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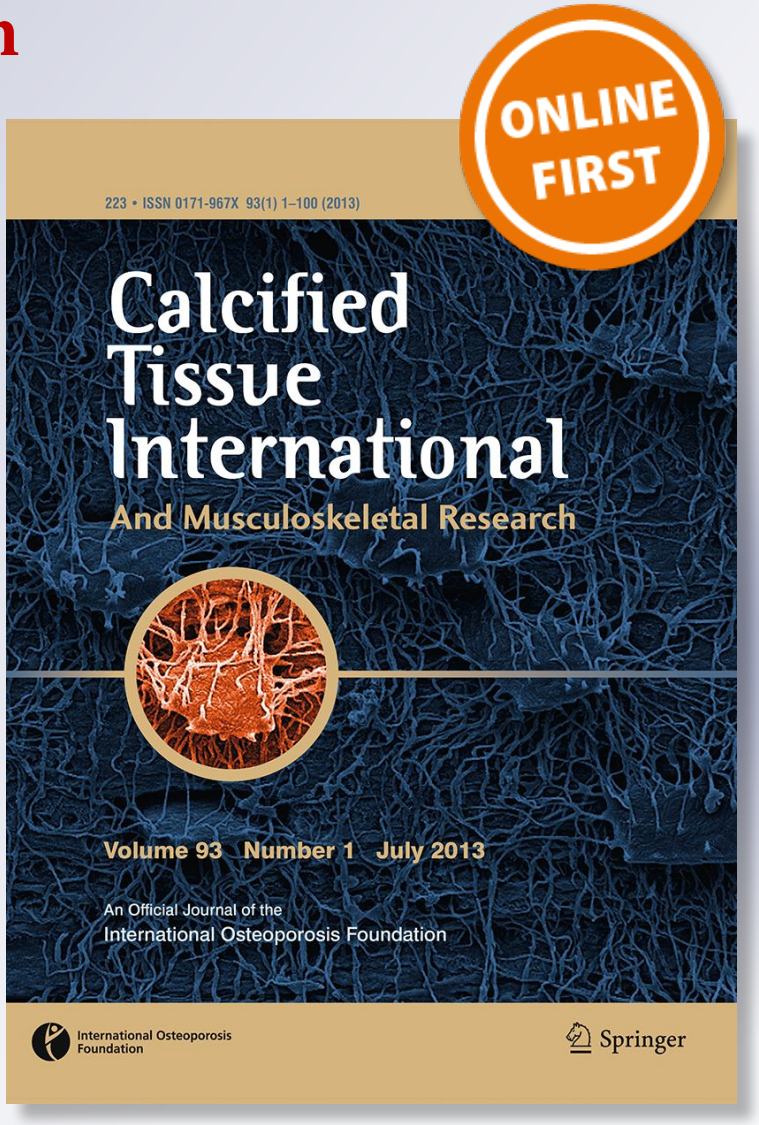

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ORIGINAL RESEARCH

A Natural, Calcium-Rich Marine Multi-mineral Complex Preserves Bone Structure, Composition and Strength in an Ovariectomised Rat Model of Osteoporosis

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Abstract Calcium supplements are used as an aid in the prevention of osteopenia and osteoporosis and also for the treatment of patients when used along with medication. Many of these supplements are calcium carbonate based. This study compared a calcium-rich, marine multi-mineral complex (Aquamin) to calcium carbonate in an ovariectomised rat model of osteoporosis in order to assess Aquamin's efficacy in preventing the onset of bone loss. Animals were randomly assigned to either non-ovariectomy control (Control), ovariectomy (OVX) plus calcium carbonate, ovariectomy plus Aquamin or ovariectomy plus Aquamin delay where Aquamin treatment started 8 weeks post OVX. At the end of the 20-week study, the trabecular architecture was measured using micro computed tomography, bone composition was assessed using Fourier transform infrared spectroscopy and the mechanical properties were assessed using nanoindentation and three-point bend testing. The study demonstrates that oral ingestion of Aquamin results in less deterioration of trabecular bone structure, mineral composition and tissue level biomechanical properties in the tibia of rats following ovariectomy than calcium carbonate. This study has shown that in an animal model of osteoporosis, Aquamin is superior to calcium carbonate at slowing down the onset of bone loss.

Keywords Osteoporosis · Bone · Bone strength · Bone composition · Calcium · Animal model

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Introduction

Osteoporosis is a disease that degrades bone mass and architecture, and impairs the ability of the skeleton to perform fundamental mechanical functions. Physiologically, osteoporosis occurs due to an imbalance in bone cell activity by which excessive resorption occurs without adequate new bone formation, thereby reducing total bone mass. As a consequence, bone strength is reduced and leads to increased risk of fractures of the vertebrae, hip or wrist potentially leading to deformity, severe pain and in some cases death. Osteoporosis causes more than 8.9 million fractures annually worldwide, or one every 3 s, approximately half of which occur in Europe, corresponding with one fracture every 8 s [1]. These 4 million osteoporotic fractures are estimated to cost the EU in the region of €31.7 billion, a figure which is expected to increase to €76.7 billion in 2050 based on the anticipated changes in the demography of Europe [2].

