Anti-VEGFs hinder bone healing and implant osseointegration in rat tibiae


Abstract
Aim: To assess the effect of anti-vascular endothelial growth factors (VEGF) on bone healing (defect volume) and implant osseointegration (bone-implant contact per cent) in rat tibia.

Materials and Methods: In Sprague–Dawley rats (n = 36), a unicortical defect was created in the right tibia and a titanium implant was placed in the left tibia of each rat. Rats were assigned into three groups and received either anti-vascular endothelial growth factor neutralizing antibody, Ranibizumab or saline (control). Two weeks following surgery, rats were euthanized and bone samples were retrieved. Bone healing and osseointegration were assessed using micro-CT and histomorphometry. One-way ANOVA followed by the Tukey’s test was used for data analyses.

Results: The volume of the bone defects in the anti-VEGF group (2.48 ± 0.33 mm³) was larger (p = 0.026) than in the controls (2.11 ± 0.36 mm³) as measured by μ-CT. Bone-implant contact percent in the anti-VEGF (19.9 ± 9.4%) and Ranibizumab (21.7 ± 9.2%) groups were lower (p < 0.00) than in the control group (41.8 ± 12.4%).

Conclusions: The results of this study suggest that drugs that inhibit the activity of vascular endothelial growth factor (i.e. anti-VEGF) may hinder bone healing and implant osseointegration in rat tibiae.

Endosseous implants are widely used to rehabilitate bone impairments (i.e. fractures) and to restore anatomical structures (i.e. lost teeth; Blanchaert 1998, Scully et al. 2007). Success of implant therapies relies on functional bone formation in direct contact with the implant, this process is defined as osseointegration (Albrektsson et al. 1981). Upon surgical placement, implants create a bone wound, and

Conflict of interest and financial disclosure
The authors revealed no conflict of interest.
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Despite its importance in osseointegration and bone healing, upregulation of VEGFs is associated with pathologic angiogenesis such as that seen in cancer- and age-related macular degeneration. Therefore, anti-VEGF therapies are very valuable in clinical oncology as a frontline of treatment for various types of cancer such as breast cancer, colorectal cancer and malignant glioma (Ferrara et al. 2004, Miletic et al. 2009). Furthermore, they are used to control aberrant angiogenesis in nonvascular eye diseases such as age-related macular degeneration (AMD; Kvant et al. 1996, Avery et al. 2006). Nowadays the standard of care for this condition is Ranibizumab, a fragment of a recombinant humanized monoclonal antibody Fab (48 kDa) that inhibits the activity of human VEGF-A (Rothenfeld et al. 2006, Lu & Adelman 2009, Mitchell 2011, Schmucker et al. 2012). For the reasons mentioned above, millions of people worldwide are currently using drugs that target VEGF, and this number is expected to reach over 500 million in the next decades (Carmeliet 2012). Anti-angiogenic drugs such as anti-VEGF and Ranibizumab will negatively affect bone healing and implant osseointegration.

Materials and Methods

The process of osseointegration and bone healing involves many cells (i.e. osteoblast, osteoclasts), growth factors (i.e. VEGF) and systems (i.e. immune system) that cannot be simulated *in vitro* and can only be assessed *in vivo*. Moreover, the European Medicines Agency and the Food and Drug Administration require preclinical investigations before human trials. For these reasons, we conducted this study to assess the effect of anti-VEGF and Ranibizumab on bone healing and implant osseointegration using rats tibiae model.

Ethical approval for this study was obtained from McGill Animal Ethics Board (#2012-7269). The study was conducted on a group of 36 female, 10 to 12-week-old Sprague Dawley rats (Charles River Laboratories, Montreal, QC, Canada) weighing 200–250 g. The rats were housed in the Genome Animal Facility of McGill University in a standard environmental 22°C with 12-hour light/dark cycles and a relative humidity of 30–70%. Every two rats were caged in a standard sterile cage bedded with wood chips that maintained them dry and clean. Rodent breeding diet and water were provided *ad libitum*. Animals were allowed to acclimatize to this environment for 2 weeks prior to surgical intervention. Animals’ condition and welfare were assessed daily for any sign of pain, infection, dehiscence, loss of appetite, weight loss or restricted movement. Analgesics (Carprofen 5–10 mg/kg) were administered when animals showed signs of pain. Prior to surgical intervention, rats were randomly divided into three groups: (i) control (*n* = 12); (ii) Ranibizumab (*n* = 12); and (iii) anti-VEGF (*n* = 12).

