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Impact of Zoledronic Acid and Denosumab Treatment on Growth Factor Concentration in Platelet Rich Fibrin of Patients With Osteolytic Bone Metastases

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Abstract. *Background/Aim: Side effects of zolendronic acid (ZA) and RANKL inhibitors (RANKL-I) include impaired wound healing and osteonecrosis of the jaw. Platelet rich fibrin (PRF) enhances wound healing and bone remodelling in vivo and in vitro. However, the topical use PRF in the surgical treatment of patients with medicament-related osteonecrosis of the jaw is relatively new and not thoroughly investigated. Furthermore, the potential attenuation of the PRF effect following antiresorptive treatment remains unclear. Therefore, we investigated the concentration of growth factors within the PRF in healthy volunteers and in patients with antiresorptive treatment. Patients and Methods: Blood samples from healthy volunteers and patients were used to produce PRF. The levels of EGF, VEGF, PDGF-BB, TGF-β1, BMP-2, and CD31 in the PRF was investigated by ELISA. Results: ZA treatment induced a significant decrease in EGF and TGF-β1 levels, whereas RANKL-I caused lower TGF-β1 levels. Conclusion: Reduced EGF levels in PRF after ZA treatment may explain the delayed wound healing and question the positive effect of PRF in these patients. PRF use in patients undergoing RANKL-I treatment seems to be more justified.*

Bone metabolism disorders such as osteoporosis as well as bone metastases are usually treated by bisphosphonates or RANKL-inhibitors (1-4). These drugs influence the interaction, differentiation and function of osteoclasts and osteoblasts via different mechanisms (5). A main side effect of these substances is medicament-related osteonecrosis of the jaw (MRONJ) that mostly occurs after dentoalveolar intervention and is associated with prolonged wound healing (6). Several studies have shown that autologous platelet rich fibrin (PRF) supports wound healing due to high concentrations of growth factors such as PDGF-BB, TGF-β1, VEGF, EGF, CD31, and Bmp2 in non-MRONJ patients (7-12). PDGF is a powerful chemotactic stimulus, TGF-β1 is a regulatory protein involved in bone remodelling and fracture healing whereas VEGF supports bone healing by promoting angiogenesis (13-15). EGF also promotes bone formation and shows a positive effect on epithelial wound closure (16-18), whereas CD31 functions as a negative regulator of osteoclastogenesis, and BMP-2 plays a key role in bone remodelling, especially in fracture healing (19-21).

While patients with MRONJ are thought to benefit from local PRF treatment, related studies assessed disease stage-dependent response to this treatment in patients undergoing anti-resorptive therapy (10, 22, 23).

To evaluate the value of PRF treatment in MRONJ patients, it is mandatory to investigate the effect of bisphosphonates and RANKL-Inhibitors on the quality of PRF in these patients in comparison to healthy population. The aim of this study was to investigate growth factor expression as a surrogate parameter for the wound and bone healing potential of PRF under the influence of zoledronic acid (ZA) or Denosumab therapy compared to PRF of healthy volunteers.

Patients and Methods

Patients. Blood samples were obtained from 10 patients treated with Denosumab and from further 10 patients treated with zoledronic acid. Patients with anticoagulative therapy or dialysis were not included due to potential impact on thrombocyte number and/or function. Ten further healthy volunteers, without any medication, provided control blood samples. Detailed patient characteristics are given in Table I.

All procedures were conducted according to the ethical standards of the Declaration of Helsinki and were approved by the institutional ethical committee of the University of Lübeck, Germany.

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Key Words: Platelet rich fibrin, zoledronic acid, denosumab, growth factor, bisphosphonates, denosumab.

