**BJR**

■ **BONE FRACTURE**

# The impact of nonosteogenic factors on the expression of osteoprotegerin and RANKL during human fracture healing

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### Objectives

The osteoprotegerin (OPG) and receptor activator of nuclear factor kappa-B ligand (RANKL) balance is of the utmost importance in fracture healing. The aim of this study was therefore to investigate the impact of nonosteogenic factors on OPG and RANKL levels.

### Methods

Serum obtained from 51 patients with long bone fractures was collected over 48 weeks. The OPG and serum sRANKL (soluble RANKL) concentrations were measured using enzyme-linked immunosorbent assay (ELISA). Smoking habit, diabetes, and alcohol consumption were recorded.

### Results

Age and sex greatly influenced preoperative serum levels of OPG and sRANKL but differences were even more pronounced during fracture healing. Statistical significance was observed for overall serum levels of OPG ($p = 0.001$) and sRANKL ($p < 0.001$) in older men and women (age greater than 50 years). Interestingly, OPG levels increased over time in older women but decreased over time in older men.

### Conclusion

These data suggest that nonosteogenic factors, most significantly age and sex, have a major impact on sRANKL and OPG levels. Given the established association of OPG and sRANKL levels and nonunion, these findings seem to be of clinical relevance.

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### Article focus

■ Serum levels of osteoprotegerin (OPG) and soluble receptor activator of nuclear factor kappa-B (NF-κB) ligand (sRANKL) were monitored during regular fracture healing in humans over a period of 48 weeks.

■ The aim of this study was to evaluate the impact of nonosteogenic factors (i.e. age, sex, diabetes, cigarette smoking, and frequent alcohol consumption) on the levels of OPG and sRANKL observed during fracture healing in humans.

### Key messages

■ Age and sex greatly influenced preoperative levels of OPG and sRANKL, but differences were even more pronounced during fracture healing.

■ Age and sex had a greater impact on OPG and sRANKL levels than smoking, diabetes, and frequent alcohol consumption.

■ However, given the modifiable nature of smoking and alcohol consumption, the effect of smoking and/or alcohol cessation may be beneficial for OPG and RANKL homeostasis.

### Strengths and limitations

■ This is, to the authors' knowledge, the first time that serum levels of OPG and RANKL have been monitored in humans during fracture healing over a period of 48 weeks.

■ The impact of nonosteogenic factors, especially age and sex, on the levels of OPG and sRANKL is striking. Nevertheless, larger numbers of patients are needed to

elucidate the impact of smoking, diabetes, and frequent alcohol consumption.

## Introduction

Bone homeostasis relies on a complex interaction of various growth factors. Particularly during fracture healing, the timely incitation of regenerative processes ensures the reunion of fractured bones.[1,2] Nonosteogenic factors such as age, sex, diabetes, frequent alcohol intake, and nicotine consumption seem to impact this complex system. In this context, osteoprotegerin (OPG) and receptor activator of nuclear factor kappa-B (NF-κB) ligand (RANKL) have been shown to be critically involved in bone healing. In particular, the osteoclast-activating effects of RANKL are counteracted by OPG, which is a decoy receptor for RANKL, allowing for intramembranous bone formation and endochondral ossification during fracture repair.[3,4] While early osteoclast activity may be disadvantageous during the phase of soft callus formation, osteoclasts ultimately orchestrate hard callus remodelling.[5,6]

The soluble form of RANKL (sRANKL) is cleaved from osteoblasts and interacts directly with its receptor on the preosteoclast surface. Osteoblasts coordinate the deposition of lamellar bone and are responsible for secretory activity (i.e. OPG and RANKL) that depends upon various factors such as micromotion. The significance of micromotion at the bone-implant interface, as well as in between fracture fragments, is largely unclear. However, Ziebart et al[7] demonstrated that enhanced micromotions at the fracture site have a major impact on human osteoclast activation and osteoclast differentiation. Micromotions, as well as static loading, were shown to decrease the OPG/RANKL ratio in human osteoblasts.[7]

