Early intermittent low dose sclerostin antibody treatment promotes surface bone formation and reduces bone loss, but also decreases osteocyte apoptosis and mechanotransduction in ovariectomized rats

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Running title
Sclerostin Antibody Therapy Reduces Secondary Mineralization

Author contribution statement
Conceptualization: SMN, LMcN; Data Curation: SMN, WA, HA, LOS; Formal Analysis: SMN, WA, HA, LOS; Funding Acquisition: LMcN; Investigation: SMN, WA, HA, LOS, JAPP, JS, LMcN; Methodology: SMN, WA, HA, LOS, JAPP, JS, LMcN; Project Administration: LMcN; Resources: LMcN; Supervision: LMcN; Validation: SMN, WA, HA, LOS, JAPP, JS; Visualization: SMN, WA, HA, LOS; Writing – Original Draft: SMN, HA, LOS, LMcN; Writing – Review & Editing: SMN, WA, HA, LOS, JAPP, JS, GH, LMcN; All authors have read and agreed to the submitted version of the manuscript.

Abstract

Neutralizing sclerostin antibodies mitigate bone loss and promote bone formation, thereby reducing fracture risk in postmenopausal osteoporosis. Clinically, this treatment targets women at high risk of fragility fractures years after menopause. However, bone loss occurs rapidly during early menopause, followed by longer-term changes in bone mineralization. These changes are associated with osteocyte apoptosis, perilacunar remodelling, and altered osteocyte mechanosensitivity. The objective of this study was to evaluate the effects of early intermittent low-dose sclerostin antibody (Scl-Ab) therapy on: (1) osteoclastogenesis and bone resorption, (2) perilacunar remodelling, (3) secondary mineralization, and (4) osteocyte mechanosensitivity. Female retired breeder Wistar rats underwent bilateral ovariectomy and received monthly low-dose Scl-Ab injections (2 mg/kg/month) from 3 to 14 weeks post-OVX, while a control group remained untreated. We report that early intermittent low-dose Scl-Ab treatment increased bone formation (calcein labelling) and reduced bone resorption, as evidenced by a decrease in TRAP+ osteoclasts and reduced expression of catabolic genes (Sost, Ctsk, Mmp9) compared to untreated rats. Treatment also decreased the percentage of empty lacunae and the number of MMP14+ osteocytes, indicating improved osteocyte survival and reduced perilacunar remodelling. Furthermore, the expression of osteocyte-mediated mineralization genes (DMP1, PHEX, OPN, ALP) was reduced. Nano-CT analysis revealed decreased perilacunar mineral density, along with smaller lacunae. Additionally, the expression of mechanotransduction-related genes (Vcl, integrins α5, αV, β1, CX43, Axin2, IFT88, Adcy6, Pkd1, Cav1) was also reduced. We propose that early intervention with intermittent low-dose Scl-Ab therapy can promote surface bone formation while attenuating osteocyte-mediated secondary mineralization following initial bone loss.

Mini Abstract

Early intermittent low-dose sclerostin antibody treatment reduced osteoclastogenesis, bone resorption, and perilacunar remodelling, while promoting bone formation, decreasing osteocyte apoptosis, and downregulating genes associated with secondary mineralization and mechanosensitivity in ovariectomized rats. These findings suggest early intervention with Scl-Ab enhances bone formation and limits osteocyte apoptosis and subsequent secondary mineralization.

Keywords

Sclerostin Antibody (Scl-Ab), Postmenopausal Osteoporosis, Ovariectomized Rats, Bone Remodelling, Secondary Mineralization, Early Intervention

Introduction

Postmenopausal osteoporosis is characterized by an imbalance in bone remodelling, primarily driven by excessive bone resorption by osteoclasts, coupled with insufficient bone formation by osteoblasts. The early phase of estrogen deficiency is marked by rapid trabecular bone deterioration, followed by secondary bone tissue mineralization [1]. Moreover, estrogen deficiency induces site-specific alterations in mineral distribution, in addition to changes in the osteocyte microenvironment, osteocyte apoptosis and perilacunar remodelling [2, 3].

Neutralizing antibodies targeting sclerostin, such as romosozumab, have been approved for the treatment of postmenopausal osteoporosis, demonstrating significant efficacy in increasing bone mineral density (BMD) and reducing fracture risk [4]. These antibodies work by inhibiting sclerostin, thereby promoting the osteogenic differentiation of progenitor cells and activating bone-lining cells to stimulate bone formation [5-8], while also indirectly suppressing bone-resorbing osteoclast activity [9, 10]. Clinically, sclerostin antibodies (Scl-Ab) are indicated for postmenopausal women with severe osteoporosis who are at high risk of, or have a history of, fragility fractures [11], events that can occur many years after menopause. This increased fracture risk is largely due to rapid bone loss resulting from estrogen deficiency during the first two years after menopause, which then slows to ~1% per year [12]. Supporting these clinical indications, a single dose of romosozumab in patients with low bone mass and no prior anti-resorptive treatment led to increased serum PINP, decreased sCTX, and significant improvements in lumbar spine BMD within three months [13].

Clinical trials have shown that monthly injections of sclerostin antibody romosozumab at a dose of 210 mg significantly increases bone mineral density (BMD) within the first year of treatment [5, 6, 11]. However, after six months of continuous treatment, markers of bone formation (P1NP, osteocalcin, and ALP) return to nearly baseline levels, indicating a dampening in the bone-forming response with repeated administration [5, 6, 8, 14]. Similarly, prolonged use of sclerostin antibody is associated with decreasing levels of serum P1NP and osteocalcin [15, 16]. Extended treatment with sclerostin antibody (Scl-Ab) normalizes Wnt target genes, while elevating the expression of secreted Wnt antagonists, including SOST [10, 16]. Additionally, prolonged Scl-Ab treatment induces changes in osteocyte gene expression, particularly cell cycle progression and mitogenesis genes [10]. Interestingly, a treatment-free period restores bone formation in rodent models [10, 16] and in humans [17]. Thus, intermittent dosing schedules, with breaks in antibody administration, may reduce the dampening effect of continuous treatment, preserving efficacy.

Administration of twice-weekly high-dose (25 mg/kg) Scl-Ab injections to OVX animals, untreated for up to 16 months, restored bone mass to control levels within 4 to 15 weeks of treatment [18, 19]. Weekly Scl-Ab treatment for up to 26 weeks demonstrated sustained increases in trabecular and cortical bone volume, associated with endocortical and periosteal bone formation rates [15, 20]. These doses surpass typical human clinical exposure, leading to strong pharmacological effects. In humans, romosozumab is administered at 210 mg monthly, roughly equivalent to 3 mg/kg weekly Scl-Ab injections in animals [10, 21, 22], as IgG clearance is faster in rodents. In cynomolgus monkeys, a once-weekly treatment at 3 mg/kg, corresponding to ~1.2 times clinical exposure, was initiated four months after OVX and was effective in reversing bone loss and enhancing bone strength [22], whereas in the lumbar vertebrae of OVX rats (3 mg/kg) weekly Scl-Ab administration increased trabecular bone volume fraction (BVF) [10]. Weekly administration of low and high-dose (3, 50 mg/kg) Scl-Ab in OVX rats, for up to 26 weeks resulted in dose-dependent increases in bone mass, with sustained benefits during and after the treatment period [10]. However, the efficacy of low-dose Scl-Ab is not yet fully understood.

The rapid bone loss triggered by estrogen deficiency in the early postmenopausal period has been well-documented in clinical studies and is further supported by preclinical models.

Preclinical studies indicate that OVX or estrogen deficiency leads to early bone loss within the first 4 weeks, followed by longer-term secondary mineralization and increased microporosity associated with perilacunar remodelling and micropetrosis [3]. Osteocytes synthesize and release sclerostin, inhibiting the Wnt/β-catenin pathway crucial for bone formation by binding to LRP5/6 proteins involved in Wnt signalling and osteoblastogenesis [23, 24]. Sclerostin antagonizes the normal response to mechanical loading by osteocytes, and may lead to micropetrosis [25, 26]. In vitro, osteocytes exhibit altered mechanosensitivity during estrogen deficiency, with reduced intracellular calcium oscillations and attenuated responses to mechanical loading, leading to exacerbated osteocyte-mediated osteoclastogenesis and resorption [27]. Interestingly, sclerostin inhibition reverts these effects , regulating osteoblast and osteocyte activity via the mechanosensitive Wnt/β-catenin signalling pathway. Microarray analysis of bone tissue from ovariectomized rats treated with Scl-Ab revealed increased expression of Wnt target and extracellular matrix genes. Similarly, cortical bone from mice treated with Scl-Ab for two weeks exhibited upregulation of ECM-related genes; Twist1, and Cgref [28]. However, the effectiveness of Scl-Ab treatment during early bone loss, before secondary mineralization, remains unexplored. We hypothesize that early Scl-Ab treatment may prevent secondary mineralization by targeting perilacunar resorption and osteocyte apoptosis in postmenopausal osteoporosis.