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Calcium is the main constituent of the hydroxyapatite mineral which provides mechanical strength to the skeleton. In addition to the skeletons' roles of providing support, protection and enabling movement, it also acts as a calcium reserve for the body. Therefore, when blood calcium levels are low, parathyroid hormone (PTH) is released in response and this triggers the release of calcium from bone by stimulating bone breakdown [3]. In a bid to avert bone loss, many pre- and post-menopausal women are prescribed calcium supplements [4, 5] with the recommendation being at least 1000–1200 mg/day of calcium for bone health and prevention of fractures [6]. However, calcium supplementation is not without controversy. A recently published systematic review found weak and inconsistent evidence that calcium supplements actually prevent fractures [7]. They also concluded that dietary calcium intake is not associated with risk of fracture, and there is no clinical trial evidence that increasing calcium intake from dietary sources prevents fractures. In a small number of cases, supplements have been linked to myocardial infarction, angina, transient ischaemic attack or stroke [8, 9]. However, these studies are far outweighed by the wealth of publications advocating calcium supplements be routinely prescribed for bone health.

Aquamin is a calcium-rich, marine multi-mineral food supplement derived from the calcified skeletal remains of the red marine algae species Lithothamnion. It is a natural supplement which undergoes minimal processing as the Lithothamnion is simply harvested, dried and milled. It is available commercially in a number of forms, which have calcium concentrations ranging up to 31 wt%. In addition to calcium and magnesium, Aquamin also contains 72 trace minerals including strontium, manganese, selenium, copper and zinc, all of which have been identified as important in bone health [10]. Unlike calcium carbonate, which is extracted from limestone or rock and is made up almost entirely of calcite, Aquamin is plant based and has a porous honeycombed vegetative cell structure with aragonite, vaterite and calcite calcium salts present. This structure gives Aquamin a number of significant benefits in its chemical behaviour and its absorption.

A series of recent trials have also identified several positive effects of Aquamin on bone health. Two double blind, placebo-controlled studies in humans measured the bioavailability of Aquamin versus calcium carbonate. These studies found that as early as 60 min following treatment, PTH levels were significantly reduced in the Aquamin group compared to calcium carbonate and placebo groups (Minnesota Applied Research Centre, USA: unpublished). In-vitro studies on osteoblast cells cultured in the presence of Aquamin demonstrated that the cells exhibited early osteogenic potential and produced more mineral than those cultured in its absence [11–13]. An in vivo study in which Aquamin was used as a dietary supplement in mice on a high-fat Western diet (HFWD) showed that bone structure and function were preserved [14]. Indeed, the bone structure of these mice was superior to that of mice on the standard low-fat diet, thus asserting the osteogenic effect of Aquamin on bone cells. In a study conducted in yearling horses, an increase in bone remodelling was measured with Aquamin suggesting that old or damaged bone could be replaced or removed which could, in turn, reduce incidents of clinical bone injury [15]. These results indicate that Aquamin has the potential to enhance bone formation.

Using an established model of osteoporotic bone loss, the primary aim of this study was to compare the efficacy of Aquamin with calcium carbonate at preventing the onset of bone loss. Specifically bone structure, composition and mechanical properties were assessed in ovariectomised rats treated with Aquamin or calcium carbonate.

Methods

Animals

This study was approved by the Animal Ethics Committee of Trinity College Dublin, Ireland and performed under licence issued by the Department of Health. All applicable institutional and national guidelines for the care and use of animals were followed. Eighty-eight female, retired breeder Wistar rats, both ovariectomised and age matched controls, were obtained from Harlan Laboratories (UK) and allowed to acclimatise for 5 days before the start of the experiment (Week 0). The rats were maintained with a cycle of 12 h light and 12 h darkness. Two diets were used in this study, a regular murine chow (RM1) which provides the recommended daily allowance of calcium in the form of calcium carbonate and a specially formulated RM1 supplemented with Aquamin (both from Special Diet Services, UK). For the Aquamin supplemented feed, calcium (in the form of calcium carbonate) was removed from RM1 and Aquamin (31 wt% calcium) was added at a concentration such that calcium levels were equal in both feeds. Both feeds were therefore providing equivalent levels of calcium, just in a different form, either as calcium carbonate or Aquamin. The rats were divided into four groups: (1) non-OVX control ($n = 28$; RM1), (2) OVX plus calcium carbonate ($n = 24$, RM1), (3) OVX plus Aquamin ($n = 24$, Aquamin supplemented RM1) and (4) OVX plus Aquamin delay ($n = 12$; RM1 for 8 weeks followed by Aquamin supplemented RM1). Using this study design, the non-OVX controls and the OVX + calcium carbonate both received RM1 and comparisons between these groups showed the efficacy of the bone loss model as the only