Previous reports have demonstrated that age also impacts fracture healing on a cellular level. Circulating OPG levels were found to be associated with markers of bone turnover in postmenopausal women.[8] Similarly, bone mineral density (BMD), as a surrogate for bone turnover, was found to be altered with type 2 diabetes mellitus (T2DM), alcohol abuse, and smoking.[9] Nicotine is suspected of not only impairing fracture healing, but also exerting inhibitory effects on BMD, and therefore increases the risk of osteoporotic fractures.[10] Moreover, direct and indirect effects of nicotine have been reported to influence the OPG and RANKL homeostasis, as low OPG levels were documented in smokers.[11,12] Furthermore, in an age- and sex-matched population of patients with periodontal disease, smoking had a significant impact on serum OPG concentrations. Smokers presented a significantly higher RANKL/OPG ratio than nonsmokers.[12] All of this strongly points to a negative effect of smoking on the OPG and RANKL homeostasis.

Previously, we found that OPG and sRANKL levels were profoundly affected after long bone fracture as compared with healthy controls.[13] Furthermore, we observed a disrupted time course of OPG and sRANKL levels in patients with nonunion after long bone fractures, suggesting their involvement in human fracture healing.[13] Within this study, we set out to evaluate the impact of nonosteogenic factors, such as age, sex, T2DM, frequent alcohol intake, and nicotine, on circulating levels of OPG and sRANKL during human fracture healing.

## Patients and Methods

**Patient cohort, follow-up, and stratification.** The recruitment parameters, sample collection schedule, matching process, patient demographics, and exclusion criteria of this study have previously been published in detail.[14] In brief, a consecutive series of 51 patients (22 male, 29 female) with closed metaphyseal/diaphyseal fractures of long bone (humerus, femur, lower leg, forearm) who underwent surgery were included (Table I). All fractures were treated according to AO principles of fracture management by intramedullary nailing, plating, or external fixation. The mean patient age was 59.3 years (18 to 90). Patients receiving antiresorptive drugs were excluded from the analysis.

Patients were stratified according to comorbidities such as T2DM, frequent alcohol intake, and smoking. Smoking status was defined based on self-reported cigarette consumption (more than five per day). Smokers who used fewer than five cigarettes per day were not included in the analysis, and 50 years of age served as the cut-off to differentiate between 'young' and 'older' patients. Frequent alcohol intake was defined based on the self-reported daily alcohol intake, together with pathological liver parameters forming the frequent alcohol intake group. We defined this group as patients who drank alcohol on a daily basis (assessed during admission of the patient) and at least one of the following parameters exceeding the upper limit of the laboratory reference: aspartate aminotransferase (AST), alanine aminotransferase 1, gamma-glutamyl transpeptidase (GGT), or bilirubin. 'Diabetic' defined those patients under oral antidiabetic or insulin therapy.

**Blood collection and processing.** Patients' serum collection followed a standardized time schedule (surgery, postoperative weeks 1, 2, 4, 6, 8, 12, 24, and 48). Collections were performed seven and 14 days after surgery for the two initial timepoints, with a maximum variation of ± one day. For later timepoints, we allowed a less stringent window of ± three days. The specimens were centrifuged immediately and the resulting supernatant was stored at -80°C until assayed.

**Measurement of OPG and sRANKL.** Serum OPG levels were measured using a commercial enzyme-linked immunosorbent assay (ELISA) kit (Biomedica Gruppe, Vienna, Austria) that detects monomeric, dimeric, and ligand-bound OPG. The published sensitivity of the

