The effect of dried plum on serum levels of receptor activator of NF-κB ligand, osteoprotegerin and sclerostin in osteopenic postmenopausal women: a randomised controlled trial

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Abstract

Although several studies have confirmed the bone-protective properties of dried plum, its exact mechanisms of action remain unclear. Recent research has shown that osteocytes may control bone formation via the production of sclerostin and bone resorption via the receptor activator of NF-κB ligand (RANKL) and its inhibitor osteoprotegerin (OPG). To investigate the mechanism of action of dried plum in reversing bone loss, we measured serum levels of RANKL, OPG and sclerostin in osteopenic postmenopausal women (*n* 160). Participants were randomly assigned to the treatment group of either 100 g dried plum/d or 75 g dried apple/d (comparative control) for 1 year. All participants received 500 mg Ca plus 400 IU (10 μg) vitamin D daily. Bone mineral densities (BMD) of the lumbar spine, forearm, hip and whole body were assessed at baseline and at the end of the study using dual-energy X-ray absorptiometry. Blood samples were collected at baseline and after 12 months to assess bone biomarkers. Dried plum significantly increased the BMD of the ulna and spine in comparison with the control group. In comparison with corresponding baseline values, dried plum increased the RANKL levels by only $+1.99$ *v.* $+18.33\%$ and increased the OPG levels by $+4.87$ *v.* -2.15% in the control group. Serum sclerostin levels were reduced by -1.12% in the dried plum group *v.* $+3.78\%$ in the control group. Although percentage changes did not reach statistical significance ($P \le 0.05$), these preliminary data may indicate that the positive effects of dried plum on bone are in part due to the suppression of RANKL production, the promotion of OPG and the inhibition of sclerostin.

Key words: Postmenopausal women: Osteoporosis: Dried plum: Receptor activator of NF-κB ligand: Osteoprotegerin: Sclerostin

Osteoporosis is a major health concern for postmenopausal women. Oestrogen reduction following menopause leads to enhanced osteoclast activity and decreased osteoblast activity resulting in bone loss[1]. Compliance with osteoporosis medications reduces the risk of fracture[2]; however, adherence is suboptimal[3,4]. Natural food products that promote bone health may be cost-effective alternatives to pharmacological treatment that could improve compliance.

Among fruits and vegetables tested, dried plum (DP) has shown promising results in the prevention and reversal of bone loss. Deyhim *et al.*[5] demonstrated that a diet with 5% of DP by weight could restore bone mass in ovariectomised female rats. When Bu *et al.*[6] compared supplementation with DP to treatment with parathyroid hormone in male rats, similar improvements in bone microarchitecture and

bone mineral density (BMD) were observed in both groups. Additionally, the bone-protective effects of DP are not limited to hormone deficiency-induced bone loss; one animal study has shown that DP (25% of diet) could also reduce bone loss due to ageing[7]. In two short- and long-term clinical trials, the effects of DP on bone biomarkers and BMD were measured in postmenopausal women. Arjmandi *et al.*[8] found that supplementation with 100 g DP over 3 months improved the markers of bone formation, and Hooshmand *et al.*[9] showed that supplementation with 100 g DP over 1 year significantly improved the BMD of the ulna and spine compared with 75 g dried apple used as a comparative control.

Various biomarkers have been measured to clarify the mechanism of action of DP in bone health. Studies have

shown elevations in the levels of bone-specific alkaline phosphatase[8,10] and insulin-like growth factor-I[5,8,10], as well as reductions in the levels of urinary deoxypyridinoline[5,6,10] and tartrate-resistant acid phosphatase-5b (TRAP-5b)[9], suggesting that DP influences bone formation and resorption. Although several bone biomarkers have been assessed, its exact mechanism of action remains unclear.

Bone remodelling is a balance between bone resorption coupled with bone formation due to the activity of osteoblasts and osteoclasts. It is now understood that osteocytes can regulate this process by controlling osteoblast and osteoclast functioning through the production of the receptor activator of NF-κB ligand (RANKL) and sclerostin[11,12]. The RANKL is a component of the RANKL/receptor activator of NF-κB (RANK)/osteoprotegerin (OPG) axis that has been identified as the primary modulator of bone resorption. The RANK is a receptor found on osteoclast precursors, and the RANKL binds with the RANK to induce osteoclast formation and activation[13,14]. OPG inhibits this process by binding with the RANKL before it can interact with the RANK to stimulate osteoclastogenesis[13,14]. The RANKL:OPG ratio is considered as a marker of bone resorption[14], and Jabbar *et al.*[15] demonstrated that elevated levels of the RANKL, OPG and RANKL:OPG ratio were associated with lower BMD in postmenopausal women. To date, only one animal study[10] has measured the effects of DP treatment on the RANKL/RANK/OPG axis. Franklin *et al.*[10] found that medium (15%) and high (25%) doses of DP reduced the gene expression levels of RANKL and OPG in bone, which decreased the RANKL:OPG ratio. However, these regulatory markers and ratios have not been evaluated in a clinical study involving DP supplementation.