difference between these groups was the OVX procedure. Comparison between the OVX + calcium carbonate and OVX + Aquamin groups were the main focus of this study as the only difference between these groups was the form of calcium received. During the acclimatisation period, all animals were fed RM1. At week 0, Aquamin treatment was started in Group 3, while Group 4 treatment started at week 8. Animals were allowed to eat and drink ad libitum and routinely weighed before being euthanised by carbon dioxide inhalation at weeks 0, 2, 8, 12 and 20.

Micro Computed Tomography (MicroCT) (Structure and composition)

Following sacrifice, the right tibia was removed for microCT scanning (μCT40, Scanco, Switzerland). To determine trabecular bone characteristics, the metaphyseal trabecular region of the proximal tibia immediately below the epiphyseal growth plate was selected and 100 adjacent slices were scanned. From these scans, the trabecular bone was manually selected and bone structural parameters were automatically determined using Scanco software. Bone volume fraction (BV/TV) (primary endpoint of this study) and trabecular number (Tb.N), trabecular thickness (Tb.Th), trabecular separation (Tb.Sp) and hydroxyapatite content (HA) (secondary endpoints) were also calculated. To characterise cortical bone, the midpoint of the tibia was identified and 10% of the tibial length was scanned around this position. From these scans, the HA content was determined. Microarchitecture was analysed at a resolution of 8 μm, at 70 kVp and 114 μA and a threshold value of 210.

Fourier Transform Infrared Spectroscopy (FTIR) (Composition)

One-hundred micron thick samples were sectioned from the distal region of the left tibia using a diamond saw (Minitom, Struers, Denmark). As water has a strong absorbance in the infrared, tissues are dehydrated before analyses, thus the water spectrum is not observed. The dehydrated samples were placed on a calcium fluoride slide and positioned on the microscope stage of the Spotlight 400 FTIR Imaging system (Perkin Elmer). The system is equipped with an AutoImage microscope system operating with a 40X Cassegrain objective. A visible white-light image was recorded of the sample and the area to be scanned using infrared was specified. FTIR images were acquired in attenuated total reflectance (ATR) imaging mode. The data were collected over the nominal free-scanning spectral range, 4000–600 cm^{-1}. Spectral measurements were acquired with a pixel size of 6.25 μm × 6.25 μm at a spectral resolution of 4 cm^{-1}. Background measurements were acquired on a region with no tissue with 120 scans per pixel, whereas 32 scans per pixel were recorded from the sample.

Nanoindentation Testing (Material Properties)

Two-hundred micron thick samples were sectioned from the distal tibia (adjacent to the FTIR samples) using a diamond saw (Minitom, Struers, Denmark) before being polished with a series of graded silica-carbide grinding sheets. A Nano indenter XP system (MTS System Corporation, Oak Ridge, TN) was used to measure tissue level mechanical properties of proximal tibia sections. The Young's modulus (E) and contact hardness (H_c) were assessed using an AccuTip™ diamond Berkovich indenter tip with defined elastic modulus of 1141GPa, a Poisson's ratio equal to 0.07 and a radius of <50 nm. A total of 20 indents were performed on each sample: 10 evenly spaced indents in the cortical shell and a further 10 indents in the trabecular bone, five in an outer ring and five in an inner ring at the centre of the sample. A permanent hardness impression was made by driving the indenter into the sample for 90 s to a maximum load of 20 mN, holding for 120 s and then unloading. This cycle was repeated 3 times at each location and the modulus was determined from the unloading segment of the final indent [16].

Three-Point Bend Testing (Mechanical Properties)

Following microCT, a standard three-point bending test was applied to the right tibia. The tibiae were placed on the lateral surface on two rounded supporting bars with a distance of 12 mm between them. A preload of 1 N was applied (Z020; Zwick, Ulm, Germany) at the medial surface of the diaphysis by lowering a third rounded bar. A constant displacement rate of 5 mm/min was applied until failure. Displacement was measured from the actuator displacement transducer of the testing machine. Maximum load, maximum displacement and stiffness were calculated for each sample.

Statistics

All results are presented in graphs as the mean ± standard deviation. For all structural parameters, the percentage change relative to the non-OVX control was calculated for each animal individually at the different time intervals. Groups were compared using analyses of variance (ANOVA) with a post hoc Bonferroni test applied for multiple comparisons. Where a significant difference was shown between groups, the results are presented as (mean difference, 95% confidence interval (CI) (lower bound,

upper bound), p value). ANOVA with a Bonferroni post hoc test was also used to determine differences between the groups in composition and mechanical results. A difference of $p \leq 0.05$ was considered significant.