Surgical intervention

The surgical part of this study was carried out on November and December, 2013. We performed this study in rats tibia because it is considered a good experimental location to assess the systemic effects of drugs on implant osseointegration (Stadlinger et al. 2012). During the surgical intervention the surgeon was blinded to group allocation and all surgical interventions were performed by one surgeon using standardized instruments (i.e. burr and Ti implants) to assure consistent bone defects and implant placement among all animals. The animals were anaesthetized with isoflurane (3–5% at induction, 2–2.5% at maintenance period). After showing signs of being fully anesthetized, the legs were shaved and disinfected with chlorhexidine scrub and the animals were covered with a sterile drape. A longitudinal full thickness skin incision was created over the tibial metaphysis. The proximal tibialis muscle and the periosteum were dissected and conserved (Fig. 1a,c). A unicortical defect was created on the medial aspect of the left tibia metaphysis, 7–12 mm distal to the knee joint, using a cylindrical burr (1.5 mmø) adapted to a handpiece drill (Stryker, Hamilton, ON, Canada) under constant saline irrigation. A custom-made (1.5 mmØ × 2.0 mm long) titanium implant was placed in the left defect (Fig. 1d). The incision was closed using 5-0 monocrystal sutures. The procedure was repeated on the right tibia creating a 2.5 mmØ defect that was left empty (Fig. 1b). Performing two surgeries in each animal allowed us to reduce the number of animals by half. Animals intervention and husbandry were refined to minimize pain and distress. Carprofen (Pfizer Animal Health, Montréal, QC, Canada) was injected (5–10 mg/kg) subcutaneously 30 min. prior to surgery and 24 h after surgery for 2 days in order to provide analgesia to the rats.
Preoperative drug administration

Following surgical intervention, the first group of rats received saline injections (control); the second group received Ranibizumab (Lucentis; Genentech Inc., San Francisco, CA, USA); and the third group received anti-VEGF (R&D Systems Inc., Minneapolis, MN, USA). Ranibizumab was administered intra-peritoneally in a single injection of 15 μg diluted in 1.5 mL of saline (equivalent to the recommended human dose 0.5 mg; Lu & Adelman 2009). Ranibizumab is a humanized antibody that might not be effective on rats, therefore, rat-specific anti-VEGF, which has similar action to Ranibizumab in rats, was included as a third group in the study. This group received intra-peritoneal injections of anti-VEGF, 4 μg diluted in 1.5 mL of saline, three times per week (a dose that shown to affect blood vessels in rats). The anti-VEGF that we used is a 150 kDa anti-VEGF antibody that neutralizes the effect of VEGF164 with some reactivity with VEGF120, VEGF165 and <2% cross-reactivity with recombinant human VEGF-B, C, and D (Io et al. 2004, Rocher et al. 2011).

After the intervention, the rats were left to heal for 2 weeks before being euthanized using CO₂ overdose and had the tibiae retrieved. Right tibiae were preserved in paraformaldehyde solution 4% in PBS (Santa Cruz Biotech, Dallas, TX, USA) while the left tibiae were preserved in 10% neutral-buffered formalin (Richard Allan Scientific, Kalamazoo, MI, USA). The retrieved samples were code labelled and samples analyses were performed by one person that was blinded to group allocation.

Assessment of bone healing

A micro-CT analysis was conducted to assess bone healing in the defects. All tibiae with empty defects were scanned using a micro-CT (SkyScan1172; Bruker, Kontich, Belgium) set at a 12.7 μm resolution, a 50 kV voltage, a 0.5 rotation step, a 10 random movement and a 0.5 mm aluminium filter. The area of the bone defect was determined and included in the region of interest (ROI). The ROI included the entire original defect (2.5 mm Ø, unicortical). The ROI was reconstructed and analysed by SkyScan CT-analyser (SkyScan1172; Bruker). Three dimensional bone parameters including tissue volume, bone volume, trabecular thickness, trabecular number and trabecular separation were obtained from the 3-D reconstructed images. The volume of the defect was determined by subtracting the bone volume from the ROI.