The objective of this study was to evaluate the efficacy of early intermittent low-dose monthly sclerostin antibody treatment, starting three weeks post-ovariectomy, during the phase of rapid bone loss but before secondary mineralization. Specifically, we sought to study the potential to mitigate changes in (1) osteoclastogenesis and bone resorption, (2) perilacunar remodelling, (3) secondary mineralization, and (4) osteocyte mechanosensitivity in estrogen deficiency.

Methods

Animal model

Female retired breeder Wistar rats (6 months old, Charles River, Ireland) underwent bilateral ovariectomy (n = 8), confirmed by necropsy. To study subcutaneous delivery of intermittent low-dose Scl-Ab [21] (rodent version of romosozumab: Scl-Ab; r13c7, UCB Pharma, UK / Amgen Inc., UK), ovariectomized rats were assigned to either (a) no treatment (Control, n = 4, body weight: 382 ± 39 g) or (b) Scl-Ab (2 mg/kg/month, n = 4, body weight: 399 ± 49 g), administered every 4 weeks starting 3 weeks post-ovariectomy. This time point corresponds to the initial phase of rapid bone loss but precedes secondary mineralization changes [1]. Euthanasia occurred 3 weeks after the final injection. Procedures followed guidelines from the University of Galway's Animal Care and Research Ethics Committee (ACREC) and the Health Products Regulatory Authority (HPRA).

Calcein Labelling

To label actively mineralizing bone surfaces animals received two intraperitoneal calcein injections (20 mg/kg) at 14 and 4 days before euthanasia. This enabled quantification of mineral apposition rate (MAR) over a sufficient interval. At week 14, vertebrae were isolated from Control (n = 4) and Scl-Ab (n = 4) rats, fixed in 4% paraformaldehyde, and processed for analysis. Vertebral sections (1.5 mm) were cut using a diamond blade low-speed saw (Isomet™, Buehler, IL, USA), embedded in epoxy, sliced and mounted on SuperFrost® Plus slides (Menzel Glaser). The mineral apposition rate was calculated.

In vivo and Ex vivo Nano-CT Imaging and Analysis

In vivo micro-CT scanning (VivaCT40, Scanco Medical AG) was conducted to investigate the effect of Scl-Ab on morphometry and mineralization during estrogen deficiency. Scans were

performed at day 0, week 4, week 8 and week 14 on the right tibia of Control (n = 4) and Scl-Ab (n = 4) groups at 15 µm resolution. The X-ray settings, region of interest, and segmentation threshold were applied according to a previously established protocol [1]. Microarchitectural parameters (Bone volume fraction (BV/TV), trabecular number (Tb.N), trabecular thickness (Tb.Th), and trabecular spacing (Tb.Sp)) were quantified from the 3D reconstruction of the trabecular volume of interest using evaluation scripts in the Scanco Image Processing Language in the proximal tibia of the treatment groups at each time point.

Ex vivo high-resolution nano-CT scanning (Zeiss Xradia Versa 620) was performed at week 14 on right tibiae from control (n = 4) and Scl-Ab (n = 4) groups. Hydroxyapatite (HA) phantoms (QRM, PTW Dosimetry) were used to calibrate intensity measurements. Scans were conducted using a 0.4X objective at 70 kV, 8.5 W, with an LE3 source filter at an isotropic resolution of 9.14 µm. A 3-mm region below the proximal tibial growth plate was analysed to assess trabecular bone microarchitecture; BV/TV, Tb.N, Tb.Th, and Tb.Sp.

Lacunae and peri-lacunar mineral density were also assessed using ultra-high-resolution scans (1.11 µm voxel size). Trabecular bone sections (0.5 mm thick) were prepared, mounted, and scanned with a 4X objective and a 4-second exposure time with same electrical parameters. Lacunae were segmented using Otsu thresholding quantification of peri-lacunar mineral density and lacunar surface area.

Histological and Immunohistochemical Analysis

At week 14, femurs from Control and Scl-Ab rats were fixed in 4% paraformaldehyde. Distal femoral metaphyseal sections (1.5 mm) were cut using a diamond blade saw and decalcified in 10% EDTA for 14 days. Decalcification was confirmed via oxalate and physical probing tests. Samples were rinsed in PBS overnight, processed using a Leica ASP300 tissue processor and embedded in paraffin (Leica EG1150H). Paraffin sections (8 µm) were cut using a Leica RM2235 microtome, mounted on SuperFrost® Plus slides, and stored at room temperature, until staining.

TRAP and H&E staining were performed to visualize osteoclast number and osteocytes within lacunae, respectively. Images were captured using a light microscope (Olympus BX43, Olympus, Japan), and quantitative analysis was conducted on ImageJ.

For immunohistochemistry, paraffin-embedded sections were incubated overnight at 4°C with primary anti-MMP14 antibody (1:100; Abcam, ab38971), followed by a secondary antibody (Goat Anti-Rabbit IgG H&L (HRP); Abcam, ab6721) for 1 hour at room temperature. Slides were developed using DAB substrate chromogen solution for 5 minutes. Control sections, received antibody diluent instead of the primary antibody, were processed alongside experimental samples to confirm specificity. The prevalence of MMP14+ osteocytes, normalized to total bone area, was assessed using ImageJ [29].

Gene Expression Analysis

Tibiae from control (n=4) and Scl-Ab (n=4) groups were dissected, washed with DNase/RNase-free PBS, and cortical bone isolated. Samples were washed to remove bone marrow, snap-frozen in liquid nitrogen, and stored at -80°C. Total RNA was extracted using the RNeasy Fibrous Tissue Mini Kit (Qiagen) and converted to cDNA with the SuperScript VILO Kit (Invitrogen). RNA quality was assessed by 260/280 and 260/230 absorbance ratios. Scl-Ab-treated samples showed acceptable 260/280 ratios (1.8–2.0), with slightly lower 260/230 ratios (<2), but RNA quality was sufficient for mRNA microarray analysis, confirmed by consistent signal intensities and reproducible biological replicates.

TaqMan® microarray analysis was performed using a StepOnePlus™ PCR system, with Rpl13a as the reference gene. The study examined gene expression related to bone resorption and matrix degradation (RANKL, OPG, Sost, NFATc-1, CtsK, MMP9, MMP13,

Ccr2, ATG7, Raptor), apoptosis (Tp53, Casp3, Lamp1), bone formation and matrix production (OPN, Col1α1, Col1α2, OCN, Alp (osteoblast-associated) and DMP1, PHEX, TGFβ1, Klf10 (osteocyte-enriched)), other regulators (Ager, Fn1 (stress- and ECM-associated)) and mechanosensation and mechanotransduction (VCL, TRPV4, ADCY6, AXIN2, ITGβ1, ITGα5, ITGαV, CX43, RYR, PKD1, CAV1). Results are expressed as relative quantitative changes normalized to age-matched OVX controls (Control), with normalization to the housekeeping gene Rpl13a. Eight microarrays were performed, equally divided between Control and Scl-Ab groups.

Statistical analysis

Statistical analyses were performed using GraphPad Prism. Differences between Control and Scl-Ab groups were assessed via Student's t-tests, with significance set at $p \leq 0.05$. CT parameters (BV/TV, Tb.N, Tb.Th, Tb.Sp) were tested for normality; normally distributed data were analysed with t-tests, while non-normal data were analysed using Mann-Whitney tests. Two-way ANOVA was conducted to assess time and treatment interactions, followed by Tukey's post hoc tests for significant interactions. Results are presented as mean ± SD.

Results

Bone formation recovery in Scl-Ab treatment of ovariectomy-induced bone loss.

To assess the longitudinal effects of Scl-Ab treatment on bone microarchitecture and mineralization, we performed both in vivo micro-CT and ex vivo nano-CT analyses (Figure 1A). Morphometric analysis by in vivo micro-CT (15 µm) indicated no significant differences in bone volume fraction and trabecular microarchitecture between Scl-Ab and Control groups at baseline (day 0), week 4, week 8 and week 14 (Figures 1B, 1C). However, there was a significant decrease in bone volume fraction and trabecular number and a significant increase in trabecular spacing in week 14 compared to day 0 for both Control and Scl-Ab groups.