Results

Trabecular Bone Structure

Aquamin Prevents a Significant Loss of Trabecular Bone Structure

Three-dimensional and two-dimensional representative micrographs from each of the groups are shown in Fig. 1. These slices and reconstructions show an intact and well-connected trabecular microstructure in the control samples. A significant loss of trabecular bone structure following 20 weeks of ovariectomy is evident in the centre of the OVX + calcium carbonate samples. In contrast, the trabecular structure is preserved when Aquamin administration began at week 0 (OVX + Aquamin). The Aquamin delay group has a structure which appears intermediate between the OVX + calcium carbonate and OVX + Aquamin group.

Quantitative analysis shows that as early as week 2, the OVX + calcium carbonate group displayed large changes in structural parameters relative to the healthy controls, indicating the development of osteopenia (Fig. 2). Significant losses in bone volume fraction (BV/TV) were observed in this group by week 8 (33, 95% CI (21, 45), $p = 0.038$). Trabecular separation (Tb.Sp) was significantly increased in the OVX + calcium carbonate group by week 12 (57, 95% CI (55, 58), $p = 0.045$), while trabecular number (Tb.N) was significantly reduced in this group by week 20 (47, 95% CI (30, 65), $p = 0.049$). No significant changes were measured in trabecular thickness (Tr.Th) between groups over the study period.

Treatment with Aquamin resulted in a significant preservation of bone volume fraction by week 20 relative to the OVX + calcium carbonate group (13, 95% CI (1,21), $p = 0.049$). No significant differences were measured in individual parameters, e.g. Tr. N or Tr. Sp between OVX + calcium carbonate and the Aquamin-treated groups.

Trabecular and Cortical Bone Composition

Aquamin Prevents a Significant Loss of Hydroxyapatite Content and Mineral in Trabecular Bone

Hydroxyapatite content measured using microCT indicated a significant reduction in the HA content of trabecular bone by week 20 as seen in the OVX + calcium carbonate group in comparison to the non-OVX controls (37.5 mg HA/cm^3, 95% CI (10, 65), $p = 0.022$) (Fig. 3). Beginning oral administration of Aquamin at week 0 prevented this HA loss in trabecular bone. Following OVX, trabecular HA content was significantly greater in the Aquamin group than in the calcium carbonate group (29.5 mg HA/cm^3, 95% CI (18, 41), $p = 0.044$). Beginning administration of Aquamin 8 weeks following ovariectomy (Aquamin delay) was also sufficient to see a significant preservation of HA

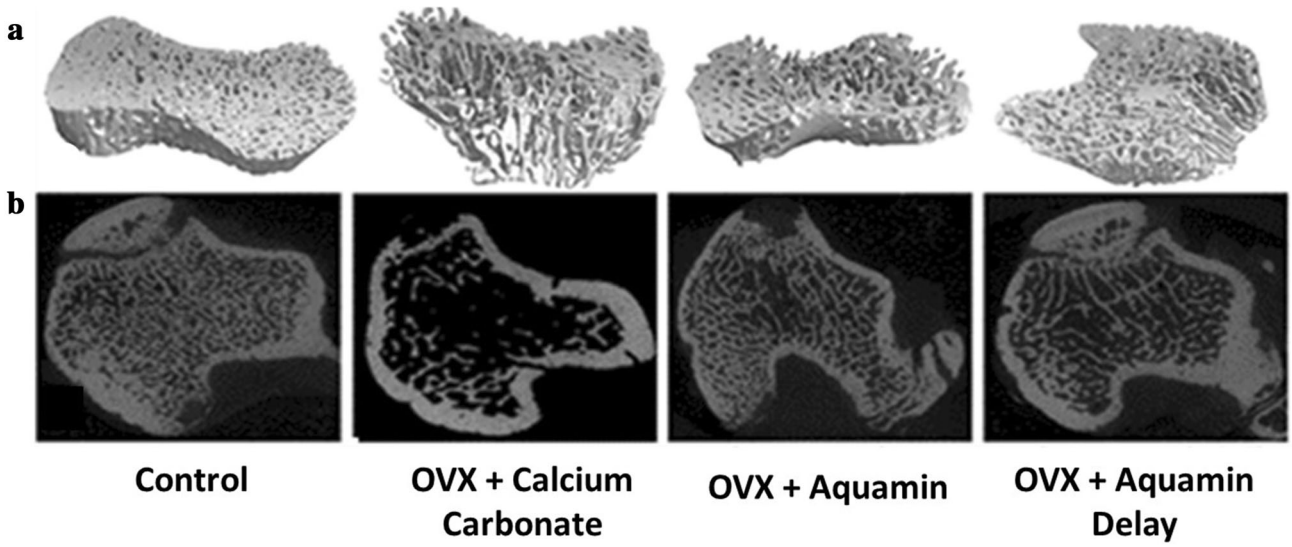

Fig. 1 Representative **a** three-dimensional microCT reconstructions and **b** two-dimensional slices of trabecular bone from the proximal tibia of rats at week 20