Sclerostin, a product of the sclerostin (*SOST*) gene, is also produced by osteocytes to alter the rate of bone remodelling[11]. Sclerostin interferes with canonical Wnt signalling, which results in reduced bone formation by the suppression of osteoblast activation and the promotion of apoptosis[16]. To date, there have been no studies analysing the effects of DP on the biomarker sclerostin.

To better understand the mechanism of action of DP in reversing bone loss, the present study measured the effect of daily consumption of DP on bone biomarkers in postmenopausal women. The primary aim of the present study was to assess the effect of DP on bone metabolism by measuring the serum levels of RANKL and OPG, and to measure the effect on the circulating levels of sclerostin.

Experimental methods

Participants

A total of 236 women who were 1–10 years postmenopausal were recruited. Of the volunteers, 160 women, with mild bone loss (those with a BMD *t*-score >2·5 SD were excluded), met inclusion and exclusion criteria, and were randomised to either the treatment (100 g DP/d; California Dried Plum Board) or control (75 g dried apple/d, Atwater Foods; LLC) group and were provided with 500 mg Ca plus 400 IU (10 μg) vitamin D

daily for 1 year. Women taking hormone replacement therapy, bisphosphonates, calcitonin, raloxifene and anabolic drugs within 3 months before the study were excluded from participating. Any other medications or supplements that could influence bone mass were also not permitted. Women with chronic diseases of the bone, liver, gastrointestinal tract, respiratory tract, kidneys or cardiovascular system, as well as those with diabetes or cancer were excluded from the study. Finally, women who drank prune juice or ate DP on a daily basis or who smoked more than twenty cigarettes/d were not included. The present study was conducted according to the guidelines laid down in the Declaration of Helsinki, and all procedures involving human subjects were approved by the Institutional Review Board at San Diego State University. Written informed consent was obtained from all subjects.

Anthropometric data

Anthropometric data, medical history, dietary intakes and physical activity patterns were measured at baseline, 3 months, 6 months and at the end of the study. The details of these measurements have been described elsewhere[9].

Bone mineral density measurements and fracture risk

BMD of the ulna, spine, hip and whole body were measured by dual-energy X-ray absorptiometry (GE Healthcare Lunar). Before data collection, the equipment was calibrated according to the manufacturer's instructions. Measurements were taken at baseline and after 12 months. The 10-year risk of hip and other major osteoporotic fractures was calculated using the Fracture Risk Assessment Tool (FRAX®)[17].

Laboratory analysis

Venous blood samples were obtained from the participants following an overnight fast. Serum was separated by centrifugation at 3500 **g** for 15 min at 4°C and then stored at −80°C. All biomarkers were assessed using commercially available ELISA kits. Baseline levels of TRAP-5b, bone-specific alkaline phosphatase, deoxypyridinoline and osteocalcin were measured with ELISA kits from Quidel Biosystems. Intra-assay (within-run) and inter-assay (between-run) coefficients of variation were 1·9–2·2 and 2·0–3·0% for TRAP-5b, 4·0–6·0 and 5·0–8·0% for bone-specific alkaline phosphatase, 5·0–12·0 and 10–17% for deoxypyridinoline and 5·0–10·0 and 5·0–10·0% for osteocalcin.

Baseline and 12-month samples were analysed for changes in the levels of RANKL, OPG and sclerostin. Serum total RANKL level was measured with an ELISA kit from ALPCO Diagnostics (intra-assay CV 0·9–3·5%; inter-assay CV 7·1–9·3%). Total OPG and sclerostin levels were measured using ELISA kits from Quidel Corporation. Intra-assay and inter-assay coefficients of variation were 2·1–3·5 and 4·2–6·1% for OPG and 1·3–1·6 and 1·8–2·7% for sclerostin.