Table I. *Characteristics of patients included in the study.*

Group	Underlying pathology	Age years	Leucocytes 10^9/l	Platelets 10^9/l	Treatment period in years	Dose mg	Interval months	Drug application method
Control	Healthy	26	5.54	227	0	0	0	0
Control	Healthy	29	6.91	260	0	0	0	0
Control	Healthy	30	7.75	306	0	0	0	0
Control	Healthy	32	6.91	255	0	0	0	0
Control	Healthy	23	7.49	206	0	0	0	0
Control	Healthy	33	7.10	269	0	0	0	0
Control	Healthy	42	4.14	254	0	0	0	0
Control	Healthy	20	5.90	279	0	0	0	0
Control	Healthy	31	7.50	280	0	0	0	0
Control	Healthy	26	7.40	247	0	0	0	0
ZA	Breast cancer	74	3.53	180	4	4	1	*i.v.*
ZA	Breast cancer	66	6.42	272	6	4	3	*i.v.*
ZA	Breast cancer	81	7.80	264	5	4	3	*i.v.*
ZA	Breast cancer	77	4.34	273	4	4	3	*i.v.*
ZA	Breast cancer	74	4.34	252	2	4	1	*i.v.*
ZA	Prostate cancer	63	13.98	334	8	4	3	*i.v.*
ZA	Prostate cancer	56	7.18	249	5	4	3	*i.v.*
ZA	Prostate cancer	49	6.28	204	4	4	1	*i.v.*
ZA	Prostate cancer	72	7.92	234	3	4	1	*i.v.*
ZA	Prostate cancer	81	5.01	284	4	4	3	*i.v.*
RANKL-I	Breast cancer	53	4.96	345	5	60	6	*s.c.*
RANKL-I	Breast cancer	61	3.25	108	5	60	6	*s.c.*
RANKL-I	Breast cancer	84	6.01	161	7	120	1	*s.c.*
RANKL-I	Breast cancer	74	6.59	225	7	60	6	*s.c.*
RANKL-I	Breast cancer	80	5.35	190	10	60	6	*s.c.*
RANKL-I	Breast cancer	56	7.80	251	4	120	1	*s.c.*
RANKL-I	Prostate cancer	63	6.09	201	5	60	6	*s.c.*
RANKL-I	Prostate cancer	81	3.91	51	3	120	1	*s.c.*
RANKL-I	Prostate cancer	63	10.38	86	5	120	1	*s.c.*
RANKL-I	Prostate cancer	57	5.80	146	5	60	6	*s.c.*

ZA: Zoledronic acid; RANKL-I: RANKL-Inhibitor; *i.v.*: intravenously; *s.c.*: subcutaneous.

Preparation of PRF. A standard vein puncture (median basilica vein, median cubital vein, and median cephalic vein) was performed to prepare PRF according to a standard protocol as previously described (24). Ten ml of blood was drawn into a tube and immediately centrifuged at 145 g for 8 min (DUO Quattro; A-PRF Mectron, Carasco, Italy) (8, 25, 26). After centrifugation, the PRF clot was removed and transferred into a new tube and frozen at −80˚C until further investigations were performed.

Growth factor quantification by ELISA test. Before evaluation of the different growth factors (GF) of PRF, samples were thawed and centrifugated at 408 g for 20 min at 18˚C as previously described (24). The levels of the growth factors TGF-β1, PDGF-BB, CD31/PECAM-1, VEGF, EGF, and BMP-2 were determined in the supernatant. The expression of growth factors was assessed using enzyme-linked immunosorbent assay tests (ELISA; R&D Systems, Minneapolis, MN, USA) and conducted according to the manufacturers' manual. Measurements were performed with benchmark plus microplate spectrophotometer set to 540 nm (Clariostar, BMG-Labtech, Ortenberg, Germany). Each ELISA sample was run in duplicate.

Statistical evaluation. Statistical analysis was performed using the statistical package IBM SPSS Statistics version 26 (IBM, Armonk, NY, USA). Values were expressed as mean±standard deviation. Differences were assessed using a *t*-test for unpaired samples as well as the Pearson correlation test. Results were considered significant when $p \leq 0.05$.

Results

Generally, the patients of the Denosumab group showed a significant lower number of platelets compared to the control (platelets $_{control}$: 258×10^9/l; platelets $_{denosumab}$ 176×10^9/l; $p=0.02$) but no difference in the number of leucocytes (leucocytes $_{control}$: 6.7×10^9/l; leucocytes $_{denosumab}$ 6.1×10^9/l) was observed. On the contrary, the mean values of platelets and leucocytes of patients treated with zoledronic acid showed no significant differences among the groups (platelets $_{control}$: 258×10^9/l; platelets $_{zoledronic\ acid}$ 254×10^9/l; leucocytes 6.7×10^9/l in both).

