Effect of Statins on Bone Mineral Density and Bone Histomorphometry in Rodents

Frans J. Maritz, Maria M. Conradie, Philippa A. Hulley, Razeen Gopal, Stephen Hough

Abstract—Statins have been postulated to affect bone metabolism. We investigated the effects of different doses of simvastatin (1, 5, 10, and 20 mg · kg⁻¹ · d⁻¹), atorvastatin (2.5 mg · kg⁻¹ · d⁻¹), and pravastatin (10 mg · kg⁻¹ · d⁻¹) administered orally for 12 weeks to intact female Sprague-Dawley rats and the effect of 20 mg · kg⁻¹ · d⁻¹ simvastatin in sham-operated and ovariectomized rats on femoral bone mineral density (BMD) and quantitative bone histomorphometry (QBH) and compared them with controls. BMD was decreased by 1 mg · kg⁻¹ · d⁻¹ simvastatin (P=0.042), atorvastatin (P=0.0002), and pravastatin (P=0.002). The effect on QBH parameters differed with different doses of simvastatin (ANOVA, P=0.00012). QBH parameters of both bone formation and resorption were equivalently and markedly increased by 20 mg · kg⁻¹ · d⁻¹ simvastatin in 2 separate groups of intact rats and were reflected by a relatively unchanged BMD. At lower doses, 1 mg · kg⁻¹ · d⁻¹ simvastatin decreased bone formation while increasing bone resorption, as reflected by a marked decrease in BMD. Ovariectomized animals receiving 20 mg · kg⁻¹ · d⁻¹ simvastatin showed no change in BMD relative to the untreated, ovariectomized controls; their increase in bone formation was smaller than in sham-operated rats receiving simvastatin, and there was no change in bone resorption. Dose-response curves of simvastatin for bone formation and resorption differed. These studies indicate that (1) statins decrease BMD in rodents, (2) high-dose simvastatin increases bone formation and resorption, (3) low-dose simvastatin decreases bone formation and increases bone resorption, (4) the effects of simvastatin on QBH differ at different dosages, (5) the effects of simvastatin seen in intact rats are not observed in ovariectomized rats, and (6) simvastatin is unable to prevent bone loss caused by ovariectomy. (Arterioscler Thromb Vasc Biol. 2001;21:1636-1641.)

Key Words: atorvastatin ■ bone histomorphometry ■ bone mineral density ■ pravastatin ■ simvastatin

The hydroxymethylglutaryl coenzyme A reductase inhibitors (statins) are widely used in the treatment of dyslipidemia in an age group that has an increased prevalence of osteoporosis. The statins, apart from reducing the intracellular cholesterol pool, also reduce other products of the mevalonate pathway, including the isoprenoids farnesyl diphosphate and geranylgeranyl diphosphate. Farnesyl diphosphate and geranylgeranyl diphosphate are attached to the carboxy terminal of numerous monomeric, small GTP-binding proteins to form cytosolic prenylated proteins. Prenylation is furthermore essential for the membrane localization and function of these prenylated proteins, including Rac and Rho. Rac and Rho are pivotal in mediating the cytoskeletal changes initiated by growth factors and integrins, leading to membrane ruffling, the formation of lamellipodia and stress fibers, and resulting in the activation of polarized and motile cells, including macrophages and osteoclasts. Alendronate, a nitrogen-containing bisphosphonate used in the treatment of osteoporosis, inhibits prenylation and thereby inhibits the osteoclast function. By reducing substrate availability, statins also inhibit prenylation and have been shown to inhibit osteoclastic bone resorption in a fashion similar to alendronate. In a study investigating the effect of lipid-clearing agents on bone, lovastatin and other lipid-lowering agents were able to prevent steroid-induced osteoporosis. It is uncertain whether prenylation plays a role in osteoblast function. However, recent animal studies have shown that statins also stimulate bone formation, which has raised the possibility that these drugs may be used as anabolic agents in the management of established osteoporosis.