Table 1. Baseline characteristics of the study participants

(Mean values and standard deviations)

	Dried apple (n 45)		Dried plum (n 55)		
	Mean	SD	Mean	SD	P
Age (years)	55·43	5·18	57·72	4·08	0·02
Age since menopause (years)	5·77	3·69	6·15	3·55	0·67
BMI (kg/cm^2)	24·18	3·70	24·79	4·69	0·48
Spinal BMD (g/cm^2)	0·99	0·10	0·93	0·09	0·01
Ulna BMD (g/cm^2)	0·45	0·05	0·43	0·05	0·04
Total hip BMD (g/cm^2)	0·88	0·10	0·85	0·11	0·09
Whole-body BMD (g/cm^2)	1·09	0·09	1·05	0·09	0·07
RANKL (pmol/l)	3770	3874	4814	4100	0·26
OPG (pmol/l)	5·65	1·27	5·61	1·52	0·88
Sclerostin (ng/ml)	0·63	0·24	0·61	0·20	0·63
Osteocalcin (ng/ml)	16·10	4·58	15·87	4·84	0·82
TRAP-5b (U/l)	3·79	1·23	3·68	1·51	0·70
BALP (U/l)	16·39	4·63	16·74	5·32	0·74
DPD (nmol/l)	7·07	2·45	8·75	7·98	0·22
P (mmol/l)	3·98	0·39	4·02	0·34	0·61
Ca (mmol/l)	10·04	0·40	10·11	4·52	0·43
10-year risk of fracture (%)	9·72	3·13	10·87	3·17	0·12
10-year risk of hip fracture (%)	1·69	0·93	2·00	1·35	0·26

BMD, bone mineral density; RANKL, receptor activator of NF-κB ligand; OPG, osteoprotegerin; TRAP-5b, tartrate-resistant acid phosphatase-5b; BALP, bone-specific alkaline phosphatase; DPD, deoxypyridinoline.

Statistical analysis

Statistical analysis of data was conducted using SPSS for Microsoft Windows (version 21.0). ANOVA was used to assess the main effects of treatment group and time, as well as the interaction effects. The data were assessed with and without outliers. When appropriate, values >3·3 SD units from the mean were excluded from the analysis. However, if the removal of outliers did not affect the statistical results, they were included in the final analysis. Unless otherwise specified, results are expressed as means and standard deviations. An α-level of <0·05 was considered to be statistically significant.

Results

Baseline characteristic data of the participants in the dried apple and DP groups are presented in Table 1. The mean age was significantly higher in the DP group; however, age since menopause was similar between the two groups. In women, most bone loss occurs within the first 10 years after menopause[18]; therefore, for the present study, we recruited women who were within 10 years of menopause and not necessarily within a certain age range. There were no significant differences in baseline BMI, biochemical markers, 10-year fracture risk and BMD of the hip or whole body between the two groups. However, spinal and ulna BMD were slightly higher in the dried apple group at baseline.

Attrition rates were not significantly different between the two groups (37·5 %). Overall, the dried fruit regimens were well accepted and considered to be palatable. Compliance to the dried fruit regimens was on average 82 % in each group. BMD increased in both groups receiving the dried fruits, yet the participants in the DP group experienced greater increases in the BMD of the ulna and spine compared with those in the dried apple group. The results are displayed in Fig. 1.