**Table I.** Patient demographics (n = 51)

| Parameter | Age group | | | Sex | | | Smoking | | | Frequent alcohol intake | | | Diabetes | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | < 50 yrs | > 50 yrs | p-value* | Male | Female | p-value* | Yes | No | p-value* | ≥ daily | < daily | p-value* | Yes | No | p-value* |
| Mean age, yrs (SD) | 37 (10.2) | 73 (10.5) | < 0.011† | 44 (16.7) | 71 (18.4) | < 0.011† | 43 (11.6) | 66 (18.0) | 0.359 | 45 (2.8) | 63 (18.2) | 0.324 | 67 (16.2) | 58 (18.1) | 0.228 |
| **Fracture site, n** | | | | | | | | | | | | | | | |
| Forearm | 3 | 3 | 0.565 | 2 | 4 | 0.606 | 3 | 3 | 0.239 | 1 | 5 | 0.947 | 1 | 5 | 0.824 |
| Diaphyseal | 2 | 1 | | 1 | 2 | | 2 | 1 | | 1 | 2 | | 0 | 2 | |
| Metaphyseal | 1 | 2 | | 1 | 2 | | 1 | 2 | | 0 | 3 | | 1 | 3 | |
| Humerus | 5 | 10 | 0.579 | 5 | 10 | 0.361 | 4 | 11 | 0.781 | 1 | 14 | 0.184 | 3 | 12 | 0.401 |
| Diaphyseal | 3 | 4 | | 3 | 4 | | 3 | 5 | | 1 | 6 | | 1 | 6 | |
| Metaphyseal | 2 | 6 | | 2 | 6 | | 1 | 6 | | 0 | 8 | | 2 | 6 | |
| Lower leg | 11 | 9 | 0.064 | 13 | 7 | 0.011† | 6 | 14 | 0.941 | 5 | 15 | 0.269 | 1 | 19 | 0.146 |
| Diaphyseal | 6 | 4 | | 7 | 3 | | 3 | 6 | | 2 | 9 | | 0 | 12 | |
| Metaphyseal | 5 | 5 | | 6 | 4 | | 3 | 8 | | 3 | 6 | | 1 | 7 | |
| Femur | 1 | 9 | 0.035† | 2 | 8 | 0.099 | 2 | 8 | 0.466 | 2 | 8 | 0.828 | 2 | 8 | 0.520 |
| Diaphyseal | 1 | 4 | | 1 | 2 | | 1 | 3 | | 2 | 3 | | 1 | 3 | |
| Metaphyseal | 0 | 5 | | 1 | 6 | | 1 | 5 | | 0 | 5 | | 1 | 5 | |
| **Treatment, n** | | | | | | | | | | | | | | | |
| Plate | 5 | 9 | 0.753 | 5 | 9 | 0.510 | 3 | 11 | 0.442 | 0 | 14 | 0.042† | 3 | 11 | 0.325 |
| Nail | 12 | 22 | 0.417 | 14 | 20 | 0.503 | 10 | 24 | 1.000 | 8 | 26 | 0.119 | 4 | 30 | 0.565 |
| External fixation | 1 | 2 | 0.830 | 2 | 1 | 0.396 | 2 | 1 | 0.144 | 2 | 1 | 0.022† | 1 | 2 | 0.309 |
| Patients, n | 20 | 31 | N/A | 22 | 29 | N/A | 15 | 36 | N/A | 9 | 42 | N/A | 7 | 44 | N/A |

*Chi-squared test
†Statistically significant
N/A, not applicable

assay was 2.8 pg/ml; the intra- and interassay coefficients of variation were 4% to 10% and 7% to 8%, respectively. The RANKL levels were assayed using an ELISA kit (Biomedica) that detects soluble, uncomplexed human RANKL in serum. The manufacturer's insert indicates that the ELISA can reliably detect values below 1.6 pg/ml using extrapolation. Further details on the design and performance of the assay have been published previously.[15,16]

**Statistical analysis.** Comparisons between independent groups of continuous variables were performed by non-parametric Mann–Whitney U test. For comparison of OPG and sRANKL concentrations between different time-points, nonparametric Wilcoxon's signed-rank test for paired samples was used. Correlation between OPG and sRANKL was analyzed by Spearman's correlation coefficient. The sRANKL/OPG ratio was calculated by dividing sRANKL levels by OPG levels. Statistical analyses were performed using SPSS for Windows (version 19; IBM Corp., Armonk, New York). The statistical significance level was set to $p \leq 0.05$.