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In the present study, we examined the effect of different dosages of simvastatin on bone mineral density (BMD) and quantitative bone histomorphometric (QBH) parameters of bone formation and bone resorption in intact female rats. In addition, the effect of simvastatin on ovariectomized rats and the effect of atorvastatin and pravastatin on intact rats were examined.
Methods

Three-month-old female Sprague-Dawley rats were acquired from the Animal Research Unit, Faculty of Health Sciences, University of Stellenbosch. The Ethics Committee and the Animal Research Committee, Faculty of Health Sciences, University of Stellenbosch, approved the treatment and study protocols. For all studies, 3-month-old female rats weighing ~250 g were obtained from similarly raised and weaned litters and housed, 5 rats per cage, in a light- (14 hours) and temperature- (23°C to 25°C) controlled environment in a pathogen-free room. The rats were allowed free access to water, were pair-fed, and were weighed weekly.

To examine the effect of different doses of simvastatin, 50 rats were randomly allocated to 5 groups. Four groups received the active drug simvastatin, in the form of 20 mg · kg⁻¹ · d⁻¹ (S20 group), 10 mg · kg⁻¹ · d⁻¹ (S10 group), 5 mg · kg⁻¹ · d⁻¹ (S5 group), or 1 mg · kg⁻¹ · d⁻¹ (S1 group) dissolved in vegetable oil as the vehicle and mixed with their feed; the fifth group served as a control and received the equivalent amount of vehicle as placebo. Drugs and placebo were administered for 12 weeks. The dosages of simvastatin were based on earlier safety and efficacy studies in rats, and these were similar to those used by other researchers who assessed the influence of statins on bone metabolism in rodents.

To examine the effect of alternative statins, 2 groups of 10 rats each were administered 2.5 mg · kg⁻¹ · d⁻¹ atorvastatin (A2.5 group) or 10 mg · kg⁻¹ · d⁻¹ pravastatin (P10 group) dissolved in vegetable oil as the vehicle and mixed with their feed; a third group served as a control and received the equivalent amount of vehicle as placebo. Drugs and placebo were continued for 12 weeks. The dosages of statins used were based on those that are clinically accepted as biologically equivalent doses in humans.

In an ovariectomy (OVX) model, 40 rats were randomly allocated to 4 groups of 10 rats each. Two weeks before administration of the study drugs, an OVX was performed under ether anesthesia on 2 groups, of which 1 group received 20 mg · kg⁻¹ · d⁻¹ simvastatin dissolved in vegetable oil as the vehicle that was mixed with their feed (OVX-S); an equivalent amount of vehicle was administered to the other group as a placebo (OVX). A sham (Sh) operation was performed under ether anesthesia on the remaining 2 groups, of which 1 group received 20 mg · kg⁻¹ · d⁻¹ simvastatin (Sh-S) and the other, placebo, as described above (Sh). The treatment was continued for 8 weeks.

In all groups of rats, 13 and 3 days before they were humanely killed, all animals received oxytetracycline hydrochloride (25 mg/kg IM). At the end of the study periods, the rats were humanely killed with an overdose of thiopental, and the tibias and femurs were harvested. In the OVX model, blood was drawn for rat follicle-stimulating hormone (rFSH) and estradiol estimations to confirm the estrus status of the animals. For bone densitometry, the femurs were preserved in 70% alcohol. For QBH, 1 tibia from each rat was removed, fixed in a modified Millonig’s solution (3.7% formaldehyde, 93 mmol/L NaH₂PO₄, 105 mmol/L NaOH, and 14.6 mmol/L sucrose) for 24 hours only, embedded in methylmethacrylate, sectioned at 5 μm, and stained by the Goldner technique. Histomorphometric analyses were performed by a single, experienced technician using a Merz-Schenk integrating eyepiece; the technician was blinded to the treatment group of the rats. Trabecular bone only was analyzed by not including sections within 2 fields (×250 magnification) of either the growth plate or the cortices. Particular care was taken to analyze this same standardized site in every animal. At least 120 fields per animal were counted. Time-spaced tetracycline labeling was assessed on unstained, 50-μm-thick sections. Histomorphometry terminology and calculations used are those described in the Report of the American Society for Bone and Mineral Research Committee on Histomorphometry Nomenclature.

For statistical analyses, the BMD and QBH parameters were analyzed by ANOVA. Differences between groups and comparisons with controls were analyzed with a post hoc analysis with Fisher’s protected least significant difference test. A correlation between different doses of simvastatin and QBH parameters of bone formation and resorption was examined by Pearson’s test.