Morphometric analysis at nano-CT resolution (9.14 µm) revealed significant differences between the Scl-Ab treated and Control groups at week 14 (Figures 1D, 1E). Specifically, there was a significant increase in BV/TV and trabecular number, and a significant decrease in trabecular spacing in the Scl-Ab group compared to the untreated OVX group. No significant differences were observed in trabecular thickness.

Fluorochrome labelling of mineral apposition (Figure 1F) confirmed that Scl-Ab therapy enhanced bone formation in OVX rats. The mineral apposition was 11.54 ± 2.194 µm in the Scl-Ab-treated group compared to 6.16 ± 2.347 µm in untreated OVX controls ($p < 0.05$). The mineral apposition rate was 0.62 ± 0.23 µm in the Scl-Ab-treated group compared to 1.14 ± 0.22 µm in untreated OVX controls ($p < 0.05$).

Osteoclastogenesis and bone resorption are reduced in OVX rats treated early with intermittent low-dose Scl-Ab.

Early administration of intermittent low-dose Scl-Ab (2 mg/kg/month) significantly reduced osteoclast activity and gene expression associated with bone resorption. By week 14, TRAP+ staining was significantly lower in Scl-Ab-treated OVX animals than in untreated OVX controls (Figure 2A).

Gene expression analysis confirmed a downregulation in key bone catabolism markers: NFATc-1 (6-fold), CtsK (12-fold), MMP13, Raptor, Sost (11-fold), MMP9 (25-fold), and ATG7 (10-fold) (Figures 2B, 2C). A trend toward a lower RANKL/OPG ratio was observed in the Scl-Ab group ($p = 0.06$), suggesting a shift toward reduced osteoclastogenesis. Interestingly, CCR2 expression was upregulated (31-fold) in Scl-Ab-treated OVX animals compared to untreated controls ($p < 0.05$, Figures 2B, 2C).

Osteocyte apoptosis and perilacunar remodelling are reduced in OVX rats treated early with intermittent low-dose Scl-Ab.

Scl-Ab treatment significantly reduced osteocyte apoptosis in OVX rats. Specifically, the percentage of empty lacunae was significantly lower in Scl-Ab-treated OVX animals compared to untreated OVX controls ($p < 0.05$, Figure 3A). Apoptosis-related genes were downregulated in Scl-Ab-treated OVX animals: Tp53 (11-fold), Casp3 (11-fold), and Lamp1 (100-fold) ($p < 0.01$, Figure 3B). Nano-CT revealed no significant difference in mean BMD between groups ($p = 0.08$; Figure 3C). However, treated animals exhibited significantly smaller lacunar surface areas (Figure 3C). The number of MMP14+ osteocytes, indicative of perilacunar remodelling, were significantly reduced in the Scl-Ab-treated group ($p < 0.05$, Figure 3D). Mineral density significantly reduced near the lacunar edge in Scl-Ab-treated OVX animals compared to controls ($p < 0.05$; Figure 3E). This reduction was localized, diminishing with distance from the lacunae (Figure 3E).

Osteocyte gene expression is reduced in OVX rats early treated with intermittent low-dose Scl-Ab.

Scl-Ab-treated OVX animals exhibited significant downregulation of several osteocyte-enriched genes, including *Klf10* (6-fold), *DMP1*, and *PHEX* (10-fold each), which are critical for osteocyte-mediated regulation of mineral homeostasis and signalling within the bone matrix. Additionally, *OPN* (osteopontin), a matrix protein expressed by both osteoblasts and osteocytes, was significantly reduced (30-fold), suggesting a broader suppression of mineral-associated signalling within the osteocytic network. Importantly, expression of *AGER*—a receptor known to be upregulated in osteocytes under oxidative or inflammatory stress—was significantly increased (82-fold) in the Scl-Ab-treated group compared to untreated OVX controls ($p < 0.05$; Figures 4A, 4B), pointing to potential activation of stress response pathways in osteocytes.

Osteoblast gene expression is reduced in OVX rats early treated with intermittent low-dose Scl-Ab.

In parallel, we observed suppression of genes primarily associated with osteoblast function. This included downregulation of *ALP* (100-fold), a key enzyme involved in matrix mineralization, as well as *Col1α1* (43-fold) and *Col1α2* (65-fold), which encode the major structural components of type I collagen in the bone matrix. Additionally, expression of *TGFβ1* (50-fold) and *Fn1* (55-fold), both involved in osteoblast-mediated matrix signalling and remodelling, was significantly reduced ($p < 0.01$; Figures 4A, 4B). No significant change was observed in *OCN* (osteocalcin) expression ($p = 0.24$), a late marker of osteoblast differentiation and bone formation.

Downregulation in mechanotransduction gene expression in OVX rats early treated with intermittent low-dose Scl-Ab.

To explore the impact of Scl-Ab treatment on mechanotransduction pathways, we performed a heatmap analysis of related gene expression, which revealed downregulation in the Scl-Ab-treated OVX group: Vcl (55-fold), Integrin α5 (5-fold), Integrin αV (25-fold), Integrin β1 (50-fold), CX43 (33-fold), Axin2 (11-fold), Adcy6 (10-fold), IFT88 (6-fold), Pkd1 (4-fold), and Cav1 (16-fold) ($p < 0.01$, Figure 5A, 5B). Conversely, RyR expression—a key regulator of intracellular calcium signalling—was significantly upregulated (22-fold) in Scl-Ab-treated OVX animals ($p < 0.05$, Figure 5A, 5B). TRPV4 expression remained unchanged ($p = 0.41$).

Discussion

The primary objective of this study was to assess the effectiveness of administering intermittent low-dose Sclerostin Antibody (Scl-Ab, 2 mg/kg/month) during the initial phase of rapid bone loss, prior to the onset of secondary mineralization changes [1]. Early administration of intermittent low-dose Scl-Ab reduces osteoclastogenesis and osteocyte apoptosis. It also limits perilacunar remodelling, secondary mineralization, and changes in osteocyte mechanosensitivity associated with estrogen deficiency (see Figure 6). Fluorochrome labelling revealed an increase in bone formation on trabecular surfaces following Scl-Ab therapy in OVX rats. This enhanced bone formation, combined with the suppression of osteoclastogenesis, resulted in an increase in bone volume. These results suggest that early intervention with intermittent low-dose Scl-Ab therapy can mitigate bone loss and osteocyte-mediated secondary mineralization.

Several limitations should be considered in this study. Firstly, the estrogen-deficient rat model may not fully replicate human osteoporosis, as it induces rapid bone loss through sudden estrogen depletion. However, ovariectomized rats are commonly used for postmenopausal osteoporosis research because they exhibit bone loss patterns similar to humans. They initially undergo rapid bone loss from sudden estrogen deficiency, followed by a more balanced remodelling phase. Secondly, the effect of Scl-Ab injections on bone loss was less pronounced with the low dose (2 mg/kg) compared to the reported efficacy of higher doses (25 mg/kg) in other OVX rat studies [20]. Additionally, the frequency of administration in our study (once per month) differs from other studies, which typically administer Scl-Ab once or twice per week. Nevertheless, a 2 mg/kg/month dose of Scl-Ab was sufficient to reduce osteoclast number, MMP14 osteocyte expression, and the incidence of empty lacunae in ovariectomized rats.

Our findings demonstrate that early administration of intermittent low-dose Scl-Ab in estrogen-deficient rats reduces osteoclastogenesis, consistent with its known dual effects on bone formation and resorption at higher doses [9, 10]. We observed reduced expression of NFATc-1, a key regulator of osteoclast function [30]. Additionally, levels of CtsK (critical for bone resorption) and Raptor (a component of the mTOR pathway) were decreased [31, 32]. Together, these results confirm that early low-dose Scl-Ab reduces both osteoclast formation and activity.

Significant differences in trabecular bone mass and microarchitecture by week 14 align with established effects of Scl-Ab in mitigating bone loss in younger OVX rat models at higher and more frequent doses (25 and 50 mg/kg weekly), especially when administered at later stages (e.g., 8 weeks post-OVX) or in severe osteoporosis with unloading [10, 33]. Notably, in our study, Scl-Ab treatment began at three weeks post-OVX, near the end of the rapid bone loss phase, when 10–30% of bone mass had already been lost [1].