Serum levels of RANKL, a promoter of osteoclastogenesis, increased by 18·33 % in those consuming dried apple and only by 1·99 % in those consuming DP (Fig. 2). However, the mean difference between the groups was not statistically different after 12 months (Table 2). OPG, a decoy receptor for the RANKL, decreased in the dried apple group by 2·15 % and increased in the DP group by 4·87 %, although changes were not statistically significant (Fig. 2; Table 2). The RANKL:OPG ratio increased in the dried apple group
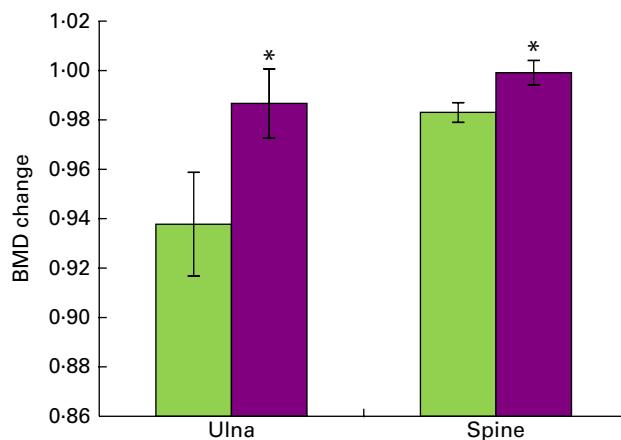
Fig. 1. Bone mineral density (BMD) changes from baseline in the ulna and spine after a 1-year consumption of dried apple (■) or dried plum (■). Values are means, with standard deviations represented by vertical bars. * Mean value was significantly different from that of the dried apple group (P<0·05). A colour version of this figure can be found online at http://www.journals.cambridge.org/bjn
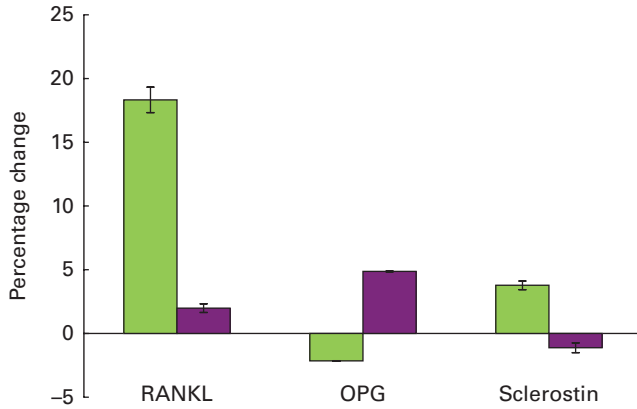
Fig. 2. Percentage changes from baseline in the serum levels of receptor activator of NF-κB ligand (RANKL), osteoprotegerin (OPG) and sclerostin after a 1-year consumption of dried apple (■) or dried plum (■). Values are means, with standard deviations represented by vertical bars. A colour version of this figure can be found online at http://www.journals.cambridge.org/bjn

and decreased in the DP group, but significant differences were not found. Sclerostin levels decreased in the DP group, but the levels were not significantly different from the dried apple group (Table 2).

Discussion

Studies conducted in hormone-deficient rats have demonstrated that DP can prevent and even restore bone loss due to hormone deficiency, as well as improve bone microarchitecture[5,6,10,19–21]. As described in the present study and elsewhere[8,9], DP also demonstrates bone-protective effects in postmenopausal women. Studies of bone biomarkers have shown that DP may influence the activity of both osteoblasts and osteoclasts by modifying bone formation[5,8,10,21] as well as bone resorption[10,21], yet the exact mechanism of action remains to be clarified.

We postulated that modifications of the RANKL/RANK/OPG pathway may explain the mechanism of action of DP in bone health. In the present study, serum levels of OPG decreased by 2·15 % in the dried apple group and increased by 4·87 % in the DP group. Total RANKL levels increased by 18·33 % in those consuming dried apple and by 1·99 % in those consuming DP. However, the levels were not significantly different from baseline. In an earlier animal study that assessed the effect of DP on the RANKL/OPG pathway, mRNA levels of RANKL and OPG were measured in orchidectomised male rats supplemented with DP[10]. The study showed that medium and high doses of DP significantly reduced the gene expression levels of both RANKL and OPG and led to a non-significant reduction in the RANKL:OPG ratio. This suggests that DP has an impact on bone turnover by downregulating the expression levels of RANKL and OPG. Similar reductions in the RANKL:OPG ratio were demonstrated in the present study, although changes did not reach a significant level. This could be in part due to the measuring of serum levels of RANKL and OPG in the present study, whereas Franklin *et al.*[10] measured gene expression in bone. Unfortunately, serum levels are difficult to interpret due to the limitations of available ELISA kits[14,15] and conflicting results among studies because of the complexity of the relationship between RANKL, OPG and bone metabolism[22]. Many different cell types express RANKL and OPG, and how circulating levels reflect activity in the bone microenvironment is uncertain[23]. Findlay *et al.*[24] found that gene expression of RANKL in bone, as measured by RT-PCR, correlated with the measurements of BMD, yet serum levels were inversely related. This was only true for the male population as the relationship was not seen in the female participants. Overall, mRNA levels of RANKL may better reflect RANKL activity in bone; unfortunately, due to the invasive nature of measuring bone mRNA, we chose to measure serum levels in the present study.