Simvastatin (Zocor; Merck, Sharpe & Dohme), atorvastatin (Lipitor, Parke-Davis), and pravastatin (Pravachol, Bristol-Myers Squib) were obtained commercially. The rFSH assay system (Biotrak, rFSH [125I], code RPA550; Amersham Life Science) was obtained from AEC Amersham. The estradiol kit (estradiol double antibody) was supplied by Diagnostic Product Corp. The rat feeds (rat and mouse breeder feed, Animal Specialties [PTY] Ltd: phosphorus (minimum) 8 g/kg, calcium (maximum) 18 g/kg) were provided by the Animal Research Unit, Faculty of Health Sciences, University of Stellenbosch. Oxytetracycline hydrochloride (Terramycin 100; Pfizer Animal Health) was obtained commercially.

Results

Bone Densitometry

Administration of 2.5 mg · kg⁻¹ · d⁻¹ atorvastatin (P = 0.0002), 10 mg · kg⁻¹ · d⁻¹ pravastatin (P = 0.002), and 1 mg · kg⁻¹ · d⁻¹ simvastatin (P = 0.042) resulted in significant reductions in BMD when compared with control groups (Figures 1 and 2). There was a trend toward a decrease in BMD with decreasing doses of simvastatin (20, 10, 5, and 1 mg · kg⁻¹ · d⁻¹), and the decrease was the greatest with the smallest dose (Figure 2).

In the Sh-OVX model, OVX produced the expected marked reduction in BMD when compared with their Sh-operated controls (Figure 3) and thus, supports the validity of the model. The BMD data on rats in the Sh-OVX model obtained from the 2 different centers were similar and did not differ statistically. The addition of 20 mg · kg⁻¹ · d⁻¹ simvastatin to the Sh-S animals (Figure 3) and to the intact

Figure 1. Effect on BMD of atorvastatin and pravastatin. C indicates control; A2.5, atorvastatin at 2.5 mg · kg⁻¹ · d⁻¹; P10, pravastatin at 10 mg · kg⁻¹ · d⁻¹. Values are mean±SE. *Significant vs control.

Figure 2. Effect on BMD of different doses of simvastatin. C indicates control; S20, simvastatin at 20 mg · kg⁻¹ · d⁻¹; S10, simvastatin at 10 mg · kg⁻¹ · d⁻¹; S5, simvastatin at 5 mg · kg⁻¹ · d⁻¹; S1, simvastatin at 1 mg · kg⁻¹ · d⁻¹. Values are mean±SE. *Significant vs control.
rats (S20; Figure 2) produced similar and decreasing trends in BMD when compared with their respective controls, but neither reached statistical significance. The addition of simvastatin to the OVX group (OVX-S) produced no change or trend in the BMD when compared with their controls (OVX; Figure 3).

Quantitative Bone Histomorphometry

The different dosages of simvastatin had a significant overall effect on the QBH parameters (ANOVA, \( P = 0.00012 \)). The QBH parameters of bone formation and resorption were increased by 20 mg · kg\(^{-1} \cdot d^{-1} \) simvastatin (S20 group; Figure 4). This dose of simvastatin produced marked percent increases in osteoid volumes, osteoid surfaces, and osteoblast numbers when compared with the control group. A similar trend was observed in the rate of bone formation, although this did not reach statistical significance. Similar effects on bone formation were seen in the Sh-operated animals that also received 20 mg · kg\(^{-1} \cdot d^{-1} \) simvastatin (Sh-S group; Figure 5). In the group that received 20 mg · kg\(^{-1} \cdot d^{-1} \) simvastatin (S20), an increase in the QBH parameters of bone resorption was demonstrated (Figures 4 and 5), and eroded surfaces, as well as those occupied by osteoclasts, were significantly increased by simvastatin. These increases in bone resorption were again reflected in the Sh-operated rats that received 20 mg · kg\(^{-1} \cdot d^{-1} \) simvastatin (Sh-S; Figure 5).

Simvastatin at 20 mg · kg\(^{-1} \cdot d^{-1} \), a dosage that was used in 2 separate models, therefore caused a similar increase in the parameters of both bone formation and resorption, and the net effect of this is reflected in the minor change seen in BMD at this dosage (Figure 2).

