Clin Oral Invest (2011) 15:105–111
DOI 10.1007/s00784-009-0365-2

ORIGINAL ARTICLE

Bisphosphonates: restrictions for vasculogenesis and angiogenesis: inhibition of cell function of endothelial progenitor cells and mature endothelial cells in vitro

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Received: 3 July 2009 / Accepted: 2 December 2009 / Published online: 19 December 2009

Abstract Bisphosphonate-associated osteonecrosis of the jaws (BP-ONJ) is one of the main side effects in patients treated with bisphosphonates for metastasis to the bone or osteoporosis. BP-ONJ usually occurs in patients treated with highly potent nitrogen-containing bisphosphonates. The exact mechanism of action and etiopathology is still unknown. In addition to inhibition of bone remodelling, an anti-angiogenetic effect has become the focus of research. The aim of these study was to investigate the effect of different bisphosphonates on human umbilicord vein endothelial cells (HUVEC) and endothelial progenitor cells (EPC), which play an important role in angiogenesis. Using varying concentrations, the impact of one non-nitrogen-containing bisphosphonate (clodronate) and three nitrogen-containing bisphosphonates (ibandronate, pamidronate and zoledronate) on HUVEC and EPC was analysed. The biologic behaviour of HUVEC after incubation with different bisphosphonates was measured in a Boyden migration assay as well as in a 3D angiogenesis assay. The number of apoptotic cells was measured by Tunnel assay. To underline the importance of neoangiogenesis in the context of BP-ONJ, we measured the EPC number after incubation with different bisphosphonates in vitro. HUVEC and EPC were significantly influenced by bisphosphonates at different concentrations compared with the non-treated control groups. The nitrogen-containing bisphosphonates pamidronate and zoledronate had the greatest impact on the cells, whereas clodronate followed by ibandronate was less distinct on cell function. These results underline the hypothesis that inhibited angiogenesis induced by bisphosphonates might be of relevance in the development and maintenance of BP-ONJ. The increased impact by highly potent bisphosphonates on HUVEC and EPC may explain the high prevalence of BP-ONJ in patients undergoing this treatment.

Keywords Bisphosphonate-associated osteonecrosis of the jaws · Bisphosphonate · HUVEC · EPC · Angiogenesis · Vasculogenesis

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Introduction

The benefit of bisphosphonates in the treatment of malignant bone neoplasias like multiple myeloma, bone metastases (e.g. due to primary breast cancer or prostate cancer) or metabolic bone diseases like Paget's disease and severe osteoporosis is without controversy. Their antiresorptive mechanism has resulted in a significant reduction in skeletal-related events such as pain, hypercalcaemic episodes or fractures with the need of radiation therapy or stabilising operations, thus increasing quality of life.

However, besides these explicit benefits, the side effects of bisphosphonates should be considered especially for high-dosage, long-term treatment with highly potent derivatives. Traditional, rather unspecific side effects can be categorized into acute phase reactions [1], gastrointestinal effects [2]

and renal side effects [3]. Since 2003, a new specific side effect, bisphosphonate-associated osteonecrosis of the jaws (BP-ONJ), has increasingly become the focus of clinical and preclinical investigations, particularly because it clearly has increased in frequency since then. According to the American Association of Oral and Maxillofacial Surgeons [4], BP-ONJ is defined as exposed necrotic bone in the maxillofacial region for a period of at least 8 weeks in connection with current or previous bisphosphonate therapy and a lack of head and neck radiation in the patient's history. Described incidences for BP-ONJ range from 1% to 11% in breast cancer patients [5], from 3% to 17% in multiple myeloma patients [6] and from 3% to 19% in prostate cancer patients [7].

Employed bisphosphonates significantly differ in part due to biological effects, clinical potency as well as in seriousness of side effects. Highly potent, nitrogen-containing bisphosphonates such as pamidronate and zoledronate are more often associated with BP-ONJ compared to the less potent ibandronate and the non-nitrogen-containing clodronate.