Assessment of Blood vessels density and osteoclast number

All right tibiae samples (tibiae with bone defects) were decalcified using 10% EDTA for 3 weeks. The decalcified bone samples were dehydrated in ascending concentrations of ethanol (70–95%) using the automated paraffin tissue processor (ASP300-Leica, Wetzlar, Germany) and cleared with Xylene. The dehydrated samples were pre-infiltrated with Paraplast X-Tra wax at 58°C. The pre-infiltrated samples were embedded in paraffin block wax using an embedding center (EG1160-Leica) and cut into 5 μm thick sections using a microtome (RM2265-Leica). At least five coronal sections were obtained from each experimental defect. Two sections per defect were stained with von Willebrand Factor (vWF) (Millipore’s Blood Vessels Staining Kit, Millipore, Billerica, MA, USA) to assess the blood vessels density in the bone defects. The vWF-stained histological sections were recorded using a digital optical microscope (Carl Zeiss Microscopy, Jena, Germany). The total number of blood vessels stained by vWF were quantified manually, the average blood vessels density per defect was obtained, and the data were presented as blood vessels density per mm² mean value ± standard deviation. Another two sections per defect were stained with Tartrate Resistance Acid Phosphatase (TRAP) to assess the number of osteoclasts. The rational for this assessment is that VEGFs stimulate bone osteoclastic activity (Aldridge et al. 2005), accordingly anti-VEGF could have an negative effect bone healing and implant osseointegration through osteoclast. The histological sections were recorded and the total number of osteoclasts was quantified using an imaging software (ZEN 2012 SP2, Zeiss, Jena, Germany). The average number of osteoclasts per defect were obtained and the data were presented as osteoclast numbers per mm² of mineralized tissue mean value ± standard deviation.
Assessment of osseointegration

Histology and histomorphometry were conducted to assess osseointegration. All left tibiae (with implants) were dehydrated in ascending concentrations of ethanol (70–100%) before embedding them in polymethyl methacrylate histological resin (Technovit 9100, Heraeus Kulzer, Wehrheim, Germany). After polymerization, the osseointegrated implants were sectioned into histological slides (30 μm thickness) using a diamond saw (SP1600, Leica Microsystems GmbH, Wetzlar, Germany) and stained using basic fuchsin and methylene blue. Due to the small size of the implants, only one histological section through the middle of each implant was obtained and scanned (Carl Zeiss Microscopy). Histomorphometrical measurements were performed using the ImageJ software (Wayne Rasband; National Institute of Health, Bethesda, MD, USA). Implant osseointegration was defined as bone-implant contact area (BIC) and was calculated by dividing the bone-covered implant perimeter (BIP) by the total implant perimeter (TIP) as showed in this equation: BIC = BIP/TIP%. Two analyses of BIC were performed; first BICtotal included the cortical and trabecular peri-implant area and; second BICtrabecular included the trabecular peri-implant area only. All histomorphometric measurements were calculated as percentage values ± standard deviation.

Statistical analysis

Sample size was calculated to achieve a power of 80% at significance level of 5% in order to be able to reject the null hypothesis that there is no difference between Ranibizumab, anti-VEGF and control in terms of bone healing and implant osseointegration. A 10% difference between groups was considered to be clinically relevant, and a 12% potential standard deviation was assumed based on a previous study (Du et al. 2009). Accordingly, a total of 11 rats per group were determined to be sufficient. However, one rat was added to each group to compensate for 10% potential dropouts (animal die out). Descriptive statistics were conducted and data distribution was

Fig. 2. Micro-CT Scans. Coronal, sagittal, trans-axial sections and 3-D reconstructions of the micro-CT scans of bone defects shows larger defects in Ranibizumab and anti-VEGF treated rats compared to the control group. Scale bar = 200 μm.

Fig. 3. Micro-CT Analyses. Micro-CT data analysis of bone defects in Ranibizumab and Anti-VEGF treated rats compared to the control group: (a) Defect volume, (b) Trabecular thickness, (c) Trabecular number (d) Trabecular separation. Statistical analysis by one-way ANOVA and Welch F test, n control = 11 n Ranibizumab = 11, n Anti-VEGF = 12.