These effects were not seen with lower doses of simvastatin, which caused smaller and opposing effects on both bone formation and bone resorption (Figure 4). Simvastatin at 1 mg · kg\(^{-1} \cdot d^{-1} \) decreased the parameters of bone formation while simultaneously increasing the parameters of bone resorption (Figure 4). The net effect of these changes caused by 1 mg · kg\(^{-1} \cdot d^{-1} \) simvastatin are reflected in the significant change in BMD at this dose. All of the parameters of bone formation changed in parallel at the different dosages of simvastatin, as did the parameters of bone resorption (Figure 4). A significant correlation between simvastatin dose and QBH parameters of bone formation and resorption was demonstrated (osteoid volume/bone volume, \( r = 0.369, P = 0.0208 \); osteoid volume/total volume, \( r = 0.449, P = 0.004 \); osteoid surface, \( r = 0.403, P = 0.011 \); osteoblast surface, \( r = 0.490, P = 0.001 \); bone formation rate, \( r = 0.445, P = 0.004 \); eroded surfaces, \( r = 0.438, P = 0.005 \); and osteoclast surface, \( r = 0.362, P = 0.023 \)). Furthermore, it is evident that the dose-response curves for the histomorphometric parameters of bone formation and resorption are not the same (Figure 4).

OVX, as expected, resulted in a significant decrease in bone volume and significant increases in bone resorption and formation when compared with the untreated Sh-operated group. The addition of 20 mg · kg\(^{-1} \cdot d^{-1} \) simvastatin to the Sh-operated group (Sh-S) resulted in QBH changes similar to those seen in the S20 group (Figures 4 and 5). However, equivalent changes were not seen in the OVX animals. The addition of 20 mg · kg\(^{-1} \cdot d^{-1} \) simvastatin to the OVX group (OVX-S) resulted in smaller percent increases in the parameters of bone formation that were not significant, and no effect was observed on the parameters of bone resorption (Figure 6). These effects are furthermore reflected in the lack of any effect on BMD by treatment with simvastatin in the OVX groups (Figure 3). Furthermore, it is evident that
simvastatin was unable to prevent the loss of BMD in the OVX group.

In the Sh and Sh-S groups, the rFSH levels were 0.6±0.07 and 0.51±0.05 ng/mL, respectively, and the estrogen levels were 63±17.22 and 52.3±14.41 pmol/L, respectively. In the OVX animals, the rFSH level increased in the OVX and OVX-S groups (6.5±0.44 and 5.46±0.25 ng/mL, respectively), and the estrogen level decreased (15.4±2.36 and 11.81±1.68 pmol/L, respectively), indicating that the OVX had been successful. The weight of the rats increased in all groups by a mean of 22.2 g, and the weight gain per group did not differ statistically between groups. One rat from the 1 mg·kg⁻¹·d⁻¹ simvastatin group died after 9 weeks of unknown causes. No other illnesses occurred in the remaining rats.

Discussion
There are sound reasons to believe that statins may have a beneficial effect on bone health. Existing data demonstrate that prenylation is important in osteoclast function and that inhibition of prenylation impairs this function. The target of the nitrogen-containing bisphosphonates is the osteoclast, where they cause apoptosis by Mst1 cleavage and the activation of caspases, effects that can be mimicked by statins. It might therefore be surmised that statins could have an effect on bone similar to alendronate. Further support of a beneficial effect of statins on bone comes from data demonstrating that statins increase bone formation. Mundy et al showed that statins are able to increase bone formation in neonatal murine calvarial bones in organ culture, as well as when injected into the subcutaneous tissue overlaying the murine calvaria. Furthermore, the effect of systemic administration of statins was investigated in OVX and intact rats as measured by histomorphometric parameters. Simvastatin in dosages ranging from 1 to 10 mg·kg⁻¹·d⁻¹ increased bone formation and trabecular bone. Others have since confirmed the induction of BMP-2 by some statins. Those authors concluded that the effect of statins was predominantly on bone formation. In vivo, lovastatin has also been shown to counteract the inhibitory effect of steroids on osteoblastic gene expression and consequently, to inhibit steroid-induced osteonecrosis and steroid-induced osteoporosis.