In most cases, clinically manifest BP-ONJ is triggered by injury to the oral mucosal integrity, as occurs during dental surgical procedures such as tooth extractions, pressure denture sores or periodontal disease. In these patients, physiological wound healing with *restitutio ad integrum* is substantially impaired, which requires elaborative and often inefficient treatment strategies [8]. To date, the exact etiopathology of BP-ONJ has not been investigated in detail. However, research hints at a multifactorial genesis affecting different tissues and cell types. The reduced bone remodelling in BP-ONJ patients is attributed to bisphosphonate-induced inhibition of osteogenic cells and osteoclasts in a dose-dependent manner [9]. Furthermore, bisphosphonates have been proven to reduce viability of oral keratinocytes [10], which corresponds with impaired mucosal wound healing. Next to this, a reduction of extracellular matrix protein production has been described [11].

Sufficient tissue vascularity and vessel formation is indispensable for tissue homeostasis, local immunity and adequate regeneration. Bone tissue is particularly vascularised, and endothelial cells have been proven to play an essential role during bone remodelling [12]. In this context, the term *angiogenesis* refers to sprouting from preexisting vessels of rather mature endothelial cells. Of great scientific interest is the fact that bone-marrow-derived, circulating endothelial progenitor cells (EPC) bear the potential of de novo creation of primordial vessels, called *vasculogenesis* [13–15]. EPC have the potential to differentiate into mature endothelial cells [16]. Furthermore, EPC show strong paracrine effects by production of several cytokines and growth factors which increase neovascularisation such as vascular endothelial growth factor (VEGF), stromal cell-derived factor-1, insulin-like growth factor-1 and hepatocyte growth factor [16]. EPC have been shown to support the neovascularisation in the short term as well as in the long term [15].

However, histopathological analysis of BP-ONJ bone specimens revealed markedly reduced density of vessels or even avascular necrosis. Investigations of BP-ONJ patients showed both a reduction of circulating endothelial (progenitor) cells [17] as well as a significant and lasting decrease in VEGF serum levels [18], indicating that the interactions between bisphosphonates and the endothelial cell lineage with vasculogenesis/angiogenesis hold a key function in the understanding of BP-ONJ.

The focus of the present study was to monitor the influence of four differently potent bisphosphonates on EPC viability, migration and apoptosis rate. Furthermore, mature endothelial cells (human umbilical vein endothelial cells, HUVEC) were investigated accordingly to monitor possible influence of the maturation stage of the endothelial lineage.

Materials and methods

Cell culture

Cell cultures were prepared and maintained according to standard cell culture procedures. HUVEC were cultured in an endothelial basal medium (EBM) supplemented with 1 μg/mL hydrocortisone, 12 μg/mL bovine brain extract, 50 μg/mL gentamicin, 50 ng/mL amphotericin-B, 10 ng/mL epidermal growth factor and 10% foetal calf serum (FCS) until the third passage.

EPC culture assay

Mononuclear cells (MNCs) were isolated by density gradient centrifugation with Biocoll (Biochrom KG, Berlin, Germany) from peripheral blood of healthy human volunteers as previously described [15]. Immediately following isolation, total MNCs (8×10^6 cells/mL medium) were plated on 25 cm^2 culture flasks coated with human fibronectin (Sigma, Steinheim, Germany) and maintained in EBM supplemented with EGM SingleQuots, VEGF (100 ng/mL), and 20% FCS.

Apoptosis assay

For the apoptosis assay, HUVECs (5×10^4) in EBM-2 medium supplemented with EGM-2 kit were seeded in six-well plates; after 24 h, HUVECs were incubated with bisphosphonates (clodronate, ibandronate, pamidronate and zoledronate) at increasing bisphosphonate concentrations (0, 5, 50, 100, 200 and 500 μmol) for 24 h. Next, cells were dissolved by trypsin–EDTA and centrifuged onto coverslips at 2,000 rpm for 5 min. Then, cells were fixed with 3%

paraformaldehyde for 10 min at room temperature (RT), permeabilised with 0.1% Triton X-100, 0.1% sodium citrate for 2 min on ice, washed twice with phosphate-buffered saline (PBS), pH 7.4, and incubated for 1 h at 37°C in the dark with a dUTP nick end labelling (TUNEL) reaction mixture (Boehringer Mannheim, Indianapolis, IN, USA) for in situ detection of cell death. After washing twice with PBS, pH 7.4, cells were incubated at RT with the Hoechst solution for 5 min. All Hoechst-positive nuclei as well as TUNEL-positive nuclei were visualised using a Zeiss Axioplan fluorescence microscope. Then, apoptosis was expressed as per cent of fragmented Hoechst-positive nuclei versus total Hoechst-positive nuclei and as per cent of TUNEL-positive nuclei versus total Hoechst-positive nuclei and was reported as fold increase versus control.