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tested for normality using the Kolmogorov–Smirnov test; the test showed that all outcomes were normally distributed. The Levene statistic test was used to assess homogeneity of variance. One-way ANOVA followed by the Tukey’s test, were used when the variance was homogenous, which was the case for bone defect volume, blood vessels density, osteoclast number and osseointegration, and data were presented as mean ± standard deviation. When homogeneity of variance was rejected, the Welch F test followed by Games Howell test were used and data were presented as mean ± standard deviation, median and range values. This was the case for trabecular number, thickness and separation. Data analyses were carried out using SPSS 17 (SPSS Inc., Chicago, IL, USA). Statistical significance was set at p < 0.05.

Results

Surgeries proceeded without complications, however, one rat from the Ranibizumab group and one from the control group were excluded because of post-operative bone fracture. No other animal drop-out was registered.

Micro-CT: bone healing

The size of the bone defect was significantly different among groups as indicated by one-way ANOVA (p = 0.026). Moreover, Tukey’s test indicated that the bone defect volume was significantly larger in the anti-VEGF group (2.48 ± 0.33 mm³, p = 0.022) than in the control group (2.11 ± 0.36 mm³). However, no significant differences were observed between Ranibizumab (2.35 ± 0.23 mm³) and control (p = 0.152) as well as between Ranibizumab and anti-VEGF groups (p = 0.66; Figs 2 and 3a).

Trabecular thickness was different among groups as determined by Welch F test (p = 0.024). Games Howell test showed that trabeculae were thinner in the anti-VEGF group (0.20 ± 0.04 mm, median = 0.20 mm, range: 0.19–0.21 mm, p = 0.041) than in the controls (0.288 ± 0.096 mm, median = 0.30 mm, range: 0.18–0.36 mm). No significant differences were detected between Ranibizumab (0.23 ± 0.05 mm, median = 0.24 mm, range: 0.20–0.27 mm) and control groups (p = 0.253) as well as between Ranibizumab and anti-VEGF groups (p = 0.184; Fig. 3b).

Trabecular number was different among groups (Welch F test; p < 0.001). The number of trabeculae was higher (Games Howell test) in the anti-VEGF group (2.60 ± 0.394/ mm, median = 2.64/mm, range: 2.34–2.91/mm, p = 0.007) and Ranibizumab group (2.23 ± 0.477/mm, median = 2.06/mm, range: 1.81–2.52/ mm, p = 0.035) than in the control group (1.26 ± 1.21/mm, median = 0.77/mm, range: 0.31–2.29/mm). However, no significant difference was observed between Ranibizumab and anti-VEGF (p = 0.073; Fig. 3c).

Trabecular separation was also different among groups as determined by Welch F test (p < 0.001). Games Howell test indicated that the mean trabecular separation was lower in the anti-VEGF group (0.148 ± 0.039 mm, median = 0.15 mm, range: 0.12–0.18 mm, p = 0.013) and the Ranibizumab (0.215 ± 0.074 mm, median = 0.20 mm, range: 0.14–0.28 mm, p = 0.027) than in the controls (0.6 mm ± 0.420 mm). No significant difference was observed between the Ranibizumab and anti-VEGF groups (0.097; Fig. 3d).

Angiogenesis and osteoclastogenesis

Ranibizumab and anti-VEGF inhibited the formation of new blood vessels as determined by one-way ANOVA (p < 0.001). Tukey’s test showed that blood vessel density was lower in the anti-VEGF group (7.8 ± 4.0/mm², p < 0.001) and Ranibizumab group (11.7 ± 4.3/mm², p < 0.001) than in the control group (28.0 ± 6.5/mm²; Fig. 4). No significant difference was observed between anti-VEGF and Ranibizumab groups (p = 0.90). Osteoclast numbers in the anti-VEGF group (28.5 ± 11.4 osteoclast/mm²) and Ranibizumab group (21.5 ± 11.9 osteoclast/mm²) were not significantly different from the control group (19.2 ± 5.2 osteoclast/mm²; one-way ANOVA p = 0.929 (Fig. 5).