## Results

We observed that sRANKL continuously increased during fracture repair until postoperative week 12, and then started to decline slowly until postoperative week 48 (Fig. 1a). In contrast, OPG levels were found to differ substantially in postoperative dynamics; after an initial increase at week 1, OPG declined continuously until postoperative week 48, returning to preoperative levels at week 12 (Fig. 1b).

As we had observed a very distinct time course of OPG and sRANKL during fracture healing, we further analyzed the impact of sex or age. We detected distinct differences in absolute values as well in the time course of OPG and sRANKL (Supplementary Figs aa to ad, ba, and bb). Mean overall OPG levels were significantly higher in older and female patients (age: $p < 0.001$; sex: $p < 0.001$), while mean overall sRANKL levels were significantly lower in older patients and tended to be lower in female patients (age: $p = 0.016$; sex: $p = 0.061$).

Given the significant impact of age and sex on OPG and sRANKL levels, we sought to subclassify patients further with respect to these characteristics (Fig. 2). Interestingly, younger women had significantly lower overall OPG levels than older women ($p < 0.001$) and older men ($p < 0.001$). However, the most striking difference was detected between older women and older men ($p = 0.001$). Similarly, also for sRANKL, younger women were found to have significantly lower overall levels than older women ($p = 0.002$). Again, the same results were observed for men ($p < 0.001$). The most notable difference, however, was observed between older men and older women ($p < 0.001$).

We analyzed these subgroups further, focusing on the OPG and sRANKL time course (Fig. 2). Of note, while young men and women did not differ substantially in terms of their OPG and sRANKL levels during fracture healing, sex had a major effect on circulating factors in older patients (Figs 2a and 2b). Specifically, OPG was significantly decreased in older men, while initial levels did not differ in these subgroups (Fig. 2b). In clear contrast,

Fig. 1a

Fig. 1b
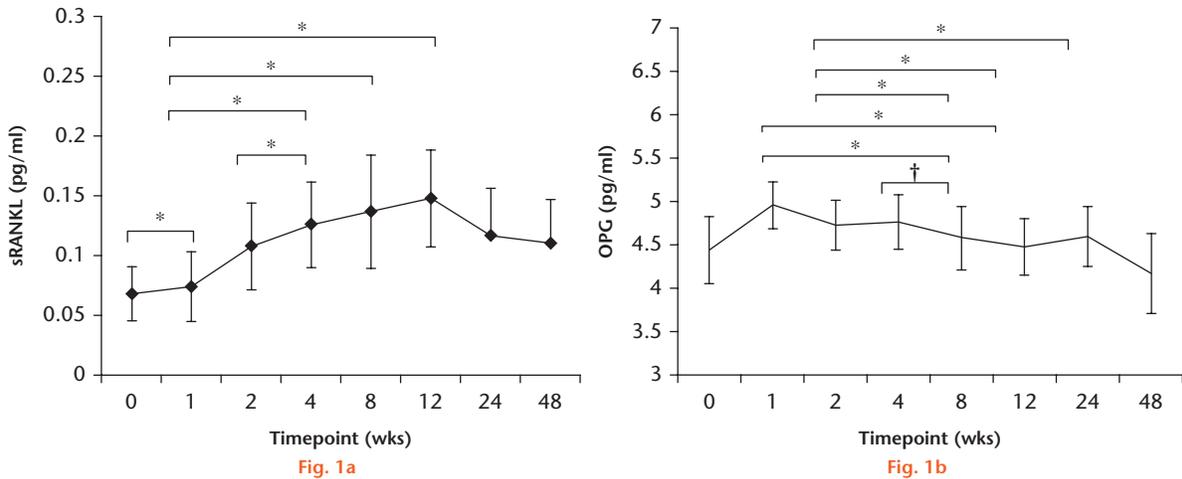
Time course of osteoprotegerin (OPG) and soluble receptor activator of nuclear factor kappa-B (NF-κB) ligand (sRANKL) during fracture healing. Time course of a) sRANKL and b) OPG is illustrated as mean and standard error of the mean (SEM). Significant changes are illustrated according to paired analyses. *$p < 0.05$; †$p < 0.005$.