Migration assay

To examine the effect of bisphosphonates on HUVEC migration, we used a 24-well Boyden chamber assay system (ThinCert™) according to the manual (Greiner, Germany). HUVECs were incubated for 24 h with different concentrations of bisphosphonates. Cells were harvested, washed twice in PBS and resuspended in HUVEC medium for adjustment to a final concentration of $10^6 mL^{-1}$. HUVECs were stimulated to migrate from the upper to lower chambers by the addition of 10 ng/mL VEGF to the lower chambers. After 12 h, cells were stained with fluorescent dye calcein-AM. Thereafter, the culture medium was removed from the inserts, and the inserts were transferred to the wells of a freshly prepared 24-well plate containing 500 µL trypsin–EDTA per well. This plate was incubated for 10 min in a cell culture incubator at 37°C and 5% CO_2 with sporadic agitation. The inserts were discarded, and 200 µl of the trypsin–EDTA solution, now containing the detached migratory cells, from each well of the 24-well plate was transferred to a well of a flat-bottom, black 24-well plate. For quantification, we used a fluorescence plate reader.

In vitro angiogenesis assay

To evaluate the anti-angiogenic potential of different bisphosphonates, a commercial HUVEC angiogenesis assay (3D Angiogenesis Assay; PromoCell, Germany) was used. The assay was performed according to the manufacturer's instructions. Different concentrations of bisphosphonates were added to the test medium. The sprouting colonies were photo-documented over 48 h. For measurement of the number, length and area of the sprouts, we utilised a newly designed automatic analyzing programme (CellAnalyser).

Statistical analysis

Continuous variables are expressed as mean ± SEM. Comparisons between groups were analysed by *t* test (two-sided) or ANOVA (post hoc test: Tukey) for experiments with more than two subgroups (SPSS software). Values of $p < 0.05$ were considered as statistically significant.

Results

EPC count

Compared to the control group (set 100%), EPC count was reduced in a dose-dependent manner for all investigated

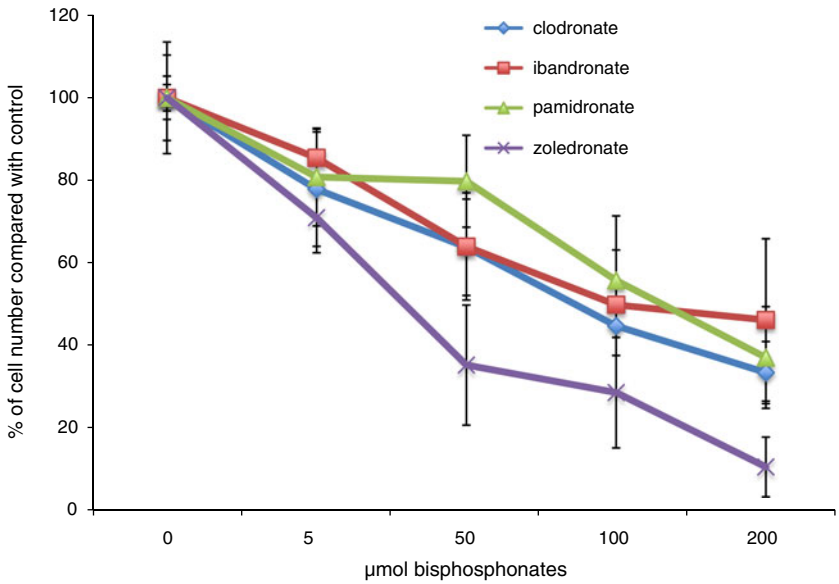

Fig. 1 Relative cell number of EPC 24 h after incubation with different bisphosphonates at different concentrations: 0, 5, 50, 100 and 200 µmol