We report that early intermittent low-dose Scl-Ab treatment also reduces osteocyte apoptosis and the prevalence of empty lacunae, consistent with prior findings in glucocorticoid-induced osteoporosis models [34]. Moreover, Scl-Ab treatment led to reduced expression of apoptosis markers (Tp53, Casp3) and decreased lysosomal activity (Lamp1), suggesting a concurrent suppression of osteocyte death and its protective cellular responses. We also observed inhibition of MMP14-dependent perilacunar remodelling.

Micropetrosis—the mineral infilling of empty lacunae—commonly follows osteocyte apoptosis, whereas perilacunar remodelling can degrade mineral surrounding osteocytes. Osteocyte loss is typically associated with smaller lacunae, while perilacunar remodelling may cause lacunar expansion. In our study, Scl-Ab treatment led to a decrease in lacunar surface area and a reduction in mineral density within the 2-micron region surrounding lacunae in OVX animals. Further research is required to elucidate how Scl-Ab balances suppression of

micropetrosis and perilacunar remodelling, and how this affects osteocyte regulation of their local microenvironment [25, 26].

Ovariectomy has previously been shown to increase osteocyte canalicular diameter [3], potentially enhancing fluid shear stress and making osteocytes more susceptible to apoptosis [3, 35-37]. In this study, Scl-Ab treatment reduced apoptosis markers (Tp53, Casp3) and lysosomal activity (Lamp1), supporting a protective mechanism against osteocyte death. Interestingly, a prior study in rats reported increased p53 expression in osteocytes following Scl-Ab treatment [10], highlighting the potential for context-dependent or time-sensitive effects of Scl-Ab on osteocyte stress responses. Adding further complexity, we observed upregulation of AGER, a receptor implicated in cellular stress [38], after early intermittent low-dose Scl-Ab treatment, suggesting a nuanced and multifaceted role for Scl-Ab in osteocyte biology.

We also observed a significant increase in mineral apposition rate, indicating enhanced osteoblast-driven surface bone formation in Scl-Ab-treated animals. Although early osteogenic genes (ALP, Col1α1, Col1α2) were downregulated at week 14, the bone formation observed likely occurred earlier in treatment, driven by initial osteoblast activity. In aged OVX rats, Scl-Ab initially promotes progenitor proliferation, leading to rapid osteoblast recruitment. Although proliferation declines over time, bone formation remains elevated due to enhanced osteoblast efficiency [39, 40]. These dynamics differ from those seen with high-dose or delayed Scl-Ab regimens, which tend to more robustly upregulate osteogenic gene expression at later disease stages [10, 20].

Importantly, while surface bone formation increased, osteocyte-mediated secondary mineralization was reduced. To our knowledge, this is the first report showing downregulation of osteocyte-enriched genes (Klf10, DMP1, and PHEX) following early intermittent low-dose Scl-Ab treatment. We also found downregulation of mechanotransduction-related genes (ItgαV, Itgβ1, and Cav1), which are essential for osteocyte mechanosensation and Wnt signalling. Short-term Scl-Ab treatment has also been associated with reduced osteocyte mechanosensitivity, possibly due to the presence of immature osteocytes following new bone formation. However, longer-term studies show a return to normal osteocyte signaling, which may explain the eventual reduction in bone formation [41]. We propose that Scl-Ab may initially reduce osteocyte sensitivity to mechanical loading—a potential protective adaptation to the altered mechanical environment following bone loss. By dampening mechanotransduction, Scl-Ab may help mitigate stress-induced osteocyte apoptosis and pathological mineralization [42].

In summary, early intermittent low-dose Scl-Ab treatment preserves osteocyte viability and prevents perilacunar remodelling, while suppressing bone resorption and promoting surface bone formation. Moreover, low-dose treatment can prevent osteocyte-driven secondary mineralization and mechanosensitivity through distinct cellular mechanisms.

Statement on conflicts or disclosures:

Syeda Masooma Naqvi, Wahaaj Ali, Hollie Allison, Laura M. O'Sullivan, Juan Alberto Panadero-Perez, Jessica Schiavi-Tritz and Laoise M. McNamara declare the following which may be considered as potential competing interests: The authors report sclerostin antibody was provided by UCB Ltd. Gill Holdsworth reports a relationship with UCB Ltd that includes employment.

References

1. O'Sullivan LM, Allison H, Parle EE, Schiavi J, McNamara LM (2020) Secondary alterations in bone mineralisation and trabecular thickening occur after long-term estrogen

421 deficiency in ovariectomised rat tibiae, which do not coincide with initial rapid bone loss.
422 Osteoporosis International 31:587-599
423 2. Emerton KB, Hu B, Woo AA, Sinofsky A, Hernandez C, Majeska RJ, Jepsen KJ,
424 Schaffler MB (2010) Osteocyte apoptosis and control of bone resorption following
425 ovariectomy in mice. Bone 46:577-583
426 3. Allison H, O'Sullivan LM, McNamara LM (2022) Temporal changes in cortical
427 microporosity during estrogen deficiency associated with perilacunar resorption and osteocyte
428 apoptosis: A pilot study. Bone Reports 16:101590
429 4. Gregson CL, Armstrong DJ, Bowden J, et al. (2022) UK clinical guideline for the
430 prevention and treatment of osteoporosis. Archives of Osteoporosis 17:58
431 5. Cosman F, Crittenden DB, Adachi JD, et al. (2016) Romosozumab Treatment in
432 Postmenopausal Women with Osteoporosis. N Engl J Med 375:1532-1543
433 6. McClung MR, Grauer A, Boonen S, et al. (2014) Romosozumab in postmenopausal
434 women with low bone mineral density. N Engl J Med 370:412-420
435 7. Costa S, Fairfield H, Farrell M, Murphy CS, Soucy A, Vary C, Holdsworth G, Reagan
436 MR (2021) Sclerostin antibody increases trabecular bone and bone mechanical properties by
437 increasing osteoblast activity damaged by whole-body irradiation in mice. Bone 147:115918
438 8. Padhi D, Allison M, Kivitz AJ, Gutierrez MJ, Stouch B, Wang C, Jang G (2014)
439 Multiple doses of sclerostin antibody romosozumab in healthy men and postmenopausal
440 women with low bone mass: a randomized, double-blind, placebo-controlled study. J Clin
441 Pharmacol 54:168-178
442 9. Ominsky MS, Boyce RW, Li X, Ke HZ (2017) Effects of sclerostin antibodies in animal
443 models of osteoporosis. Bone 96:63-75
444 10. Taylor S, Ominsky MS, Hu R, et al. (2016) Time-dependent cellular and transcriptional
445 changes in the osteoblast lineage associated with sclerostin antibody treatment in
446 ovariectomized rats. Bone 84:148-159
447 11. Saag KG, Petersen J, Brandi ML, Karaplis AC, Lorentzon M, Thomas T, Maddox J,
448 Fan M, Meisner PD, Grauer A (2017) Romosozumab or Alendronate for Fracture Prevention
449 in Women with Osteoporosis. N Engl J Med 377:1417-1427
450 12. Sowers MR, Zheng H, Jannausch ML, McConnell D, Nan B, Harlow S, Randolph JF,
451 Jr. (2010) Amount of bone loss in relation to time around the final menstrual period and follicle-
452 stimulating hormone staging of the transmenopause. J Clin Endocrinol Metab 95:2155-2162
453 13. Padhi D, Jang G, Stouch B, Fang L, Posvar E (2011) Single-dose, placebo-controlled,
454 randomized study of AMG 785, a sclerostin monoclonal antibody. J Bone Miner Res 26:19-26
455 14. Recker RR, Benson CT, Matsumoto T, et al. (2015) A randomized, double-blind phase
456 2 clinical trial of blosozumab, a sclerostin antibody, in postmenopausal women with low bone
457 mineral density. J Bone Miner Res 30:216-224
458 15. Stolina M, Dwyer D, Niu QT, et al. (2014) Temporal changes in systemic and local
459 expression of bone turnover markers during six months of sclerostin antibody administration
460 to ovariectomized rats. Bone 67:305-313
461 16. Holdsworth G, Greenslade K, Jose J, Stencel Z, Kirby H, Moore A, Ke HZ, Robinson
462 MK (2018) Dampening of the bone formation response following repeat dosing with sclerostin
463 antibody in mice is associated with up-regulation of Wnt antagonists. Bone 107:93-103
464 17. McClung MR, Brown JP, Diez-Perez A, et al. (2018) Effects of 24 Months of Treatment
465 With Romosozumab Followed by 12 Months of Denosumab or Placebo in Postmenopausal
466 Women With Low Bone Mineral Density: A Randomized, Double-Blind, Phase 2, Parallel
467 Group Study. J Bone Miner Res 33:1397-1406
468 18. Virdi AS, Irish J, Sena K, Liu M, Ke HZ, McNulty MA, Sumner DR (2015) Sclerostin
469 antibody treatment improves implant fixation in a model of severe osteoporosis. J Bone Joint
470 Surg Am 97:133-140