Fig. 2a

Fig. 2b



Fig. 2c
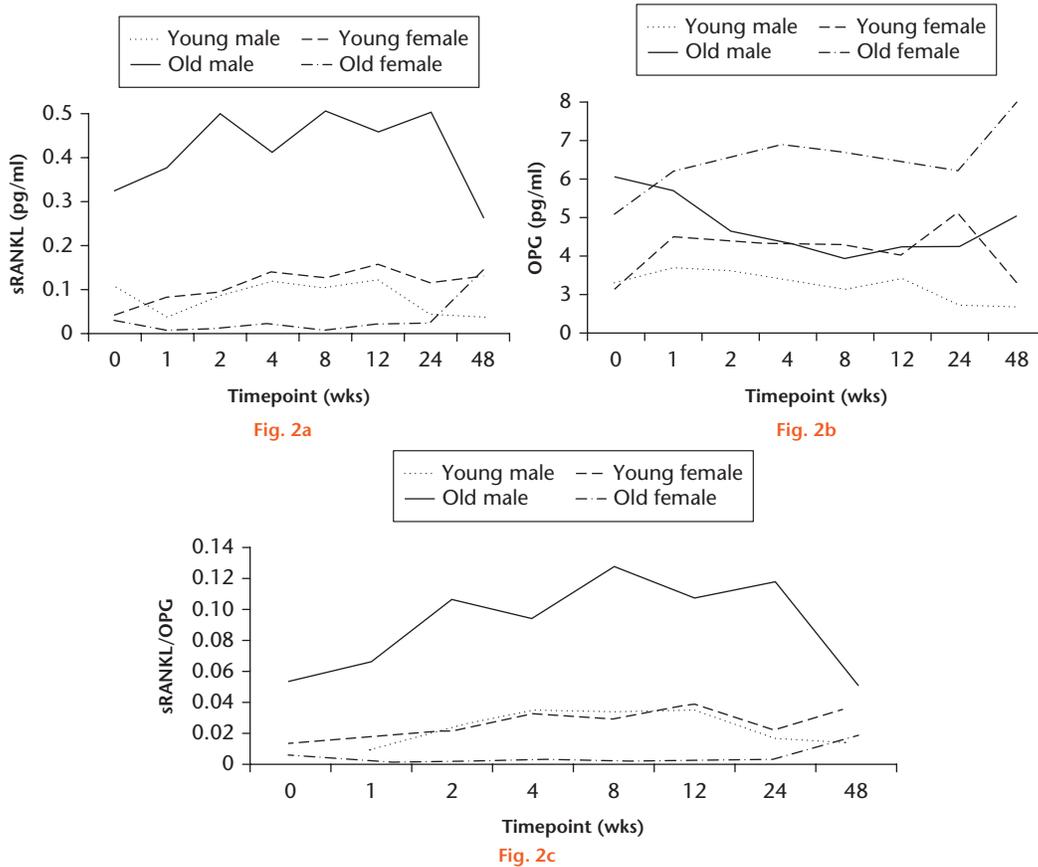
Time course of circulating osteoprotegerin (OPG) and soluble receptor activator of nuclear factor kappa-B (NF-κB ligand) (sRANKL) during human fracture healing stratified according to age and sex. Time course of a) sRANKL and b) OPG are illustrated as means according to four groups: young women, young men, older women, and older men. The age cut-off to differentiate between 'young' and 'older' patients was 50 years. c) In particular, the differences between the older men and older women are noteworthy. The ratio of sRANKL and OPG was calculated to reflect their balance.

sRANKL levels were found to be significantly elevated in older men compared with older women (Fig. 2a).