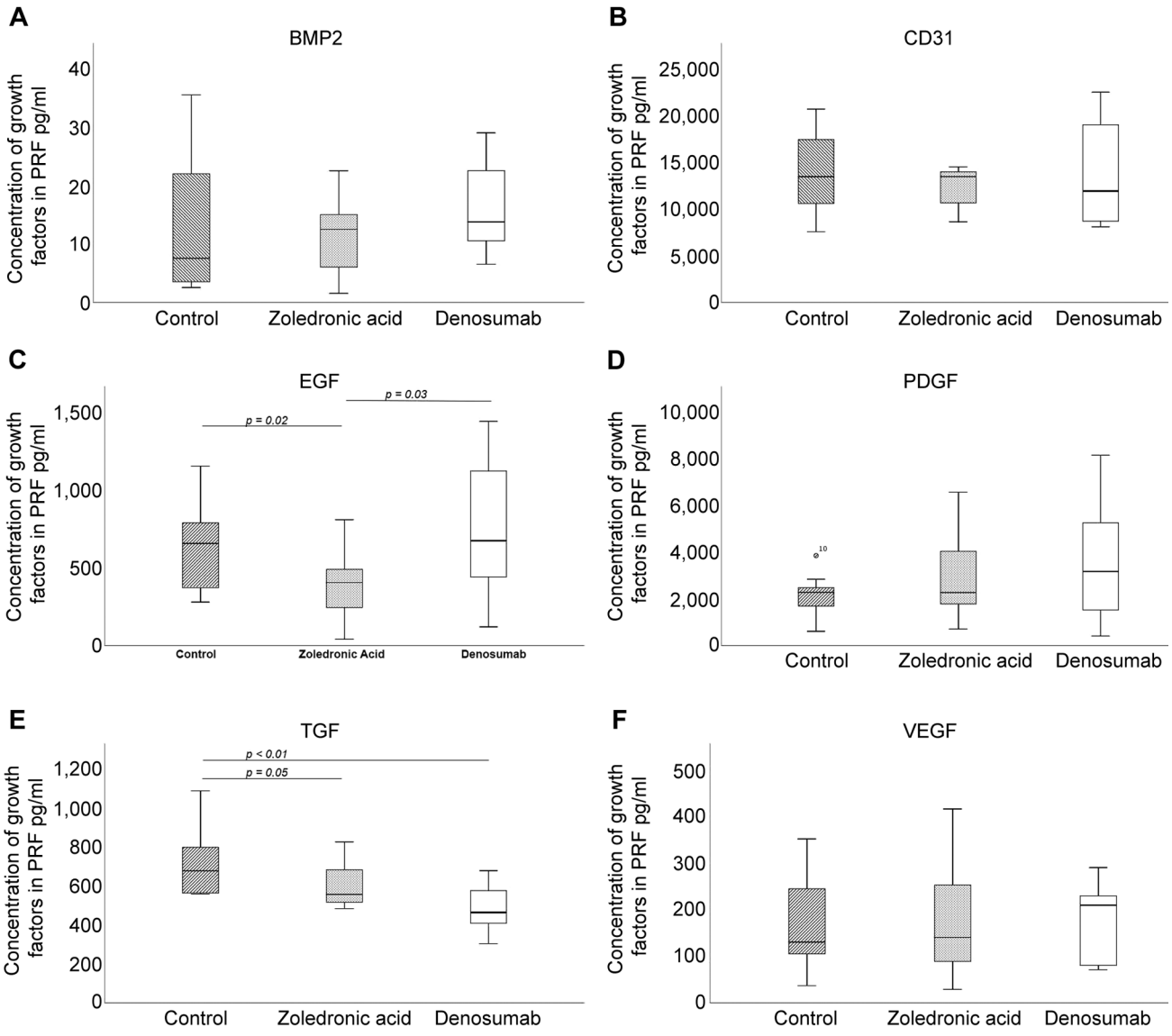

Figure 1. *Results of the ELISA assays showing the concentration of BMP2 (A), CD31 (B), EGF (C), PDGF (D), TGF (E), and VEGF (F) in platelet rich fibrin (PRF) of healthy volunteers in comparison to patients treated with zoledronic acid and denosumab.*

Impact of platelet, leucocyte number, and Denosumab and ZA treatment period on growth factor expression in PRF. Zoledronic acid treatment resulted in a significant decrease of EGF concentration in PRF in comparison to control and denosumab treatment [ZA: 391±236 pg/ml control: 658±301 pg/ml (p=0.02); denosumab: 729±427 pg/ml (p=0.03)] (Figure 1C), while TGF-beta1 concentration was significantly lower in both zoledronic acid and denosumab groups compared to the control [control: 715±168 pg/ml ZA: 608±117 pg/ml (p=0.05); denosumab: 490±119 pg/ml (p<0.01)] (Figure 1E). All other growth factor concentrations remained unchanged within the different groups (Figure 1A-F).