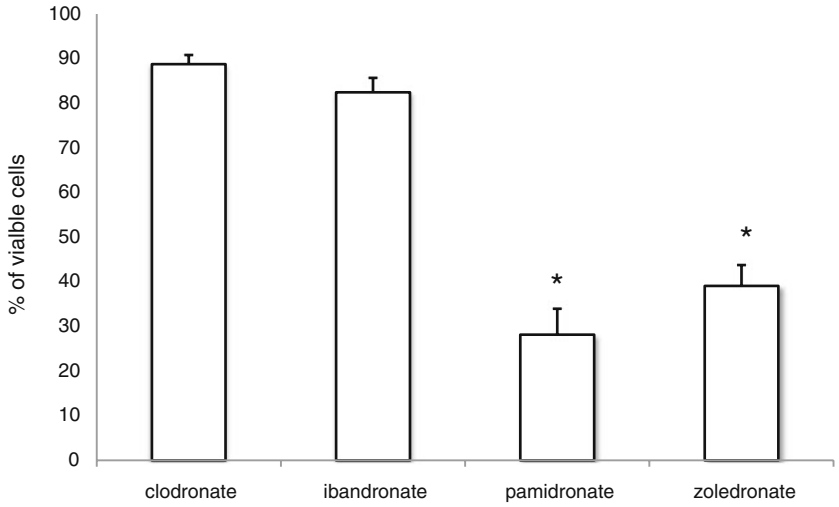

Fig. 2 TUNEL assay of HUVEC after 24 h: relative number of viable cells after treatment with different bisphosphonates at 50 µmol compared to a not treated control set to 100%. Compared to the clodronate and ibandronate group, pamidronate and zoledronate showed a significant reduction in cell viability. The differences between clodronate and ibandronate and the difference between pamidronate and zoledronate were not significant (*$p<0.0002$)

bisphosphonates after 48 h (Fig. 1). Incubation with bisphosphonates reduced the cell count, but only became significant for zoledronate at 50 µmol ($p=0.028$) compared to the control; incubation with 50 µmol clodronate, ibandronate and pamidronate resulted in no significant reduction. Incubation with 100 µmol resulted in significant reduction of cell count for clodronate ($p=0.018$), and a tendency could be shown for ibandronate ($p=0.065$) and pamidronate ($p=0.069$). The latter became significant at concentrations of 200 µmol ($p=0.012$). Between the different bisphosphonates, zoledronate had the greatest impact.

HUVEC viability

After incubation with 50 µmol bisphosphonates, HUVEC viability was significantly reduced for pamidronate ($p<0.001$) and zoledronate ($p<0.001$) after 48 h. Reduction of cell viability in cells incubated with clodronate and ibandronate was not significant (Fig. 2).

HUVEC migration

HUVEC migration capacity was significantly ($p<0.002$) reduced after incubation with 50 µmol of all investigated nitrogen-containing bisphosphonates, and a tendency could be proven for clodronate ($p=0.065$). The effect of pamidronate and zoledronate was significantly stronger compared to the other bisphosphonates (Fig. 3).

Angiogenesis assay

Figure 4 shows exemplary images of HUVEC sprouts directly after incubation with 50 µmol of the different investigated bisphosphonates and after 48 h. The images for

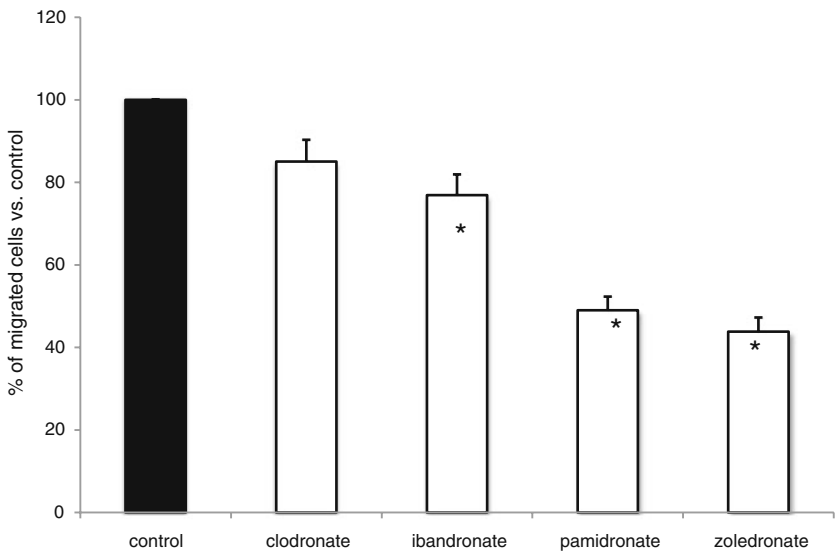

Fig. 3 Inhibition of HUVEC cell migration by different bisphosphonates. Incubation with 50 µmol resulted in a significant reduction of cell migration capacity for all investigated nitrogen-containing bisphosphonates. *$p<0.002$