Furthermore, serum RANKL exists in a free form and in a bound form with OPG[22]. We measured total RANKL levels because only a small portion of circulating RANKL is unbound and the levels can be so minute that available ELISA kits are unable to detect them[22,25]. Total RANKL levels are in higher quantities in the circulation and easier to measure. Unfortunately, interpretation of free *v.* total RANKL is poorly

Table 2. Effect of dried apple and dried plum on bone biomarkers

(Mean values and standard deviations)

| | Dried apple | | | | Dried plum | | | | |
| | Baseline | | 12 months | | Baseline | | 12 months | | |
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	P
RANKL (pmol/l)	3770	3874	4080	3927	4814	4100	4857	4101	0·117
P			0·204				0·382		
OPG (pmol/l)	5·65	1·27	5·49	1·23	5·61	1·52	5·66	1·24	0·342
P			0·124				0·765		
RANKL:OPG ratio	727	784	762	812	911	789	906	787	0·500
P			0·431				0·896		
Sclerostin (ng/ml)	0·63	0·23	0·62	0·25	0·61	0·20	0·56	0·19	0·456
P			0·797				0·133		

RANKL, receptor activator of NF-κB ligand; OPG, osteoprotegerin.

understood[22]. In the present study, there was large variability of total RANKL levels among postmenopausal women, inhibiting our ability to find statistical significance. These results are similar to those reported by Reyes-Garcia et al.[26] and Findlay et al.[24] who found high levels of variability in total RANKL levels among postmenopausal women treated with alendronate and in osteoarthritic males.

We measured the effect of DP on osteocyte activity by measuring the levels of sclerostin. To our knowledge, the present study was the first to evaluate this relationship. Sclerostin inhibits bone formation by interfering with the Wnt-signalling pathway and preventing osteoblast activity[27]. After menopause, sclerostin levels increase, suggesting that postmenopausal bone loss is in part due to a lessening in osteoblastogenesis[28]. Alternatively, reductions in sclerostin levels by alterations in the SOST gene can increase bone formation and lead to sclerosteosis and van Buchem disease, two conditions characterised by high BMD[29,30]. Therefore, osteoporosis therapies that can regulate sclerostin expression have the potential to improve BMD. In the present study, DP reduced sclerostin levels by 1·1%, while dried apple increased the levels by 3·8%. Further research with a larger sample size may help to explain whether the positive effects of DP on BMD are in part due to the decreased levels of sclerostin.

The present study had several limitations. There was a small sample size of participants in each group, and we only measured the levels of OPG, RANKL and sclerostin at two time points during the study (baseline and 12 months). Assessment of serum biomarkers at 3-month intervals throughout the experimental period would have provided a more detailed and precise view of what changes were taking place over time. In addition, as we mentioned previously, bone biopsy gives a more accurate view of changes in the expression levels of RANKL and OPG. Future research may benefit from trying to measure bone levels of these biomarkers as opposed to serum levels alone.

In conclusion, although the present study showed a possible role for the OPG, RANKL and sclerostin pathways in increasing BMD in the DP group, these changes in serum biomarkers did not reach a significant level. Further mechanistic studies are needed to evaluate more closely the role of DP treatment on these pathways.

