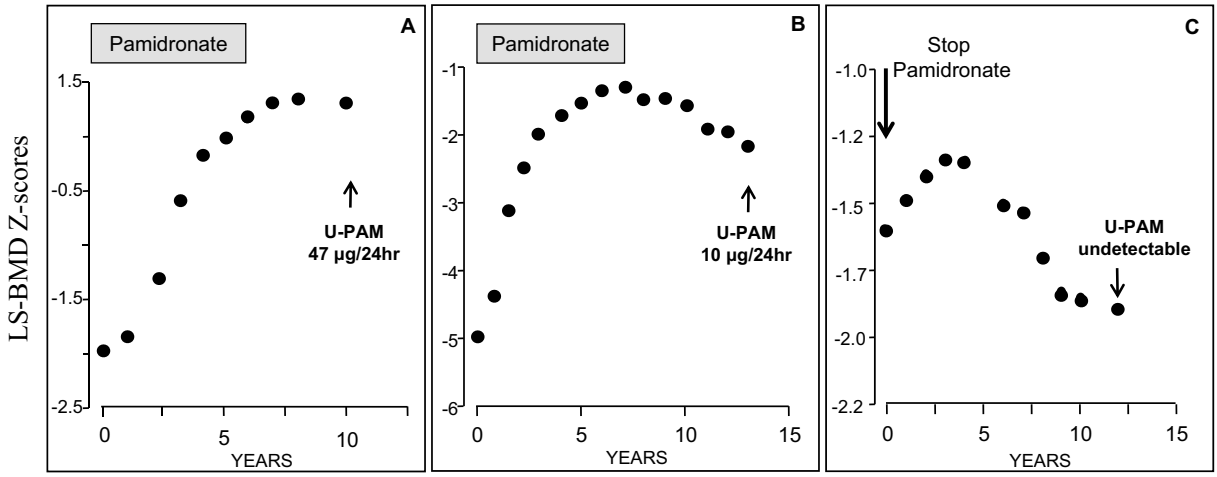

Fig. 8. Long-term sequential measurements of lumbar spine (LS) BMD *Z*-scores in 3 young patients during and after discontinuation of treatment with pamidronate (A, B) or after discontinuation of pamidronate (C). Values of urinary excretion of pamidronate (U-PAM) are also depicted.

metaphyseal sclerosis on radiographs of treated children was also indicative of effective absorption of oral pamidronate and confirmed compliance to treatment that could have been, otherwise, very difficult to objectively evaluate in clinical practice. The studies showed also that the bisphosphonate was released in the circulation up to 8 years after stopping treatment but there was no relation between the cumulative dose of the bisphosphonate and its excretion in urine [72]. This was the first direct evidence of long-term release of bisphosphonate in humans that could persist for years after discontinuation of treatment. The activity of the released bisphosphonate is unknown but may account for the absence of quick reversal of the effect of treatment on bone turnover and BMD observed after stopping treatment (Fig. 8) as also reported in adults. Intermittent administration of pamidronate, pioneered by Belgian investigators, was systematically studied by Canadian investigators in children with osteogenesis imperfecta [79,80].

4. Placebo-controlled studies

4.1. Glucocorticoid-induced osteoporosis

The first placebo-controlled study with an antiresorptive agent in glucocorticoid-treated patients was performed in New Zealand with pamidronate provided by Bijvoet. Reid et al. [81] studied 35 patients randomized to oral pamidronate 150 mg/d or placebo and followed for one year. Compared to placebo, pamidronate reduced biochemical and histomorphometric indices of bone turnover with no evidence of

osteomalacia in bone biopsies. Pamidronate significantly prevented glucocorticoid-induced trabecular and cortical bone loss, measured by quantitative computer tomography and metacarpal cortical area, respectively; volumetric BMD increased significantly by 19.6% in pamidronate-treated patients while it decreased by 8.8% in those treated with placebo after one year. In patients who consented to continue the study for another year there was further loss of bone mass in the placebo group that was prevented by pamidronate treatment [82]. Repeat bone biopsies revealed again no evidence of mineralization defect. The authors stated that the satisfactory safety profile of pamidronate was maintained with longer term use. The same group reported later results of a 2-year study of the same design in women with postmenopausal osteoporosis. Results of the efficacy and safety of pamidronate were similar to those of the glucocorticoid study [83].

4.2. Rheumatoid arthritis

Early studies had suggested an increased prevalence of osteoporosis [84] in patients with rheumatoid arthritis (RA) and in animal models of adjuvant arthritis bisphosphonates were reported to have additional anti-inflammatory properties [85]. Encouraged by early observations of a potential anti-inflammatory effect of pamidronate in patients with RA [51] short- and long-term studies in patients with RA not receiving glucocorticoids were performed in collaboration with the Department of Rheumatology in Leiden.

4.2.1. Short-term study

Thirty patients with active RA were randomized to a single intravenous infusion of placebo, 20 mg pamidronate, or 40 mg pamidronate [86]. Compared to placebo, pamidronate treatment induced a rapid and sustained reduction of bone resorption associated with significant improvement of clinical variables of disease activity in both groups of pamidronate-treated patients within 3 weeks. Erythrocyte sedimentation rate and serum C-reactive protein levels improved significantly more in patients treated with 40 mg pamidronate. No serious side-effects were documented. The study confirmed the original observations of Bijvoet et al. [51] and showed an additional, probably dose-dependent, short-term effect on disease activity by a single pamidronate infusion in patients with RA.