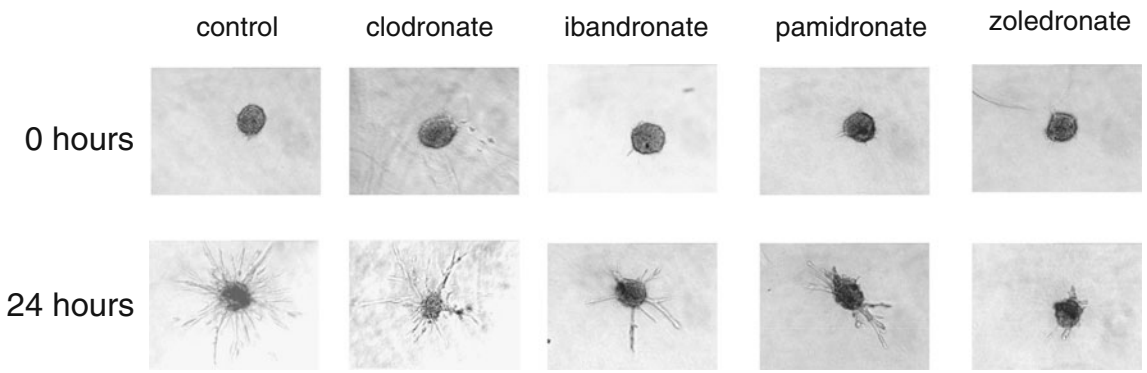

Fig. 4 Angiogenesis assay: exemplary pictures of HUVEC sprouts for the time points 0 and 48 h after incubation with different bisphosphonates at a concentration of 50 µmol (original magnfication, tenfold)

ibandronate and pamidronate showed markedly reduced sprouts after 48 h. Incubation with zoledronate basically showed no sprouts after 48 h. Figure 5 shows the mean area of the sprouts for the time points 0, 12, 24 and 48 h. After 48 h, the control group had a dense, radial sprouting out of the initial cell spheroid. Incubation with clodronate resulted in similar, however slightly reduced sprouting after 48 h which was not significant. The effect of ibandronate was not significant at any point in time either. The impact of pamidronate and zoledronate compared to the control group turned out to be significant after 48 h (p=0.007 res. p=0.017).

Discussion

Several theories on necrosis development for BP-ONJ are being discussed in the literature. Besides the influence of bisphosphonates on cell death of osteoclasts and the impact on osteoblasts through consistently disturbed bone remodelling, an anti-angiogenetic influence has become the focus of research [19–21]. The development of blood vessels is essential for healing and regeneration processes, especially in the avascular necrosis of BP-ONJ.

The influence of different concentration of bisphosphonates on the cell function and viability both on matured endothelial cells (HUVEC) and EPC was investigated in this study. Angiogenesis is defined as blood vessel development due to the development of endothelial cell bridges by the dividing of matured preexisting endothelial cells [22]. EPC are important for vasculogenesis, a mechanism of new vessel formation [15].

Nitrogen-containing bisphosphonates have an influence on the migration ability of HUVEC. Particularly pamidronate and zoledronate had a significant impact compared to the control and the clodronate group. This is of relevance since migration has a key function in angiogenesis. A similar influence was found for the apoptotic rate of bisphosphonate-treated HUVEC: HUVEC treated with pamidronate and zoledronate had a higher rate of apoptosis compared to the