When we compared the ratio of OPG and sRANKL, we observed that the sex- and age-specific differences were even more pronounced (Fig. 2c). In particular, it seemed

that reduced OPG levels were always accompanied by excessive sRANKL levels and vice versa.

Additionally, we aimed to evaluate the relevance of diabetes, smoking, and frequent alcohol intake on OPG and sRANKL levels. Accordingly, we found that overall

OPG levels were decreased by smoking (p < 0.001; Supplementary Fig. ca), as well as by frequent alcohol intake (p = 0.004; Supplementary Fig. cc). However, we did not observe substantial differences in its postoperative time course with regard to these modifiable risk factors (Supplementary Figs cb and cd). For T2DM, we did not observe any significant differences in overall levels (Supplementary Fig. ce). Also, the time course itself did not differ substantially (Supplementary Fig. cf).

We further analyzed sRANKL levels with respect to smoking, frequent alcohol intake, and T2DM. Interestingly, as for OPG, smokers were also found to have reduced overall sRANKL levels (p < 0.001; Supplementary Fig. da). Focusing on the time course, we found that only non-smokers significantly increased their respective sRANKL levels during fracture healing (p = 0.033; Supplementary Fig. db). Similarly, frequent alcohol intake was also associated with decreased overall sRANKL levels (p < 0.001). However, as for smoking, at individual timepoints, no significant differences were observed (Supplementary Fig. dc). Evaluating the time course of sRANKL, we found that only patients without frequent alcohol intake were able to increase sRANKL levels during fracture healing (p = 0.020; Supplementary Fig. dd). For T2DM, we did not observe any significant differences in overall levels (Supplementary Fig. de). Also, the time course itself did not differ substantially (Supplementary Fig. df).

## Discussion

Factors such as age, sex, cigarette smoking, diabetes, and frequent alcohol intake may impact human fracture healing.[2] To the authors' knowledge, this is the first study that reports the significant impact of nonosteogenic factors, such as age and sex, on circulating OPG and sRANKL levels in patients who underwent surgical repair for long bone fracture. We also found that smoking and frequent alcohol intake affect these growth factor levels, albeit to a lesser extent. In particular, substantial preoperative differences in OPG and sRANKL levels were observed. Furthermore, the postoperative time course during the first year after fracture repair was significantly affected by the aforementioned factors. As OPG and sRANKL have been suggested to be potentially associated with fracture healing as well as the development of nonunions,[13] these findings are of major clinical relevance, especially as therapeutic interventions may be effective only in subgroups of patients.

Regarding OPG levels in older patients, previous studies reported a positive correlation of OPG with age.[17-19] Similarly, we found significantly higher OPG levels over the entire observation period in patients aged greater than 50 years. With respect to the key feature of OPG (i.e. avoiding early soft callus resorption), elevated OPG levels in older patients may reflect an effort to enhance fracture healing in the context of BMD loss. Wagner and Fahrleitner-Pammer[20] have described this observation as a "compensatory response" that aims to counteract the bone loss associated with increased age. Given the central involvement of OPG in osteoclast maturation, it is important to emphasize its role during fracture healing. Its function comprises the pure resorption of mineralized callus and bone remodelling. Importantly, its involvement has also been documented in the very early phase of healing,[6] where we observed major differences in OPG levels in regard to age, underlining the relevance of the observed differences in OPG levels.

As for the impact of age on sRANKL levels, contrasting results are discussed in the current literature.[21-23] Pulsatelli et al[23] analyzed sRANKL levels in a healthy population aged 70 to 100 years, comparing them with 14 subjects with a mean age of 28 years. The authors found no correlation, except for individuals between 80 and 90 years of age, who presented a significant decline in sRANKL levels compared with controls. In contrast, we observed no significant difference in overall sRANKL levels in our patients with respect to age. However, the time course of sRANKL during fracture healing revealed that older patients had a distinct induction of sRANKL immediately after, as well as during, the course of fracture healing, while sRANKL levels of younger patients remained broadly constant. Accordingly, in terms of age, there seemed to be a disrupted response to fracture healing in older patients, who may require a greater "compensatory response" to allow for fracture repair.