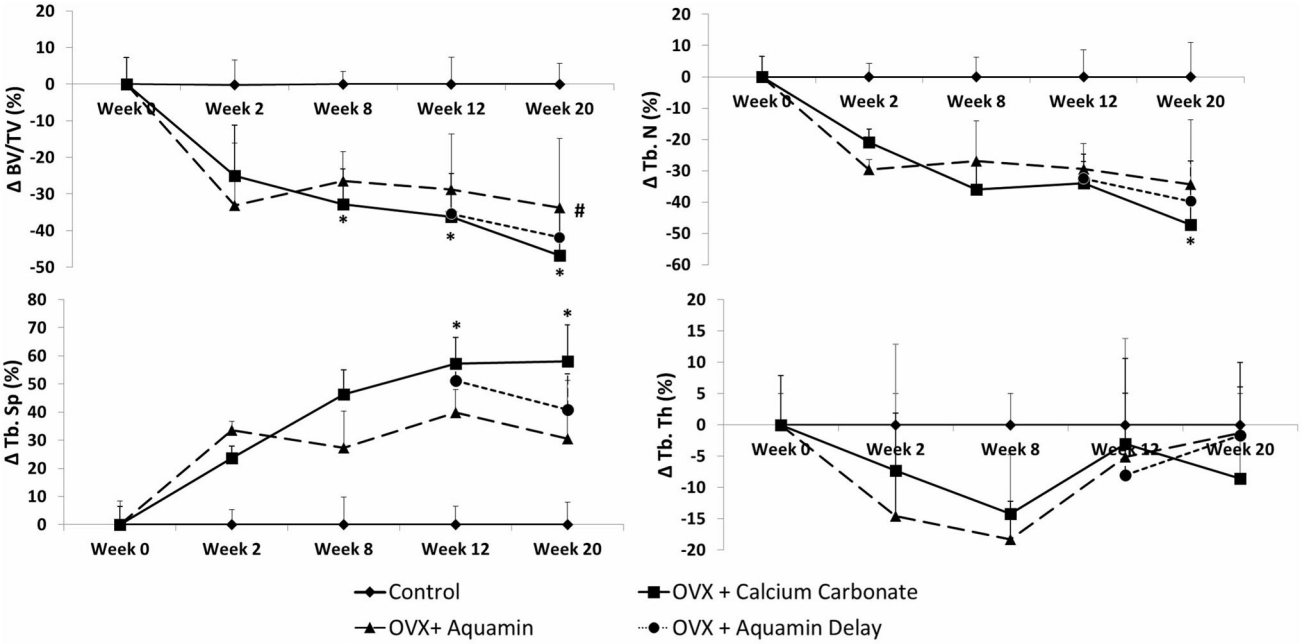

Fig. 2 Percentage change in structural parameters in the metaphyseal proximal tibia for all groups over all time points. $N = 6$ per group (*asterisk* indicates $p < 0.05$ relative to control, *number sign* indicates $p < 0.05$ relative to OVX + calcium carbonate)