471 19. Wu J, Cai XH, Qin XX, Liu YX (2018) The effects of sclerostin antibody plus
472 parathyroid hormone (1-34) on bone formation in ovariectomized rats. Z Gerontol Geriatr
473 51:550-556
474 20. Li X, Niu QT, Warmington KS, et al. (2014) Progressive increases in bone mass and
475 bone strength in an ovariectomized rat model of osteoporosis after 26 weeks of treatment with
476 a sclerostin antibody. Endocrinology 155:4785-4797
477 21. Turk JR, Deaton AM, Yin J, et al. (2020) Nonclinical cardiovascular safety evaluation
478 of romosozumab, an inhibitor of sclerostin for the treatment of osteoporosis in postmenopausal
479 women at high risk of fracture. Regul Toxicol Pharmacol 115:104697
480 22. Ominsky MS, Boyd SK, Varela A, et al. (2017) Romosozumab Improves Bone Mass
481 and Strength While Maintaining Bone Quality in Ovariectomized Cynomolgus Monkeys. J
482 Bone Miner Res 32:788-801
483 23. Notsu M, Kanazawa I, Takeno A, Yokomoto-Umakoshi M, Tanaka KI, Yamaguchi T,
484 Sugimoto T (2017) Advanced Glycation End Product 3 (AGE3) Increases Apoptosis and the
485 Expression of Sclerostin by Stimulating TGF-β Expression and Secretion in Osteocyte-Like
486 MLO-Y4-A2 Cells. Calcif Tissue Int 100:402-411
487 24. Bonewald LF (2011) The amazing osteocyte. J Bone Miner Res 26:229-238
488 25. Kogawa M, Khalid KA, Wijenayaka AR, Ormsby RT, Evdokiou A, Anderson PH,
489 Findlay DM, Atkins GJ (2018) Recombinant sclerostin antagonizes effects of ex vivo
490 mechanical loading in trabecular bone and increases osteocyte lacunar size. Am J Physiol Cell
491 Physiol 314:C53-C61
492 26. Kogawa M, Wijenayaka AR, Ormsby RT, Thomas GP, Anderson PH, Bonewald LF,
493 Findlay DM, Atkins GJ (2013) Sclerostin Regulates Release of Bone Mineral by Osteocytes
494 by Induction of Carbonic Anhydrase 2. Journal of Bone and Mineral Research 28:2433-2435
495 27. Allison H, McNamara LM (2019) Inhibition of osteoclastogenesis by mechanically
496 stimulated osteoblasts is attenuated during estrogen deficiency. Am J Physiol Cell Physiol
497 28. Kedlaya R, Veera S, Horan DJ, Moss RE, Ayturk UM, Jacobsen CM, Bowen ME,
498 Paszty C, Warman ML, Robling AG (2013) Sclerostin inhibition reverses skeletal fragility in
499 an Lrp5-deficient mouse model of OPPG syndrome. Sci Transl Med 5:211ra158
500 29. Dole NS, Mazur CM, Acevedo C, et al. (2017) Osteocyte-Intrinsic TGF-β Signaling
501 Regulates Bone Quality through Perilacunar/Canalicular Remodeling. Cell Reports 21:2585-
502 2596
503 30. Kim JH, Kim N (2014) Regulation of NFATc1 in Osteoclast Differentiation. J Bone
504 Metab 21:233-241
505 31. Dai R, Wu Z, Chu HY, Lu J, Lyu A, Liu J, Zhang G (2020) Cathepsin K: The Action
506 in and Beyond Bone. Front Cell Dev Biol 8:433
507 32. Dai Q, Xie F, Han Y, Ma X, Zhou S, Jiang L, Zou W, Wang J (2017) Inactivation of
508 Regulatory-associated Protein of mTOR (Raptor)/Mammalian Target of Rapamycin Complex
509 1 (mTORC1) Signaling in Osteoclasts Increases Bone Mass by Inhibiting Osteoclast
510 Differentiation in Mice*. Journal of Biological Chemistry 292:196-204
511 33. Zhang DY, Hu MY, Chu T, Lin LJ, Wang JY, Li XD, Ke HZ, Qin YX (2016) Sclerostin
512 antibody prevented progressive bone loss in combined ovariectomized and concurrent
513 functional disuse. Bone 87:161-168
514 34. Achiou Z, Toumi H, Touvier J, Boudenot A, Uzbekov R, Ominsky MS, Pallu S,
515 Lespessailles E (2015) Sclerostin antibody and interval treadmill training effects in a rodent
516 model of glucocorticoid-induced osteopenia. Bone 81:691-701
517 35. Ciani C, Sharma D, Doty SB, Fritton SP (2014) Ovariectomy enhances mechanical
518 load-induced solute transport around osteocytes in rat cancellous bone. Bone 59:229-234

36. Brennan MA, Haugh MG, O'Brien FJ, McNamara LM (2014) Estrogen Withdrawal from Osteoblasts and Osteocytes Causes Increased Mineralization and Apoptosis. Horm Metab Res

37. Tomkinson A, Gevers EF, Wit JM, Reeve J, Noble BS (1998) The Role of Estrogen in the Control of Rat Osteocyte Apoptosis. 13:1243-1250

38. Yang X, Liu CJ, Wang ZZ, Ding D, Shi JW, Wu XT, Sun LW, Fan YB (2021) Effects of advanced glycation end products on osteocytes mechanosensitivity. Biochem Biophys Res Commun 568:151-157

39. Boyce RW, Brown D, Felx M, Mellal N, Locher K, Pyrah I, Ominsky MS, Taylor S (2018) Decreased osteoprogenitor proliferation precedes attenuation of cancellous bone formation in ovariectomized rats treated with sclerostin antibody. Bone Rep 8:90-94

40. Ominsky MS, Brown DL, Van G, et al. (2015) Differential temporal effects of sclerostin antibody and parathyroid hormone on cancellous and cortical bone and quantitative differences in effects on the osteoblast lineage in young intact rats. Bone 81:380-391

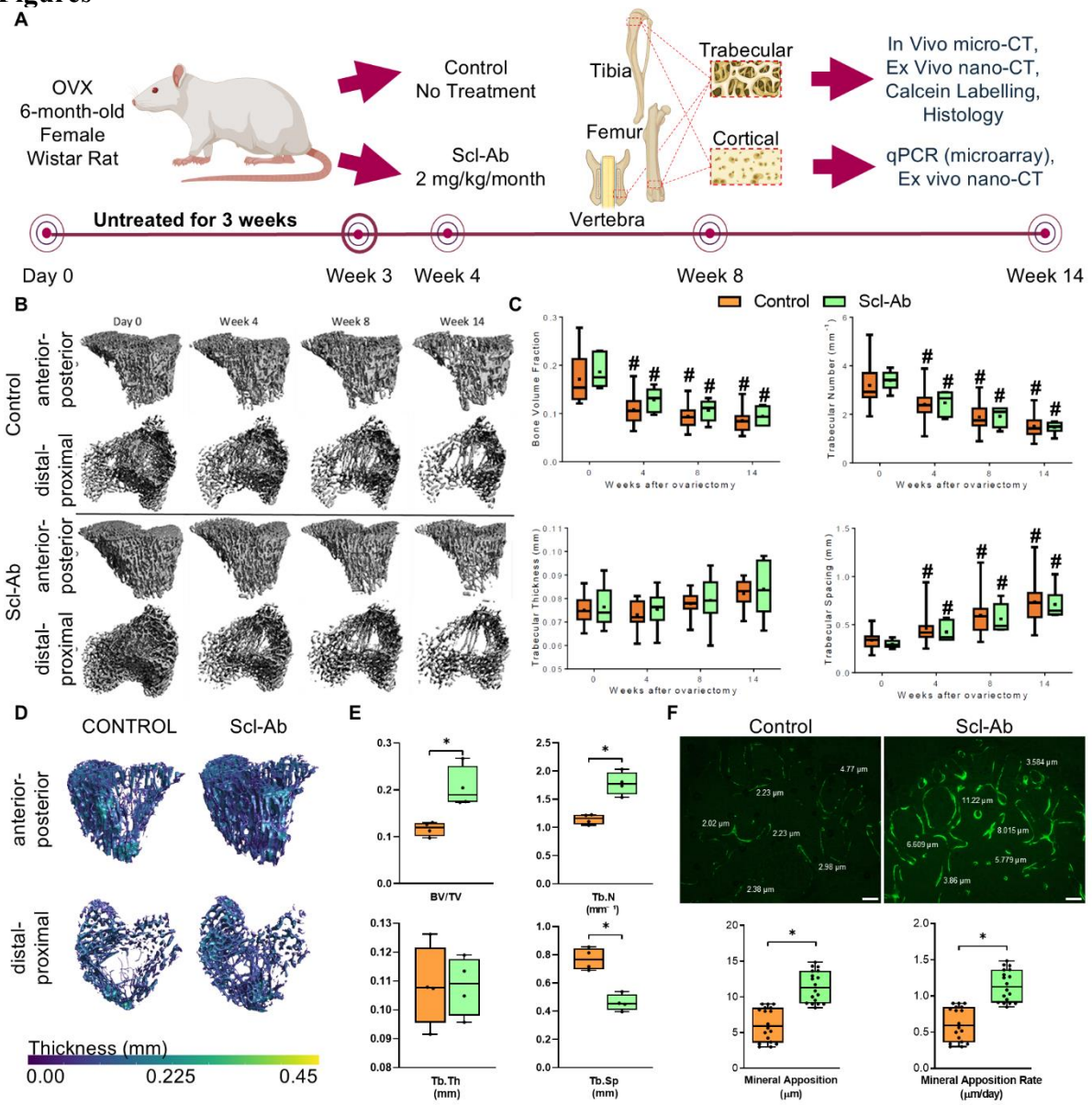
41. Morrell AE, Robinson ST, Ke HZ, Holdsworth G, Guo XE (2021) Osteocyte mechanosensing following short-term and long-term treatment with sclerostin antibody. Bone 149:115967