Osseointegration

Bone implant contact area was different among groups, the average BICtotal and BICtrabecular were significantly different among groups as determined by one-way ANOVA.
Tukey’s test indicated that the BIC total was significantly lower (\(p < 0.001\)) in the anti-VEGF (19.9/6.4, \(p < 0.001\)) and Ranibizumab groups (21.7/6.2, \(p < 0.001\)) than in the control group (41.8/6.8). Tukey’s test also indicated that BIC trabecular in the anti-VEGF (18.6/6.1, \(p < 0.001\)) and Ranibizumab groups (19.8/4.4, \(p < 0.001\)) were significantly lower than in the controls (35.3/10.7%; Fig. 6).

**Discussion**

This study provides the first evidence indicating that anti-VEGFs impair bone healing and implant osseointegration in rats tibiae. Many risk factors could influence osseointegration (Lang & Jepsen 2009, Moy et al. 2004, Misch 2008, Esposito et al. 1998, Bornstein et al. 2009, Heitz-Mayfield & Huynh-Ba 2009, Salvi & Bragger 2009). These risk factors can be categorized into (i) very high, and; (ii) significant risk factors. Very high risk factors include serious systemic diseases (rheumatoid arthritis, osteomalacia, osteogenesis imperfect, HIV) and immunosuppressive medications (corticosteroids, chemotherapy or immunosuppressive drugs) as well as drug and alcohol abuse. Significant risk factors include irradiated bone, severe and uncontrolled diabetes, bleeding disorders, drug-induced anticoagulation and heavy smoking (Buser et al. 2000). However, a recent systematic review on this topic concluded that the level of evidence indicating absolute and relative contraindications for oral implant therapy due to systemic conditions and medications is low and that no data exist for all medical conditions (Bornstein et al. 2009).

Vascular endothelial growth factors can improve bone healing by enhancing angiogenesis (Zhang et al. 2014), and by stimulating bone turnover through osteoclasts chemotaxis and activity (Niida et al., 1999, Nakagawa et al., 2000, Deckers et al. 2000, 2002, Street et al. 2002a,b, Ramazanoglu et al. 2013). Accordingly, anti-VEGF could affect bone healing by suppressing angiogenesis or osteoclasts. However, our data showed that the compromised bone healing and osseointegration among the anti-VEGF group, as well as the compromised osseointegration among the Ranibizumab group, are likely to be caused only by the down-regulation of angiogenesis since neither drugs affected osteoclast number.

Our findings are in agreement with previous work that found that anti-angiogenic drug (TNF-470) had a negative effect on peri-implant bone formation although did not significantly affect the direct BIC probably because the dose they used. In fact, TNF-470 can only inhibit the blood vessel formation at doses three times higher than the one used (30 mg/kg, every other day) which was at least three times more than the dose (10 mg/kg, three times a week) Mair’s study (Castronovo & Belotti 1996, Mair et al., 2007). And here, we saw significant impairment of BIC probably because the number of newly formed blood vessels was inhibited significantly by Ranibizumab and anti-VEGF.

In this study, anti-VEGF and Ranibizumab did not affect osteoclast numbers, probably because the anti-bodies that we used have specific affinity to only some VEGFs isoforms (i.e. 164, 165 and 121) but not to all of them. Moreover, Ranibizumab which is a fragment of a recombinant humanized monoclonal antibody Fab (48 kDa) is even more specific and it only inhibits the biological activity of human VEGF-A. Osteoclasts express two types of VEGF receptors: VEGFR-1 (Flt-1) and VEGFR-2 (Flk-1). These receptors are sensitive to many VEGF isoforms besides those inhibited by the antibodies that we used in this study (i.e. 198, 206, beside 164, 165, 121 and VEGF-A; Street et al. 2002a,b). Therefore, specific conditions
inhibition of VEGF 164, 165, 121 and VEGF-A by anti-bodies we tested might not be enough to affect osteoclasts since its receptors will still be activated by the other spared VEGF isoforms (Ferrara et al. 2003, Mitchell et al., 2011, Rosenfeld et al. 2006, Kanczler et al., 2008, Lu et al., 2009, Schmucker et al. 2012).