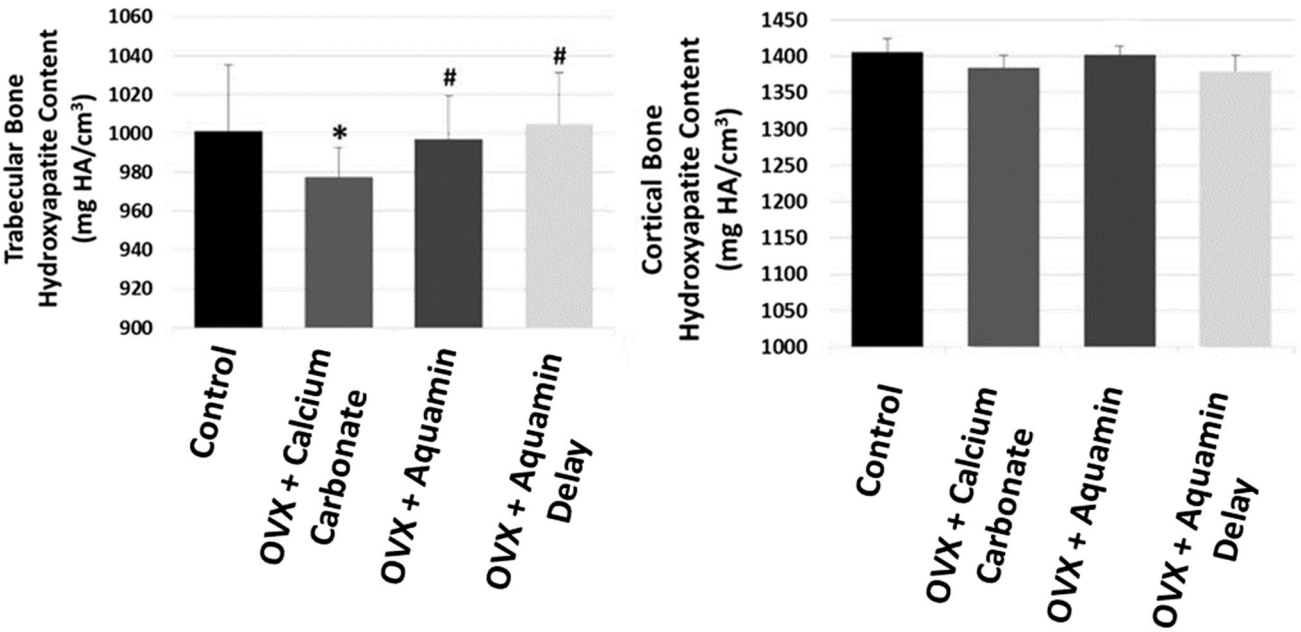

Fig. 3 Hydroxyapatite content measured using microCT indicates a significant reduction in HA content in trabecular bone following twenty weeks of ovariectomy. Aquamin administration prevented this loss of HA in trabecular bone and a significant preservation was also seen in trabecular bone in the Aquamin delay group (*asterisk* indicates $p < 0.05$ relative to control, *number sign* indicates $p < 0.05$ relative to OVX + calcium carbonate)

content in trabecular bone when the delay group was compared to OVX + calcium carbonate (32.6 mg HA/cm^3, 95% CI (25, 57), $p = 0.046$). No significant differences were measured between groups in cortical bone HA content.

Qualitative FTIR

In Fig. 4a, the week 20 representative control sample visualised with white light (centre panel) shows bone with a healthy micro architecture and good trabecular