42. Verbruggen SW, Mc Garrigle MJ, Haugh MG, Voisin MC, McNamara LM (2015) Altered mechanical environment of bone cells in an animal model of short- and long-term osteoporosis. Biophys J 108:1587-1598

Acknowledgements

This publication has emerged from research supported by SFI, co-funded under the European Regional Development Fund (14/IA/2884), and the ERC Consolidator Grant (MEMETic: 863795). The authors would like to acknowledge Servier Medical Art for their image bank, which was used to produce the figures, and UCB Pharma and Amgen Inc. for providing the Scl-Ab via a materials transfer agreement.

546 **Figures**



547
548 ***Figure 1:*** *Surface Bone formation in early low-dose Scl-Ab treatment of ovariectomy-induced*
549 *bone loss. (A) Study design: Female retired breeder Wistar rats were ovariectomized at 6*
550 *months. Three weeks post-ovariectomy, the animals were randomly assigned to (i) Control (no*
551 *treatment) or (ii) Scl-Ab (systemic delivery of 2 mg/kg/month Scl-Ab). (B) 3D micro-CT images*
552 *of proximal tibial trabecular bone at 0, 4, 8, and 14 weeks post-OVX in both groups, shown in*
553 *two orthogonal views: anterior-posterior and distal-proximal. (C) Comparison of bone mass*
554 *and microarchitectural parameters in Control and Scl-Ab groups at 0, 4, 8, and 14 weeks post-*
555 *OVX. (D) High-resolution 3D nano-CT images of metaphyseal trabecular bone from the*
556 *proximal tibia of a representative Control and Scl-Ab group at 14 weeks post-OVX, shown in*
557 *two orthogonal views: anterior-posterior and distal-proximal. (E) Comparison of bone mass*
558 *and microarchitectural parameters between the Control and Scl-Ab groups 14 weeks post-*
559 *OVX. (F) Calcein labelling of surface bone formation in Control and Scl-Ab groups, mineral*
560 *apposition (µm) and rate (µm/day) quantified (n = 4/group). Box plots show median, IQR, and*
561 *range. *$p < 0.05$; $^{\#}p < 0.05$ vs. baseline (day 0). Scale = 50 µm.*
562

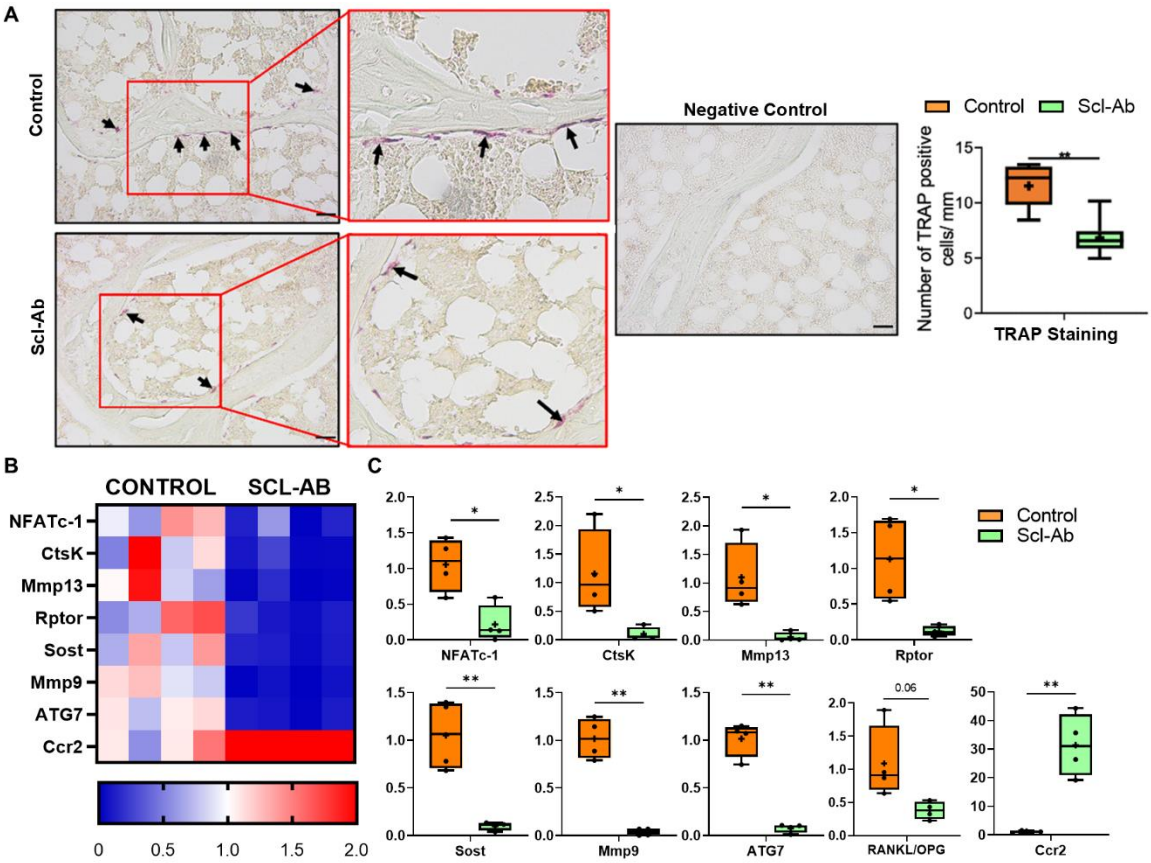


Figure 2: *Osteoclastogenesis and bone resorption are reduced in OVX rats treated early with intermittent low-dose Scl-Ab. (A) TRAP+ staining is reduced in OVX animals that received subcutaneous Scl-Ab compared to untreated OVX animals (Control) at week 14. Representative images show TRAP staining (black arrows indicate TRAP+ osteoclasts) and fast green counterstaining of cortical bone in Control and Scl-Ab (magnified images in red boxes). ImageJ analysis was performed to quantify the number of TRAP+ cells per mm. (B) Heat map showing expression of genes involved in catabolic activity and matrix degradation in cortical bone from OVX rats at week 14, comparing untreated controls to those treated with 2 mg/kg/month Scl-Ab starting 3 weeks post-OVX. (C) Scl-Ab treatment significantly lowers expression of Sost, Nfatc1, Ctsk, Mmp9, Ccr2, and Rankl/Opg. *p < 0.05, **p < 0.01; n = 4/group. Scale = 20 µm.*