Regarding the impact of sex on OPG, preoperative levels were comparable in both groups. This is in line with Trofimov et al,[24] who detected no significant sex-related differences in OPG levels in a healthy population. However, monitoring the time course revealed noticeable trends. In particular, we found that the development of OPG values over the entire observational period was almost oppositional when comparing men and women (Fig. 2b). This observation might be explained by in vitro findings, providing evidence that oestrogen boosts OPG production, inhibits RANKL expression, and modulates the responsiveness of osteoclast precursors. In contrast, testosterone appears to have the opposite effect and decreases OPG production.[25-27] Our data are in line with these observations, and the impact of sex hormones may be even more pronounced during human fracture healing. In this context, the subgroup analysis of older women and men compared with younger women and men revealed striking differences in overall OPG levels (Fig. 2). Our findings are therefore partly in line with Khosla et al,[21] who found serum OPG levels to increase with age in women. However, Khosla et al[21] also found this association in men and consequently did not observe any significant difference regarding OPG level between postmenopausal women and men aged over 50 years,

which is in contrast to our findings. These latent differences regarding OPG levels become apparent during fracture repair, suggesting very distinct mechanisms of fracture healing in these subgroups. Nevertheless, the reasons for these age- and sex-related differences in these subgroups are probably various. For example, they may be caused by a subgroup-specific increase in OPG production, increased release from the skeleton, or decreased clearance with age, which future research should seek to elucidate. Ultimately, the more fragile bone of older women may need the significant OPG increase to allow for fracture healing and remodelling during the entire first year, while this process of bone remodelling seems to be active for only two to four weeks in older men. This theory is also supported by the time course of the osteoclast activator sRANKL. While Trofimov et al[24] observed significantly higher sRANKL values in women, our study revealed no sex-related differences at study entry. However, regarding time course, we observed a more striking sRANKL induction in men, again suggesting that bone resorption after fracture is more distinct in these patients. This was substantiated further in the subgroup analysis of young patients and older patients stratified by sex. In this context, it should be noted that administration of testosterone significantly decreases sRANKL levels.[28] This would suggest that older men may have significantly increased sRANKL levels, as they have lower testosterone levels than young men.

When looking at the sRANKL/OPG ratio, these differences were even more pronounced. In particular, it seemed that reduced OPG levels were always accompanied by significantly elevated sRANKL levels and vice versa. Reduced OPG levels, combined with the significantly elevated sRANKL levels, left older men in a highly osteoclast-activating state, while older women seemed to have a comparably limited activation. This is of specific relevance given the central involvement of osteoclasts in fracture healing, as mentioned above. Accordingly, our findings not only outline the potential impact of sex hormones and age on preoperative OPG and sRANKL levels in regular bone homeostasis, but also suggest significant pathophysiological differences in the context of human fracture healing. These results seem to be of special interest in terms of medications that interact with bone resorption, such as alendronate or denosumab. Bisphosphonates, in particular, are believed to affect fracture healing negatively as they inhibit bone resorption, which is a crucial phase during fracture healing.[29] Correspondingly, Gerstenfeld et al[30] observed a delay in healing in mice treated with medications that interfere with bone resorption, but the strength at the fracture site was still improved compared with controls. However, our results suggest that potential side effects may be very different in specific patient groups. The effects on fracture healing in older men (very high sRANKL levels during fracture healing) may be far more striking than in older women (very low sRANKL levels). In fact, some patients may even benefit from these therapies during the anabolic window,[31] as their sRANKL levels are very low, and this could explain why denosumab has no negative effect on fracture healing in postmenopausal women.[32]