Interestingly, we could not observe any correlation between the number of platelets or leucocytes with the concentration of growth factors in PRF in the different treatment groups and the control (Figure 2A-F). Similarly, the treatment time had no significant impact on growth factor concentration in PRF in both treatment groups (Figure 2H-I).

Discussion

Drugs affecting bone metabolism such as zoledronic acid and RANKL-Inhibitors can lead to osteonecrosis of the jaw since they influence bone formation and turn-over, resulting in

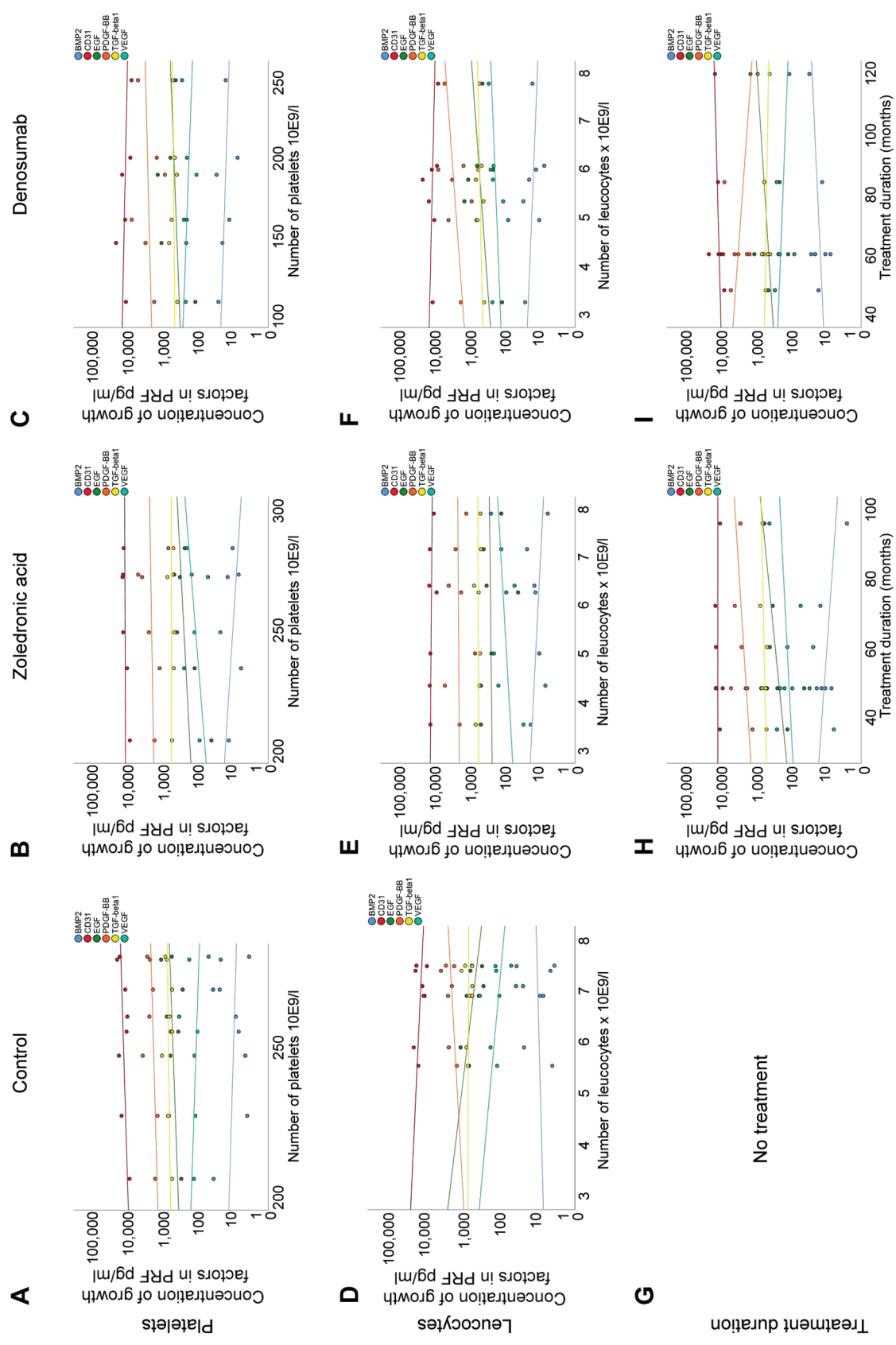

Figure 2. *Correlation of the number of platelets, leucocytes as well as of treatment duration of the different groups with the concentration of BMP2, CD31, EGF, PDGF, TGF, and VEGF in their platelet rich fibrin (PRF). No significant correlation was found.*

bone exposure with wound dehiscence, fistulas and chronic inflammation (27). Due to its high concentration of growth factors, PRF supports wound and bone healing (7-12). The use of PRF is conceivable for the treatment of MRONJ, but its effectiveness as local therapy in these patients cannot be definitively proven unless the crucial levels of growth factors in PRF under antiresorptive treatment have been assessed (28-30). Therefore, the aim of this study was to investigate PRF quality in patients undergoing ZA and RANKL-I treatment compared to control population.

Patients of the ZA group had a comparable platelet and leucocyte number to those in the control group, a finding that justifies the comparison of growth factor concentration in the PRF of both groups. On the contrast, patients in the RANKL-I group displayed significantly lower number of platelets whereas the number of leucocytes was not affected This result is in accordance with Weibrich *et al*. (31) who reported a low correlation between the baseline platelet number from whole blood and related growth factor levels. However, it is known that reduction of the centrifugal force during preparation of PRF results in higher cell number and increased growth factor release (32).

When the concentration of individual growth factors was assessed, a significant decrease of EGF in the ZA group compared to the RANKL-I and control group was found. Given the known impact of EGF on epithelial migration and thus on wound healing, the toxic effect of ZA on the oral mucosa and the disturbance in the subsequent wound healing can be understood. This effect has been also shown in vitro using human oral keratinocytes; ZA treatment resulted in reduced viability, impaired migration ability, and increased apoptosis rate (33-35). Reversible effects were also demonstrated by EGF stimulation (35, 36).