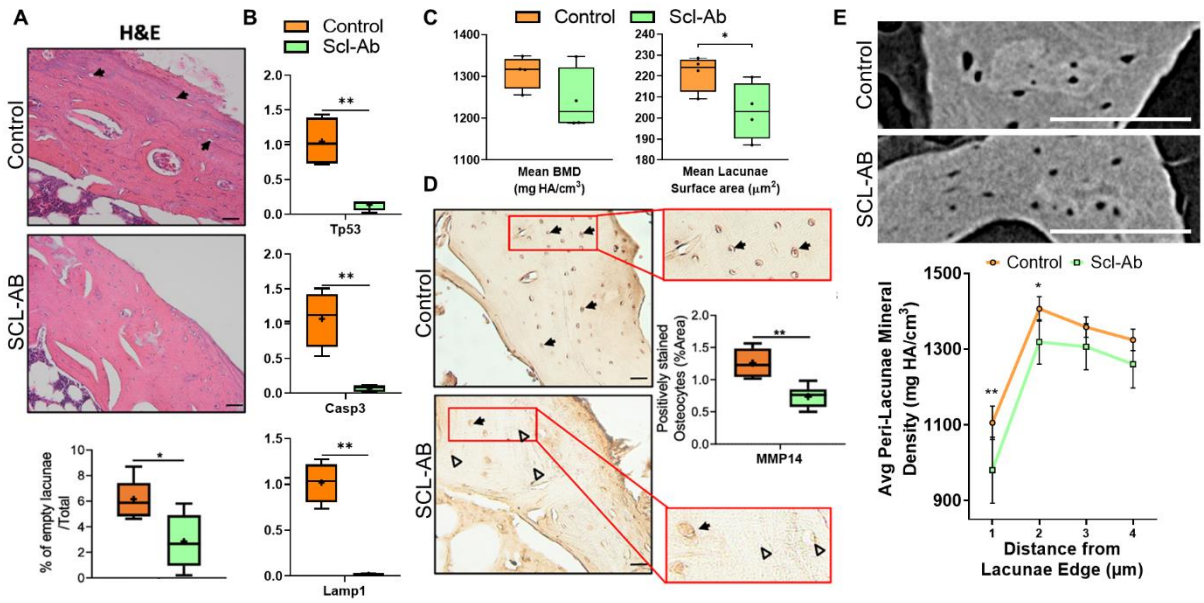


Figure 3: *Osteocyte apoptosis and perilacunar remodelling are reduced in OVX rats treated early with intermittent low-dose Scl-Ab. (A) Scl-Ab administration results in a decrease in unoccupied lacunae. Representative images show Haematoxylin and Eosin (H&E) staining of cortical bone from OVX rats at week 14, comparing untreated controls to those treated with 2 mg/kg/month Scl-Ab starting 3 weeks post-OVX. ImageJ analysis was performed to quantify the percentage of empty lacunae (black arrows indicate empty lacunae). (B) Scl-Ab administration results in a decrease in gene expression associated with apoptosis (Tp53, Casp3, and Lamp1). (C) Mean BMD (mg HA/cm³), and Mean lacunae surface area (mm³) quantified using ultra-high-resolution nano-CT images. (D) Scl-Ab administration results in a decrease in MMP14+ osteocytes. Representative images show MMP14 staining in Control and Scl-Ab (magnified images, red boxes). Black arrows indicate MMP14+ osteocytes, and the white triangle indicates MMP14- osteocytes. ImageJ analysis was performed to assess the prevalence of positively stained osteocytes, normalized to total bone area. (E) Representative nanoCT images of metaphyseal trabecular bone from the proximal tibia from OVX rats at week 14, comparing untreated controls to those treated with 2 mg/kg/month Scl-Ab starting 3 weeks post-OVX, which were evaluated to determine the average peri-lacunae mineral density (mg HA/cm³), Scale bar = 20 µm (A and D) and 100 µm (E). *p < 0.05, **p < 0.01.*

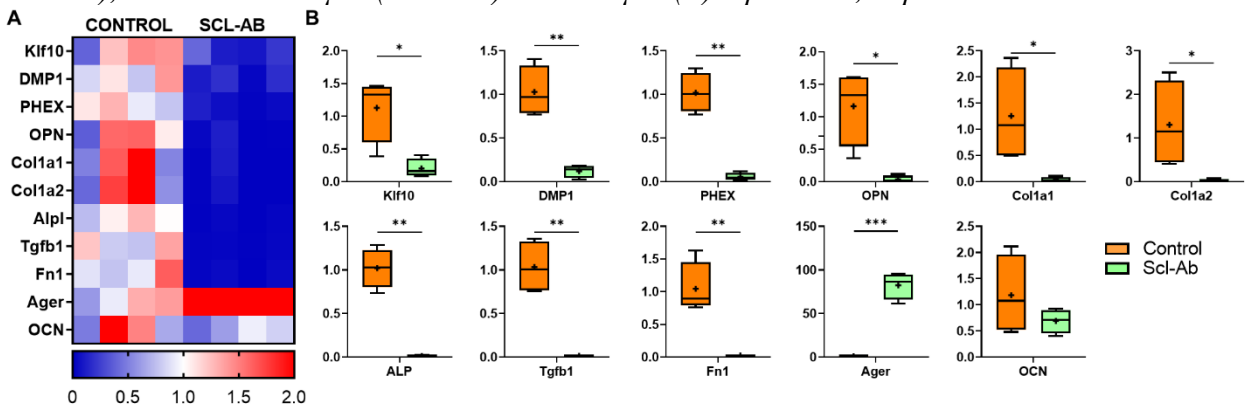


Figure 4: *Scl-Ab administration decreases gene expression related to anabolism and bone matrix formation. (A) A heat map displaying anabolic and bone matrix formation gene expression in cortical bone from OVX rats at week 14, comparing untreated controls to those treated with 2 mg/kg/month Scl-Ab starting 3 weeks post-OVX. (B) Boxplots indicate a significant decrease in markers of anabolism and bone matrix formation (OPN, ALP, DMP1,*

*PHEX) following Scl-Ab administration. n = 4 rats/group. *p < 0.05, **p < 0.01, ***p < 0.001.*

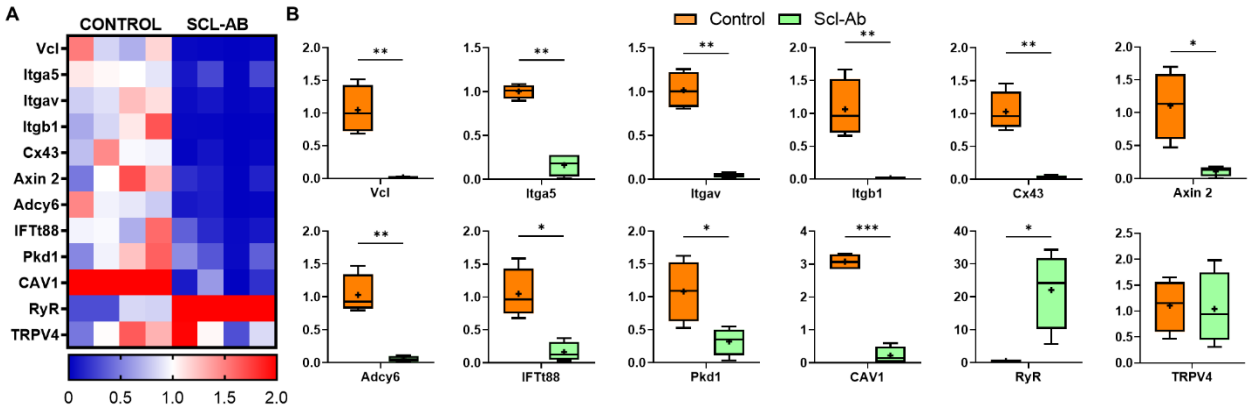


Figure 5 *Scl-Ab Administration Downregulates Gene Expression Related to Mechanotransduction and Mechanosensation (A) A heat map displaying gene expression in cortical bone from OVX rats at week 14, comparing untreated controls to those treated with 2 mg/kg/month Scl-Ab starting 3 weeks post-OVX. (B) Boxplots reveal a significant decrease in markers of mechanotransduction and mechanosensation following Scl-Ab administration. n = 4 rats/group. *p < 0.05, **p < 0.01, ***p < 0.001.*