The present study examined the effects of statins in intact and OVX female rats. Employing dual-energy x-ray absorptiometry, we showed that chronic (12 weeks) administration of atorvastatin (2.5 mg·kg⁻¹·d⁻¹), pravastatin (10 mg·kg⁻¹·d⁻¹), and simvastatin (1 mg·kg⁻¹·d⁻¹) resulted in a significant reduction in femoral BMD. Measurements of BMD of the same specimens performed at 2 different centers were found to be identical. The changes in BMD of the rats treated with 20 mg·kg⁻¹·d⁻¹ simvastatin in the simvastatin dosage study (S20) and the Sh-operated rats (Sh-S) in the OVX model that also received 20 mg·kg⁻¹·d⁻¹ simvastatin were similar. Moreover, the expected decline in BMD in OVX animals was demonstrated. The average weights of the animal groups at the start of the study were comparable, and the weight gain for any particular group was not significantly different from the others. Differences in body and skeletal size could therefore not explain our observation that statins caused a decrease in BMD. There is also no reason to believe that the statins used in our study caused osteomalacia or an increase in bone marrow fat, known to result in an underestimation of BMD. Our data showing that the administration of statins is associated with a reduced BMD therefore seem convincing.

By using bone histomorphometry, the present study showed that OVX resulted in a significant decrease in bone turnover at lower doses differed from that seen at higher doses. With different dosages of simvastatin, an inverse correlation is suggested between dose and decrease in BMD. The reason(s) why a lower simvastatin dose decreased BMD more than a higher dose remains speculative but suggests that 2 processes are operative in bone remodeling with differing dose-response curves. In fact, BMD as measured at the end of a 12-week study reflects the net balance of drug effects on bone formation.

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By using bone histomorphometry, the present study showed that OVX resulted in the expected increase in bone turnover and a significant decrease in bone volume associated with the observed decrease in BMD in the OVX group compared with the Sh-operated control group, further validating the model. Simvastatin at 20 mg·kg⁻¹·d⁻¹ increased static histomorphometric parameters of bone formation in the S20 group as well as in the Sh-operated rats (Sh-S). These results are in agreement with and support the findings of Mundy et al.

With the use of time-spaced tetracycline labeling, no significant increase in the rate of bone formation could be demonstrated in our simvastatin-treated rats. In addition, our data show an increase in the parameters of bone resorption in the 20 mg·kg⁻¹·d⁻¹ simvastatin–treated rats in the S20 and Sh-S groups. However, the effects of simvastatin on bone turnover at lower doses differed from that seen at higher doses. With 1 mg·kg⁻¹·d⁻¹ simvastatin, the parameters of bone formation were decreased, and bone resorption increased. Furthermore, it is evident that the dose-response curves of the parameters of bone formation differ from those of the parameters of bone resorption and were statistically validated.

With different dosages of simvastatin, an inverse correlation is suggested between dose and decrease in BMD. The reason(s) why a lower simvastatin dose decreased BMD more than a higher dose remains speculative but suggests that 2 processes are operative in bone remodeling with differing dose-response curves. In fact, BMD as measured at the end of a 12-week study reflects the net balance of drug effects on both osteoclastic bone resorption and osteoblastic bone formation. It is known that different bone marrow cells differ in their sensitivity to statins like lovastatin, and we hypothesize that osteoblasts and osteoclasts may also differ in their sensitivity to statins like lovastatin, and consequently, to inhibit steroid-induced osteonecrosis and steroid-induced osteoporosis.

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With different dosages of simvastatin, an inverse correlation is suggested between dose and decrease in BMD. The reason(s) why a lower simvastatin dose decreased BMD more than a higher dose remains speculative but suggests that 2 processes are operative in bone remodeling with differing dose-response curves. In fact, BMD as measured at the end of a 12-week study reflects the net balance of drug effects on both osteoclastic bone resorption and osteoblastic bone formation. It is known that different bone marrow cells differ in their sensitivity to statins like lovastatin, and we hypothesize that osteoblasts and osteoclasts may also differ in their sensitivity to statins like lovastatin, and consequently, to inhibit steroid-induced osteonecrosis and steroid-induced osteoporosis.
sensitivity to these agents. If osteoclasts were more sensitive to statins than osteoblasts, bone resorption would predominately occur at lower statin doses, resulting in a decreased BMD. At higher doses, osteoblasts may now be preferentially stimulated, resulting in a normal or increased BMD. Indeed, our data support this hypothesis: simvastatin at 20 mg · kg⁻¹ · d⁻¹ equally increased bone formation and resorption with little net change in BMD, whereas simvastatin at 1 mg · kg⁻¹ · d⁻¹ decreased bone formation while stimulating bone resorption, with a resultant, marked net decrease in BMD. Differing dose-response curves for bone formation and resorption were also demonstrated.