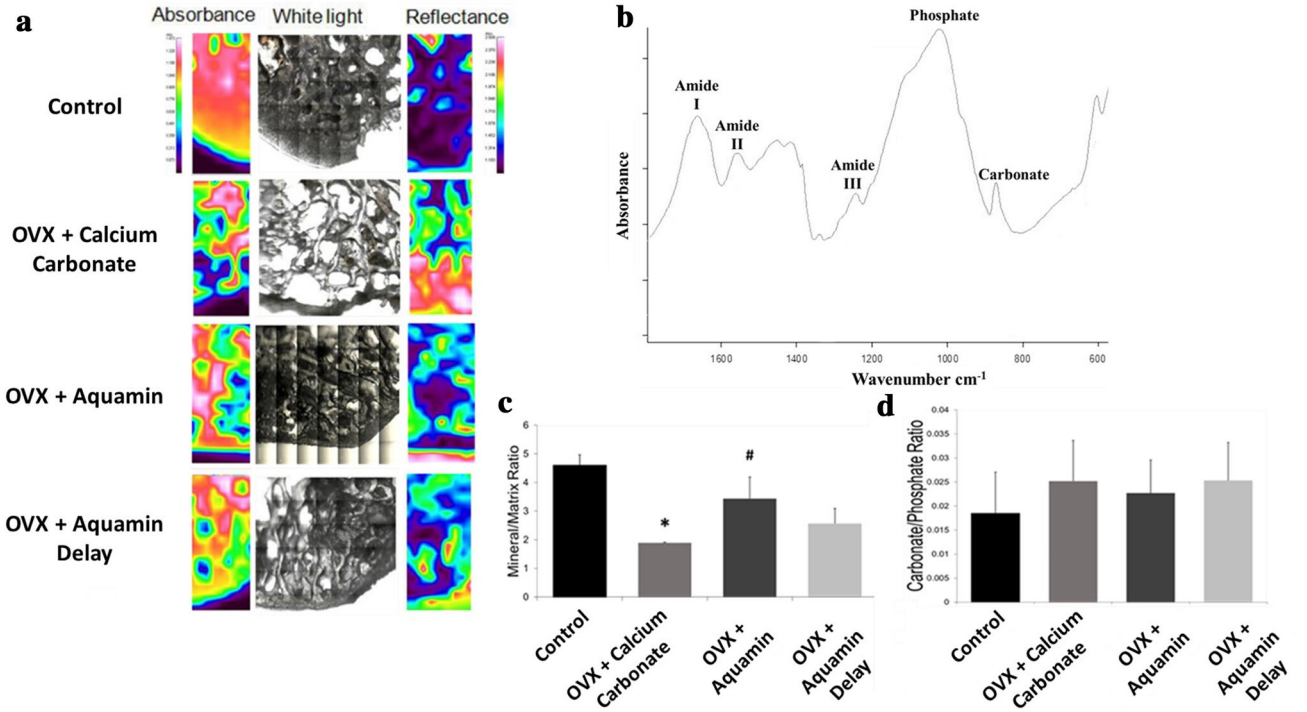

Fig. 4 **a** FTIR absorbance (*left*) and reflection (*right*) heat maps with white-light image (*centre*) from a representative sample of each group at week 20. In the absorbance maps, areas of *red* indicate increased absorbance (mineralisation) while in infrared reflection maps, *purple* indicates increased mineralisation. **b** Typical absorbance spectrum of bone indicating peaks used to calculate mineral/matrix ratio and carbonate/phosphate ratios. **c** The mineral/matrix ratio was significantly reduced following 20 weeks of oestrogen deficiency (*asterisk* indicates $p < 0.05$ relative to control). This loss of mineral was prevented by administering Aquamin from week 0 (*number sign* indicates $p < 0.05$ relative to OVX + calcium carbonate). **d** No significant changes were measured in the carbonate/phosphate ratio between groups

interconnectivity. The ATR spectrum which is recorded can be represented as an absorbance spectrum. The same region of bone visualised as an absorbance heat map (left hand panel) displays an abundance of red/orange indicating increased absorption on the surface of the sample. In ATR mode (right hand panel), blue/purple indicates increased reflection at the surface. Visual inspection of the white-light and infrared maps for the OVX + calcium carbonate sample shows significant microarchitecture deterioration and less absorption and reflection than in the control group. Treatment with Aquamin shows high absorbance represented by the presence of red in absorbance and purple/blue in the ATR heat maps. The Aquamin delay group displays absorbance and reflection profiles which are intermediate to the OVX +calcium carbonate and the OVX + Aquamin groups as indicated by red and green in the absorbance and purple and green in the ATR maps. This suggests that a delayed treatment of Aquamin has the potential to restore bone.

Quantitative FTIR

The infrared spectrum of bone shows the presence of the major molecular species, phosphate (from the mineral hydroxyapatite), carbonate (from carbonate substitution for hydroxyl and phosphate groups), and amide I, II and III from the protein constituents of bone (mainly type I collagen) [17]. These peaks are shown in a representative spectrum from a healthy control sample in Fig. 4b. The mineral/matrix ratio is linearly related to the mineral content and the carbonate/phosphate ratio is related to the chemically measured carbonate/phosphate content. In the trabecular region from each absorbance spectrum, the mineral-to-matrix ratio was assessed as the ratio of the area of the phosphate band (1170–980 cm^{-1}) to the area of the amide I band (1712–1592 cm^{-1}). The carbonate/phosphate ratio was assessed as the ratio of the area of the phosphate band to the area of the carbonate band (890–840 cm^{-1}) [17]. Analysis of the ATR spectra indicates a significant reduction in the mineral/matrix ratio in the OVX + calcium carbonate group relative to the healthy control group at week 20 (1.95, 95% CI (0.7, 3.2), $p = 0.016$) (Fig. 4c). This reduction of mineral/matrix ratio was prevented by providing calcium in the form of Aquamin from week 0. In comparison to the calcium carbonate group, the Aquamin group had a significantly higher mineral/matrix ratio (1.45, 95% CI (0.83, 2.26), $p = 0.048$). The late administration of

Aquamin did not significantly prevent the change in mineral/matrix ratio. No significant changes were measured in the carbonate/phosphate ratio between groups (Fig. 4d). These FTIR results confirm both qualitatively and quantitatively Aquamin's ability to preserve mineral composition.

Trabecular and Cortical Bone Biomechanical Properties

Aquamin Prevents a Significant Loss of Material Strength in Trabecular and Cortical Bone

Nanoindentation Material Test

The Young's modulus and hardness data are presented in Table 1. In the healthy control group, the value of the Young's modulus is lowest in the cortical shell, increasing significantly in trabeculae near the edge of this cortical shell and increasing further in trabeculae at the core of the sample. A similar pattern is seen for the hardness. In the OVX + calcium carbonate group, this stepwise increase disappears and there is only a significant difference between the core trabeculae and the edge/cortical shell when the modulus is assessed and there are no differences between locations when hardness is analysed. In the OVX + Aquamin group, trabecular bone moduli and hardness values are significantly greater than the cortical shell values which is more akin to the healthy control group. No significant differences were measured in the OVX + Aquamin delay group.

Analysis between groups of trabecular bone from the core found the modulus for the healthy control group was significantly higher than the OVX + calcium carbonate and the OVX + Aquamin delay groups. The OVX + Aquamin group was not significantly different from the control group. Similar results were observed for hardness. For trabeculae located around the edge of the sample, both the modulus and hardness in the OVX + Aquamin group was significantly greater than that for the OVX + calcium carbonate group. For cortical bone, both moduli and hardness in the OVX + Aquamin delay group were significantly higher than in the healthy control group. Hardness in cortical bone from the OVX + Aquamin group was also higher than the healthy control group.