Hypothetically, the observed reduction of EGF in ZA patients may reflect the known EGFR-based anti-neoplastic effect of ZA, which inhibits cancer cell proliferation directly and modulates stroma cells in the tumor microenvironment (37, 38). In other words, the decreased concentration of EGF in PRF during ZA treatment is a sign of its anti-neoplastic effect.

TGFβ-1 concentration was reduced in both ZA and RANKL-I groups compared to the control. Recent studies demonstrated that ZA intervenes with the TGFβ-signalling pathway and - especially in low drug concentration - inhibits TGF expression and related wound closure, possibly through suppression of Smad2/3 signalling (39).

Similar to EGF, the pro-migratory transforming growth factor TGF-β1 is considered a key factor contributing to tumor progression (40) since the blockade of TGF-β signalling has been shown to effectively prevent osteolytic bone metastasis (41). This emphasizes the antineoplastic effect of ZA and indicates that patients undergoing this therapy may carry the humoral risk of disturbed wound healing (42-44).

There is a crucial lack of information on the impact of ZA and RANKL-I therapy on wound healing in MRONJ patients (45). However, along with the results presented in the current study, a reasonable difference in the composition and certainly in the potential regenerative effect of PRF from ZA and RANKL-I patients should be postulated.

Future studies should investigate this feature in a therapeutic context and should consider application of immunologically neutral allogeneic PRF in the surgical treatment of MRONJ patients.

Conclusion

While the concentration of PDGF, CD31, VEGF, and BMP-2 showed no significant differences in PRF from ZA and RANKL-I patients, EGF and TGF in PRF from ZA-treated patients as well as TGF from RANKL-I-treated patients were significantly reduced, indicating possible negative effects on the regenerative quality of their PRF.

Conflicts of Interest

The Authors declare that they have no competing interests in relation to this study.

Authors' Contributions

D. Steller and S.G. Hakim designed the study and wrote the main manuscript. R. Simon performed the measurements and described the results. R. von Bialy designed the Figures. R. Simon and D. Steller equally contributed to the study.

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