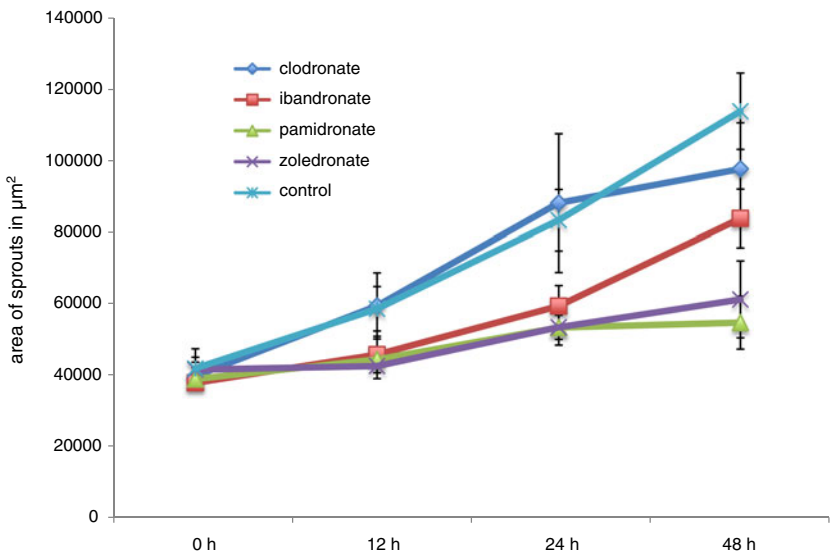

Fig. 5 HUVEC angiogenesis assay: area of spheroid with sprouts (µm^2) after incubation with different bisphosphonates. Compared to the control group, treatment with ibandronate, pamidronate and zoledronate resulted in reduced sprouting areas after 12, 24 and 48 h; only pamidronate and zoledronate had a significantly negative impact at 48 h

groups treated with clodronate or ibandronate. In the 3D angiogenesis model, pamidronate and zoledronate had significantly reduced numbers and areas of sprouts. This effect could be explained by the increased apoptosis rate and the disability of migration in HUVEC treated with the highly potent bisphosphonates. In addition to angiogenesis, vasculogenesis is influenced by bisphosphonates as well. EPC cultures treated with bisphosphonates, especially zoledronate, had significantly reduced cell numbers. Due to the accumulation of bisphosphonates in bone and its long half-life, it may interact with the release of EPCs from bone marrow niches [23] and therefore support the development and maintenance of BP-ONJ.

The biological ability, cell function and viability of HUVEC and EPC is influenced by the nitrogen-containing bisphosphonates ibandronate, pamidronate and zoledronate. Interestingly, clodronate has only a minor effect. Similar findings showing a significant influence on tumours for zoledronate but not for clodronate have been described previously [24]. This effect is used in tumour treatment [25]. In vitro studies on mature endothelial cells have shown impairment of cell proliferation [26], promotion of programmed cell death [27] and inhibition of capillary tube formation [28, 29] by bisphosphonates. In a recent in vitro investigation, Yamada et al. [30] showed that zoledronate inhibited EPC differentiation at low drug levels and induced EPC apoptosis at high concentrations. Further trials on EPC migration and sprout forming would supply further insights regarding the role of bisphosphonates in vasculogenesis and wound repair.

Important in this context might be the different modes of action of nitrogen-containing and non-nitrogen-containing bisphosphonates. Non-nitrogen-containing bisphosphonates such as clodronate are built into non-hydrolysable analogues of adenosine triphosphate (ATP) that inhibit many different ATP-dependent intracellular enzymes [31]. Therefore, very high concentrations are needed to completely inhibit special pathways. In contrast, the nitrogen-containing bisphosphonates, e.g. pamidronate and zoledronate, inhibit a key enzyme of the mevalonate pathway: the farnesyl pyrophosphate synthase [32]. Among others, this pathway is important for the production of small G-proteins, such as Ras, Rac and Rho proteins, which are important for intracellular structure and mechanisms such as intracellular transport [33, 34] This may contribute to the greater impact of nitrogen-containing bisphosphonates. For pamidronate and zoledronate, a reduction of extracellular matrix protein production such as collagen has been described for fibroblasts and osteoblasts [11]. This might be important for angiogenesis as well.

With the natural limitations of in vitro studies, these data support the theory of the anti-angiogenetic component (angiogenesis and vasculogenesis) in the development and maintenance of BP-ONJ. Therefore, these findings may also explain the higher occurrence of BP-ONJ in patients receiving nitrogen-containing bisphosphonates as compared to patients receiving non-nitrogen-containing bisphosphonates like clodronate. To evaluate these results, in vivo studies should be conducted.

Conflict of interest statement Professor Dr. W. Wagner, PD Dr. B. Al-Nawas and Dr. Ch. Walter gave speeches for Roche. Dr. Ch. Walter received a research fund for another project by Novartis.

Role of the funding source This study was not funded.

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