Acknowledgements

References

1. Weitzmann MN & Pacifici R (2006) Estrogen deficiency and bone loss: an inflammatory tale. J Clin Investig 116, 1186–1194.
2. Weycker D, Macarios D, Edelsberg J, et al. (2007) Compliance with osteoporosis drug therapy and risk of fracture. Osteoporos Int 18, 271–277.
3. Gogakos AI, Cheung MS, Bassett JHD, et al. (2009) Bone signaling pathways and treatment of osteoporosis. Expert Rev Endocrinol Metabol 4, 639–650.
4. Huybrechts KF, Ishak KJ & Caro JJ (2006) Assessment of compliance with osteoporosis treatment and its consequences in a managed care population. Bone 38, 922–928.
5. Deyhim F, Stoecker BJ, Brusewitz GH, et al. (2005) Dried plum reverses bone loss in an osteopenic rat model of osteoporosis. Menopause 12, 755–762.
6. Bu SY, Lucas EA, Franklin M, et al. (2007) Comparison of dried plum supplementation and intermittent PTH in restoring bone in osteopenic orchidectomized rats. Osteoporos Int 18, 931–942.
7. Halloran BP, Wronski TJ, VonHerzen DC, et al. (2010) Dietary dried plum increases bone mass in adult and aged male mice. J Nutr 140, 1781–1787.
8. Arjmandi BH, Khalil DA, Lucas EA, et al. (2002) Dried plums improve indices of bone formation in postmenopausal women. J Womens Health Gend Based Med 11, 61–68.
9. Hooshmand S, Chai SC, Saadat RL, et al. (2011) Comparative effects of dried plum and dried apple on bone in postmenopausal women. Br J Nutr 106, 923–930.
10. Franklin M, Bu SY, Lerner MR, et al. (2006) Dried plum prevents bone loss in a male osteoporosis model via IGF-I and the RANK pathway. Bone 39, 1331–1342.
11. Galli C, Passeri G & Macaluso GM (2010) Osteocytes and WNT: the mechanical control of bone formation. J Dent Res 89, 331–343.
12. Xiong J & O'Brien CA (2012) Osteocyte RANKL: new insights into the control of bone remodeling. J Bone Miner Res 27, 499–505.
13. Boyle WJ, Simonet WS & Lacey DL (2003) Osteoclast differentiation and activation. Nature 423, 337–342.
14. Kostenuik PJ (2005) Osteoprotegerin and RANKL regulate bone resorption, density, geometry and strength. Curr Opin Pharmacol 5, 618–625.
15. Jabbar S, Drury J, Fordham JN, et al. (2011) Osteoprotegerin, RANKL and bone turnover in postmenopausal osteoporosis. J Clin Pathol 64, 354–357.
16. Moester MJ, Papapoulos SE, Lowik CW, et al. (2010) Sclerostin: current knowledge and future perspectives. Calcif Tissue Int 87, 99–107.
17. Watts N (2011) The fracture risk assessment tool (FRAX®): applications in clinical practice. J Womens Health 20, 525–531.
18. Finkelstein J, Brockwell S, Mehta V, et al. (2008) Bone mineral density changes during the menopause transition in a multiethnic cohort of women. J Clin Endocrinol Metab 93, 861–868.
19. Arjmandi BH, Lucas EA, Juma S, et al. (2001) Dried plums prevent ovariectomy-induced bone loss in rats. J Am Nutraceutical Assoc 4, 50–56.
20. Arjmandi BH, Johnson CD, Campbell SC, et al. (2010) Combining fructooligosaccharide and dried plum has the greatest effect on restoring bone mineral density among select functional foods and bioactive compounds. J Med Food 13, 312–319.

21. Johnson CD, Lucas EA, Hooshmand S, *et al.* (2011) Addition of fructooligosaccharides and dried plum to soy-based diets reverses bone loss in the ovariectomized rat. *Evid Based Complement Alternat Med* **8**, 362–367.

22. Findlay DM & Atkins GJ (2011) Relationship between serum RANKL and RANKL in bone. *Osteoporos Intern* **22**, 2597–2602.

23. Rogers A & Eastell R (2005) Circulating osteoprotegerin and receptor activator for nuclear factor κB ligand: clinical utility in metabolic bone disease assessment. *J Clin Endocrinol Metab* **90**, 6323–6331.

24. Findlay D, Chehade M, Tsangari H, *et al.* (2008) Circulating RANKL is inversely related to RANKL mRNA levels in bone in osteoarthritic males. *Arthritis Res Ther* **10**, R2.

25. Drake MT, Srinivasan B, Mödder UI, *et al.* (2010) Effects of parathyroid hormone treatment on circulating sclerostin levels in postmenopausal women. *J Clin Endocrinol Metab* **95**, 5056–5062.

26. Reyes-Garcia R, Muñoz-Torres M, Garcia DF, *et al.* (2010) Effects of alendronate treatment on serum levels of osteoprotegerin and total receptor activator of nuclear factor κB in women with postmenopausal osteoporosis. *J N Am Menopause Soc* **17**, 140–144.

27. Guo B, Peng S, Liang C, *et al.* (2012) Recent developments in bone anabolic therapy for osteoporosis. *Expert Rev Endocrinol Metab* **7**, 677–685.

28. Mirza FS, Padhi ID, Raisz LG, *et al.* (2010) Serum sclerostin levels negatively correlate with parathyroid hormone levels and free estrogen index in postmenopausal women. *J Clin Endocrinol Metab* **95**, 1991–1997.

29. Costa AG & Bilezikian JP (2012) Sclerostin: therapeutic horizons based upon its actions. *Curr Osteoporos Rep* **10**, 64–72.

30. Silverman S (2010) Sclerostin. *J Osteoporos* **2010**, 941419.