Our study did not attempt to compare the effect of different statins on bone metabolism. However, marked reductions in BMD were noted after treatment with atorvastatin and pravastatin. There are sound reasons to believe that various statins might have differing effects on BMD. Atorvastatin has a long half-life compared with other statins, and this results in continuously increased drug levels with atorvastatin versus intermittently increased levels with other statins. Similar differences in the effect on BMD between continuous treatment and intermittent treatment have been demonstrated for parathyroid hormone. The hydrophilic pravastatin has a lower first-pass extraction compared with other statins, and the amount of statin reaching the systemic circulation may be important for its effect. Differing effects of pravastatin, compared with other statins, on vascular smooth muscle cells have been demonstrated, and pravastatin was shown not to increase BMP-2, whereas compactin and simvastatin did increase this expression. Differences in the effect on bone between fluvastatin and pravastatin have been suggested in humans. The seemingly different effects between statins in our study are therefore not entirely unexpected.

In our study, the effect of 20 mg · kg⁻¹ · d⁻¹ simvastatin on the histomorphometric parameters of bone turnover in OVX animals differred from that seen in the Sh-operated group. Simvastatin had a small and variable effect on the parameters of bone formation in the OVX animals, whereas there was no effect on bone resorption. This finding contrasted with the increase in both formative and resorptive parameters seen in the Sh-operated rats after treatment with the same dose of simvastatin. The reason for this is unclear but may suggest a permissive effect for estrogens in the action of statins on bone.

Previously published in vitro data suggest that if statins, like the bisphosphonates, inhibit prenylation, then osteoclast function would be expected to be impaired by these agents. Although smaller doses of simvastatin did decrease parameters of bone resorption, our findings of increased bone resorption with 20 mg · kg⁻¹ · d⁻¹ simvastatin suggest that these agents may have additional in vivo effects other than the inhibition of prenylation observed in vitro and may also explain the differing effects seen with different doses of simvastatin. A bisphosphonate has also been shown to have varying effects on osteoclast function that appear dependent on dosage. EB-1053 clearly inhibited osteoclast function but at very small doses increased osteoclast function.

Differences in statins, dosages, methods of administration, duration of exposure, experimental animal model (ie, bone cell sensitivities to these drugs), and/or experimental design may explain some of the contrasting results obtained in our study and those of Mundy et al. Those authors used Swiss ICR mice for their studies, whereas we used Sprague-Dawley rats in our in vivo experiments. Rats in our study were OVX at 3 months of age and subjected to 12 weeks of exposure to statins, whereas an OVX was performed at 1 month of age and statins were administered for 8 weeks in the other studies. The bioavailability of the statin with our method of administration, the amount reaching the systemic circulation, and hence, the blood-bone interface might differ from that used by Mundy et al. and because dosage possibly plays a role in the effect of statins, this might also explain some of the differences observed.

Recent data indicate that the use of statins in humans may be associated with an increased BMD and reduced fracture rate. Others have been unable to confirm these findings, and other pleiotropic effects of statins may be operative, including the effect of statins on QT dispersion and reduction of arhythmias with a consequent reduction in fall rates. Clearly, the last word has not yet been written regarding the effect of statins on bone health.

As this study and others have shown, there can be little doubt that statins have a profound effect on bone metabolism, at least in rodents. The statins are potent drugs, which not only lower serum cholesterol but also affect an indispensable prenylation pathway and have other pleiotropic effects with far-reaching consequences, including those affecting the arterial wall. These drugs are widely and increasingly used in a population in which osteoporosis is of concern. We support the finding of others that bone formation may be increased, but we also provide evidence to support an increase in bone resorption, differing effects on formation and resorption at different dosages, and especially with long-term use, a decrease in BMD in rats exposed to these agents. This should sound a note of caution before an overall beneficial effect of currently available statins on bone health is accepted, and further studies are required to clarify these issues.

References


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