4.2.2. Long-term study

The 3-year efficacy and safety of oral pamidronate 300 mg/d was examined in a randomized, double-blind, placebo-controlled study of 105 patients with RA not receiving glucocorticoids; the primary efficacy point was the difference of BMD measured yearly [87]. Treatment increased significantly BMD at all measured sites (spine, hip, forearm) by 8.4%, 2.6% and 5.4%, respectively, after 3 years; the corresponding changes of placebo-treated patients were 0.6%, −4% and −1.2%. Treatment reduced urinary hydroxyproline excretion and the pattern of changes was similar to that previously observed during long-term treatment with oral pamidronate. Joint damage, assessed radiologically, increased in both groups while clinical and biochemical parameters of disease activity improved significantly in both groups but these changes were not significantly different between the two groups. Changes in SAARDs, considered necessary for disease control, were significantly higher in placebo-treated patients. Fifteen pamidronate-treated patients vs 4 placebo-treated patients discontinued the study because of gastrointestinal side-effects, mainly nausea and vomiting. While the study met its primary end point, it did not show a significant effect of pamidronate on disease activity. In both previous studies, that showed positive effects of pamidronate treatment in patients with RA, doses higher than 300 mg/d (about 3 mg iv) were used. Results of the studies in patients with RA suggested that if the aim of treatment includes an effect on disease activity, pamidronate should be given intravenously.

4.3. Osteporosis

Despite the positive results of the long-term studies of the efficacy and safety of oral pamidronate in the treatment of osteoporosis it took a number of years before the first placebo-controlled study was performed. The reason being that the drug had already been obtained by Ciba-Geigy (Novartis) that focused mainly on the development of intravenous pamidronate for clinical use. In addition, considerable time was devoted by the chemists of the company to prepare an improved oral preparation. During a phase 2 study in the US oral pamidronate caused oesophagitis in 4 of 49 enrolled patients. American investigators, in contrast to those in Europe or South America, had at that time no experience with aminobisphosphonates and they administered pamidronate before bed time [88]. They hypothesized that this was the optimal time to give the bisphosphonate because bone resorption increases at night. This approach failed to consider the irritative effect of aminobisphosphonates after prolonged contact with the oesophageal epithelium; this being also the reason of instructing patients not to lie down after taking aminobisphosphonates. This adverse effect led to discontinuation of the development of oral pamidronate and the company focused only in the development and marketing of intravenous pamidronate. Leiden and Amsterdam investigators initiated a randomized, placebo-controlled trial in patients with established osteoporosis using the Ciba preparation which, after the decision of the company to stop the studies, was continued with enteric-coated pamidronate and identical placebo tablets prepared in Leiden. This was a 3-year study with a 2-year open extension in women with postmenopausal osteoporosis and men with idiopathic osteoporosis with at least one prevalent vertebral fracture [89]. Peptic ulcers or gastrointestinal diseases, other than malabsorption syndromes, were not exclusion criteria. Trial medication was administered on an empty stomach at least 30 min before breakfast, lunch, or dinner with a full glass of water leaving the choice of time to the patients. Compared with placebo, pamidronate increased LS-BMD of the spine and FN-BMD by 14.3% and 2.9%, respectively after 5 years. In these patients with severe osteoporosis, pamidronate decreased the incidence of new vertebral fractures by 67% during the 3-year blinded period of the study. Bone biopsies taken after one or two years of treatment showed bone of normal architecture, no marrow fibrosis and no evidence of impaired mineralization; parameters of bone turnover decreased significantly in pamidronate-treated patients, with no differences between the first and the second year, and did not change in those treated with placebo [90]. Six patients, 3 in each treatment group (6%), stopped treatment during the 3-year blinded period because of gastrointestinal complaints. Three of these patients underwent endoscopy because of severe symptoms. Esophagitis was diagnosed endoscopically in 2 patients on placebo, one of whom had a known hiatus hernia. One patient on pamidronate with epigastric complaints before the trial was found to have two duodenal ulcers and esophagitis. These, currently of historical significance, results confirmed the efficacy and safety of oral pamidronate 150 mg/d in the treatment of established osteoporosis in men and women. Oral pamidronate prepared by Gador SA was marketed in South America.

5. Epilogue

Observations during the unconventional development of pamidronate described here, particularly at its early stages, raised doubts among many experts about the place of this and other N-BPs in the management of patients with osteoporosis and other benign skeletal disorders. Concerns included the gastrointestinal toxicity of oral administration, the acute phase reaction and the long skeletal retention of bisphosphonates. Consequently, there was a period that systematic clinical research with N-BPs in such disorders was continued in only few European Centres. This attitude changed following Merck's acquisition of alendronate from the Italian company Istituto Gentili Spa and the systematic development of this N-BP by Gideon Rodan and Merck

scientists that helped to position the whole class as the most frequently used treatment of osteoporosis. Intravenous pamidronate was approved word-wide for the treatment of MAH, Paget's disease and metastatic bone disease including multiple myeloma. Pamidronate has also been extensively used off-label over the years in the treatment of numerous common or rare disorders of bone and mineral metabolism illustrating the wide spectrum of diseases that might benefit from N-BP therapy. It took only a short trip from Leiden in The Netherlands to Dusseldorf in Germany.

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