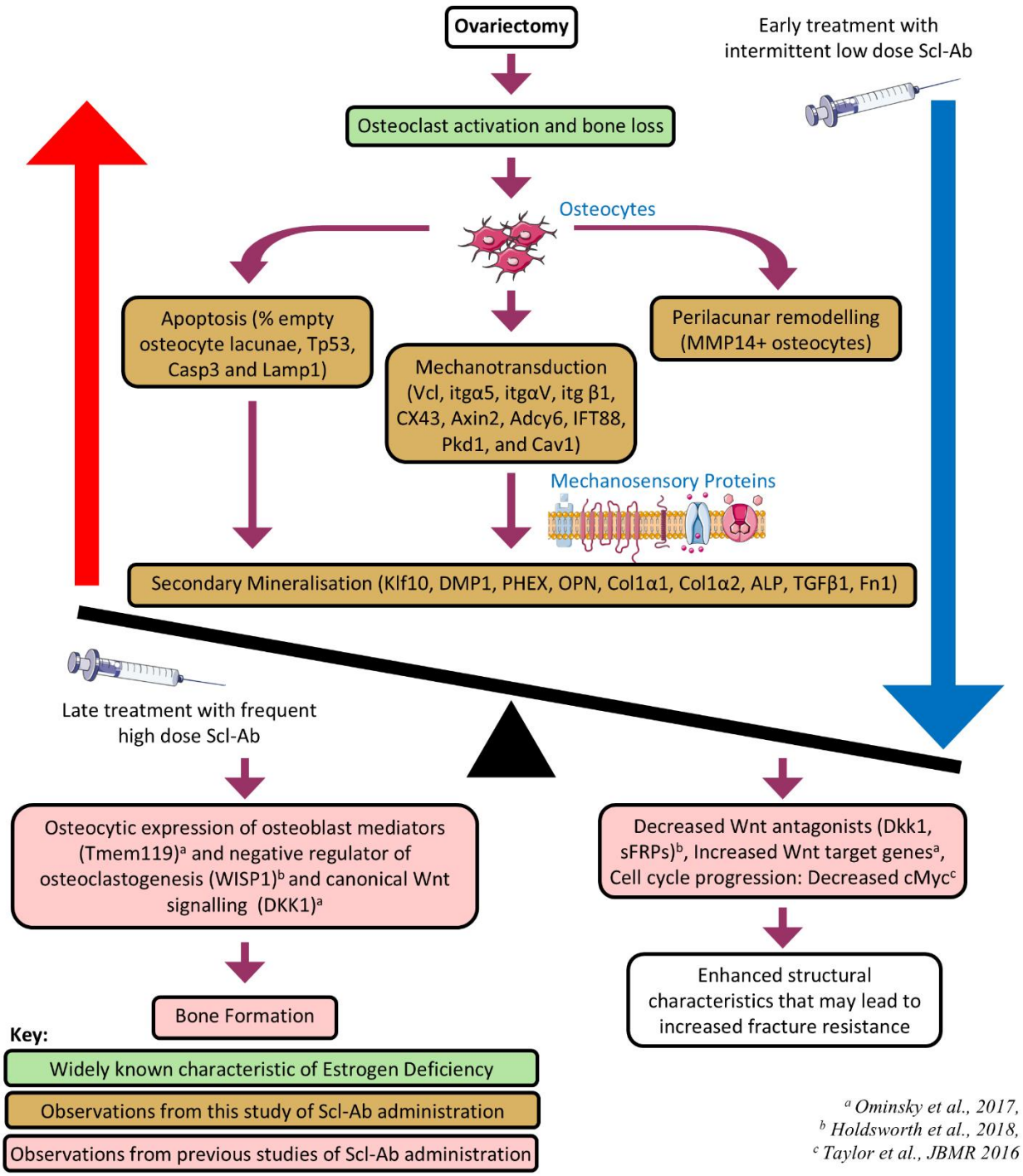
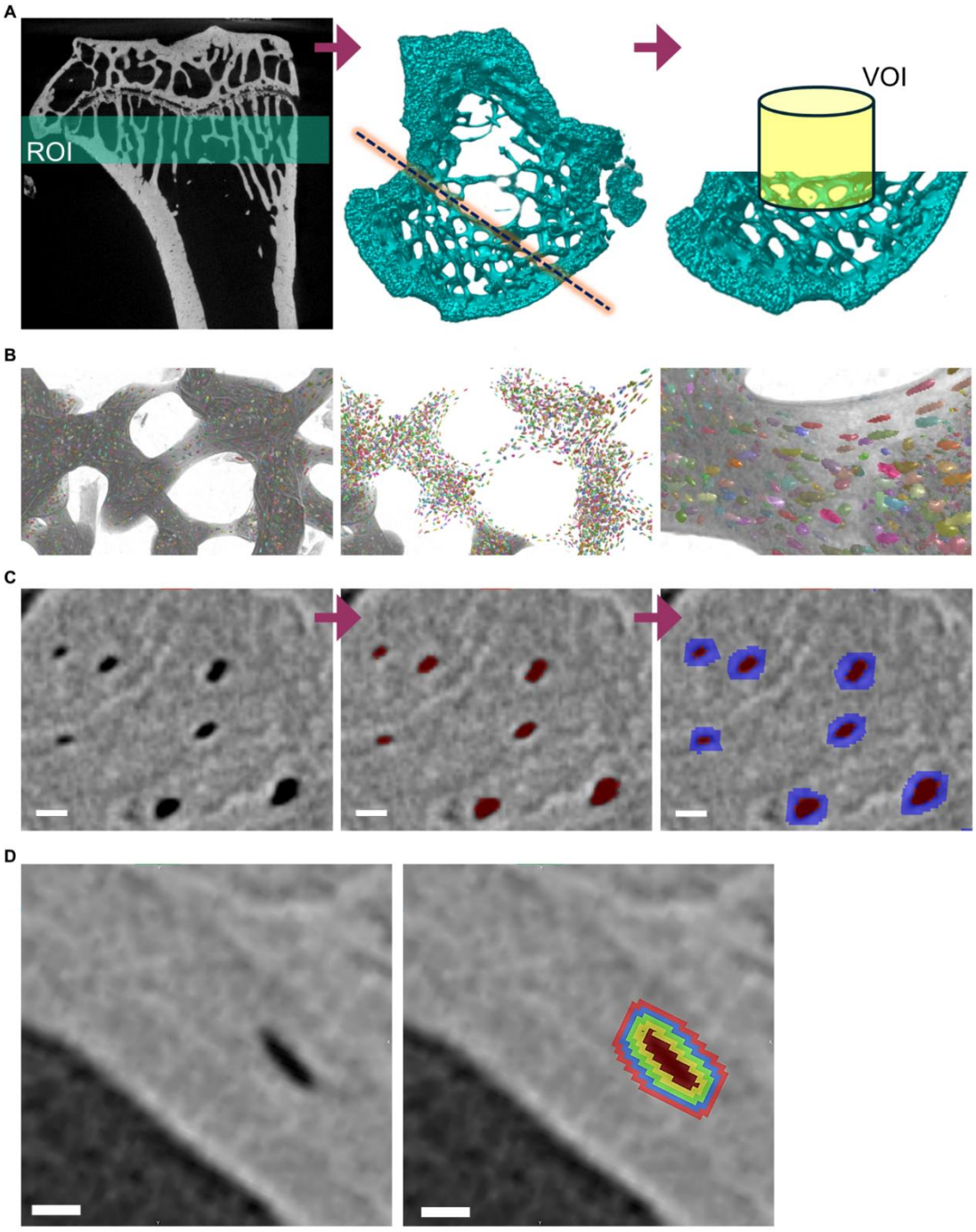


Figure 6: *Flowchart depicting the research from this study in the context of a proposed theory of the sequence of events during estrogen deficiency, with early administration of intermittent (monthly) low-dose Scl-Ab antibody and late administration of frequent (weekly) high-dose Scl-Ab.*



Supplementary Figure 1: *(A) Sample preparation for nano-CT imaging. A ~0.5 mm slice was extracted from the whole bone using a diamond blade. (B) A rendered VOI from a representative sample, with segmentation and labelling of individual trabecular structures. (C) Peri-lacunar mineral density assessment. Lacunae ROIs were dilated by 2 pixels, with the inner lacunar volume removed to isolate the peri-lacunar region for each lacuna. Lacunae are segmented in red, and peri-lacunar regions are segmented in blue. (D) Peri-lacunar mineral density variation. A similar strategy was used to analyse peri-lacunar mineral variation. New ROIs were defined by dilating the original ROI by 1 pixel at 1.1-micron spacing. Lacunae are segmented in red, with peri-lacunar regions visualized in sequentially expanded layers.*