Regarding other nonosteogenic factors, we observed less pronounced but seemingly relevant effects on OPG and sRANKL. Direct and indirect effects of nicotine were suggested to influence the OPG and RANKL homeostasis, and low OPG levels are documented in smokers.[11,12] This accords with our results, measuring decreased OPG levels in smokers at weeks 4 and 48. Similar observations were made by Lappin et al,[12] who studied RANKL and OPG concentrations in serum of an age- and sex-matched population of patients with periodontal disease. When comparing smokers and nonsmokers, the authors detected a significant difference in OPG concentrations. Moreover, smokers presented a significantly higher ratio of RANKL to OPG than nonsmokers.[12] Consistent findings have been reported by Tang et al,[11] who studied the impact of smoking on the concentration of RANKL and OPG in gingival crevicular fluid of periodontal disease. Tang et al[11] identified an exposure-dependent reduction in OPG concentrations as well as an increase in sRANKL/OPG ratio in periodontitis patients. All of this suggests that smoking has a negative effect on OPG and RANKL homeostasis, which is also documented in our study. As smoking is well known to be associated with a higher nonunion rate, its impact on the RANKL and OPG system may contribute to this clinical effect. This highlights the relevance of smoking cessation in order to avoid systemic disruption of pathophysiological processes during fracture healing.

The long-term effects of diabetes include microvascular complications, such as BMD loss and risk of nonunion.[33] Diabetic animals have been reported to form significantly less callus that contains decreased numbers of RANK-, RANKL-, and OPG-positive cells.[34] In our cohort, diabetes was not observed to have a significant effect on OPG and sRANKL levels.

Osteoprotegerin has previously been shown to be increased in alcohol-dependent individuals, which is the opposite of what was observed in our patients.[35] Levels were higher in patients who did not frequently consume alcohol at almost all observation points, as well as the overall OPG level ($p < 0.001$). Interestingly, those patients with self-reported daily alcohol consumption presented an increased sRANKL/OPG ratio, which was previously described by Santori et al.[35] It is tempting to speculate that our findings of an elevated sRANKL/OPG ratio in patients with frequent alcohol intake could reflect the organism's effort to prevent bone loss in this subgroup of patients during the course of fracture healing.

In this study, we aimed to gather a homogeneous cohort of patients with long bone fractures. However, we must point out that, despite strict inclusion and exclusion

criteria, a given heterogeneity within the patient group represents a potential bias. In particular, not only do fracture morphology and location vary to some extent between patients, but the corresponding type of fracture healing may also differ with respect to the operative procedure, such as nailing or plating (Table I). This could affect OPG and sRANKL levels, and should be identified as a potential weakness of our analysis. Nevertheless, one should note that, by and large, fracture location, as well as subsequent treatment, was distributed equally within our groups. Therefore, despite the fact that we have some heterogeneity within our groups, it is worth noting that we still observed significant differences in regard to nonosteogenic factors, suggesting that these effects seem to overcome some heterogeneity within the cohort. Since this is the main focus of the analysis, this gives us confidence that our conclusions are still meaningful despite these potential differences. However, larger analyses will have to determine if the observed differences within our groups do impact OPG and sRANKL levels significantly.

In conclusion, we observed substantial differences in preoperative levels and subsequent time course of OPG and sRANKL with respect to nonosteogenic factors. In particular, we documented for the first time in the human setting that age, sex, and other nonosteogenic factors affect circulating OPG and sRANKL levels tremendously during the course of fracture healing, suggesting substantial pathophysiological differences within patient subgroups.

## Supplementary Material

ë Figures showing the effect of sex, age, diabetes, smoking, and alcohol intake on the time courses of osteoprotegerin (OPG) and soluble receptor activator of nuclear factor kappa-B (NF-Kb) ligand (sRANKL) during human fracture healing.

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**Author contributions**
■ J. Starlinger: Conceptualized the study, Wrote the manuscript.
■ G. Kaiser: Collected the data, Revised the manuscript.
■ A. Thomas: Performed the statistical analysis.
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**Ethical review statement**
■ This study was approved by the Ethics Committee of the Medical University of Vienna (#466/2005).