Anti-VEGF and Ranibizumab also affected bone quality in the healing defects by reducing trabecular thickness and volume while increasing the trabecular number. These observations indicate that inhibition of angiogenesis seems to hinder trabecular development and thickening, and this is probably compensated by increasing the trabecular number. This observation could be explained by the fact that among the cytokines and growth factors involved in bone healing and growth, VEGFs are the key regulators of angiogenesis (Kon et al. 2001, Schliephake 2002, Dimitriou et al. 2005). Therefore, VEGF inhibition during bone formation would suppress angiogenesis and consequently reduces trabecular bone volume, probably due to deprivation of oxygen and nutrients (Maes et al. 2002), but it would have a lesser effect on osteoblast chemotaxis and differentiation. Indeed, inhibition of VEGFs suppresses capillaries invasion, reduces chondroclasts recruitment and increases the hypertrophic cartilaginous zone in growth plates, without affecting bone mineralization around the cartilaginous zone (Gerber et al. 1999). These key observations along with our findings suggest that bone formation in bone defects of animals treated with anti-VEGFs gets started but it is then interrupted due to the deprivation of nutrients and oxygen caused by a deficient blood supply (Gerber et al. 1999, Kanczler & Oreffo 2008).

The average BIC on the machined Ti implants used in this study was 42% among control rats. This BIC value is consistent with previous studies using similar materials, and it is suitable for functional loading (Wong et al. 1995). However, anti-VEGFs treatment decreased BIC almost by half, reaching levels that are not compatible with successful mechanical functionalization of implants (Wong et al. 1995). This magnitude of BIC reduction is comparable to that observed in animal exposed to radiation therapy which is a well known cause of angiogenesis deterioration and failure of dental implants (Moy et al., 2004, Weinlaender et al. 2006, Kaigler et al. 2006). Accordingly, it could be speculated that anti-VEGF therapies could be problematic in treatments with osseointegrated implants, however, future studies are needed to confirm this.

The anti-VEGF had stronger effects on bone healing than Ranibizumab. This observation was expected because the anti-VEGF used in this study was rat specific; whereas, Ranibizumab is human specific (Rosenfeld et al. 2006, Lowe et al. 2007, Lu & Adelman 2009, Mitchell 2011, Schmucker et al. 2012). Nevertheless, Ranibizumab still had a negative effect on bone healing and osseointegration in our study, probably due to the similarity in structure between rodent and human VEGF (Carmeliet et al. 1999). Indeed, Ranibizumab has been found to inhibit angiogenesis in rats (Ostendorf et al. 1999, Arevalo 2013).

In order to extrapolate our results to different age groups, we chose young growing rats. Younger rats have faster bone healing (Bak & Andreassen 1989) and should be less susceptible to drugs that delay bone healing (Aguirre et al. 2010). Accordingly, since anti-VEGF and Ranibizumab did have a negative effect on young animals, we would expect an even stronger effect on older animals that already have lower serum level of VEGF (Lin et al. 2008), although future studies will have to be performed to confirm this hypothesis.

As with any animal model, there are some limitations to be acknowledged. These include inherent variation between animals, however, this issue was compensated by sufficient sample size. Also extrapolation of our results to clinical practice could be limited by potential differences between humans and rats (e.g. bone pattern and bone quality). Moreover, the effects of anti-VEGFs on bone healing and implant

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osseointegration were assessed only at one time point (two weeks following surgery), thus it did not allow to evaluate the long-term effects of these antibodies on bone healing and implant osseointegration, these issues will have to be addressed in future studies. The 2-week time point was selected because healing defect neoangiogenesis and VEGFs expression are high in this period (Dimitriou et al. 2005). Although this study showed that anti-VEGF drugs affected bone healing and osseointegration by compromising blood vessels formation, a future knockout mouse model may be needed to confirm this hypothesis. One more limitation in this study is that the injections were given intraperitoneally instead of intravitreally, however, intravitreal-administered drugs (bevacizumab) do migrate from the vitreous cavity (Bakri et al. 2007) and cause systemic complication (Shima et al. 2008).

Conclusions
Anti-VEGFs inhibit osseointegration of Ti implants and delay bone healing.

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the vascular endothelial growth factor isoforms VEGF 
and VEGF. Principal findings: Drugs that hinder the activity of vascular endothelial growth factors, such as anti-VEGFs delayed bone healing and compromised implant osseointegration in rats' tibiae. Practical Implications: Anti-bodies that use to treat age-related macular degeneration of the eye and cancer might expose patients to higher risk of implant and bone surgery complications. Therefore, these medications should be considered as potential risk factors for implant osseointegration and bone healing in dental and orthopedic surgical interventions.