Three-Point Bend Test

Analysis of the cortical shell by three-point bend testing revealed a significant reduction in the maximum load sustained by the OVX + calcium carbonate (OVX control) group between week 2 and week 20 (Table 2). In contrast, no significant difference was seen in the OVX + Aquamin group over the study duration. No significant differences were measured in maximum displacement or stiffness between any of the groups or over the study duration.

Discussion

In this study, we used an established model of osteoporotic bone loss to compare the efficacy of Aquamin and calcium carbonate at preventing the onset of trabecular bone loss. The results of the study indicate that Aquamin results in less deterioration of trabecular bone in the proximal tibia of ovariectomised rats following 20 weeks of treatment than calcium carbonate. This protection of structure was accompanied by a preservation of the mineral composition and follows through to maintaining the mechanical integrity of the treated bone. Analysis of the samples from animals subject to the delayed Aquamin treatment was inconclusive for a number of reasons which are discussed

Table 1 The Young's modulus and hardness measured in the cortical shell and in trabecular bone from the core and the edge of the proximal tibia at week 20

Location	Control	OVX + calcium carbonate	OVX + aquamin	OVX + aquamin delay
Modulus (GPa)				
Core	7.61 ± 3.67^{a}	$5.44 \pm 2.53^{a,b,*}$	5.99 ± 1.20^{b}	5.13 ± 0.97*
Edge	5.56 ± 3.87^{b}	3.71 ± 1.94	$5.48 \pm 0.95^{b,\#}$	3.91 ± 0.71
Cortical shell	3.35 ± 0.51	3.86 ± 2.55	4.10 ± 0.55	4.70 ± 0.67**
Hardness (GPa)				
Core	0.43 ± 0.10^{a}	0.37 ± 0.07	0.42 ± 0.08^{b}	$0.29 \pm 0.05^{*,\&}$
Edge	$0.37 \pm 0.08^{b,\#}$	0.28 ± 0.06	$0.38 \pm 0.07^{b,\#,\%}$	0.24 ± 0.04
Cortical shell	0.24 ± 0.04	0.25 ± 0.03	0.27 ± 0.04^	0.27 ± 0.04^

Mean ± standard deviation. Significant difference of $p \leq 0.05$ between locations: [a] >edge, [b] >cortical shell. Between groups: * <control, # >OVX + Calcium carbonate, ** >control, & <OVX + Aquamin, % >OVX + Aquamin delay, ^ >control

Table 2 Maximum load, maximum displacement and stiffness measured using three-point bend testing of the tibia

Mechanical property	Group	Week 0	Week 2	Week 20
Maximum load (N)	Control	129.62 ± 2.02	128.03 ± 20.19	121.12 ± 10.20
	OVX + calcium carbonate	–	141.80 ± 9.41	122.23 ± 12.10 *
	OVX + aquamin	–	132.26 ± 19.36	131.85 ± 13.96
	OVX + aquamin delay	–	–	136.80 ± 12.11
Maximum displacement (mm)	Control	0.55 ± 0.14	0.54 ± 0.09	0.55 ± 0.13
	OVX + calcium carbonate	–	0.53 ± 0.15	0.54 ± 0.14
	OVX + aquamin	–	0.54 ± 0.24	0.51 ± 0.2
	OVX + aquamin delay	–	–	0.47 ± 0.06
Stiffness (N/mm)	Control	244.39 ± 59.76	247.86 ± 72.58	292.93 ± 66.37
	OVX + calcium carbonate	–	297.35 ± 128.59	257.95 ± 67.10
	OVX + aquamin	–	296.39 ± 140.21	368.25 ± 86.11
	OVX + aquamin delay	–	–	293.19 ± 51.24

Significant difference of $p \leq 0.05$: Within the OVX + Calcium carbonate group, * >week 2

in further detail below. Similarly, analyses of data on cortical bone were not conclusive. The use of a non-ovariectomy control group proved the efficacy of our model. Significant losses in bone volume were observed and measured in the ovariectomy plus calcium carbonate group as early as 8 weeks when compared to the non-ovariectomy control. This loss of structure was accompanied by a degradation in the composition and mechanical properties of the remaining bone, typical characteristics of osteoporosis.

In the current study, both the regular chow (RM1) and the Aquamin supplemented chow have equal amounts of calcium. Therefore, the Aquamin-treated animals did not receive extra dietary calcium. The significant inhibition of bone loss, measured by week 20 in this study, is therefore not a result of the quantity of calcium ingested. The significant preservation of the trabecular structure in the Aquamin groups is most likely brought about by the unique form in which the calcium is presented by Aquamin. Since calcium's role in the prevention of osteoporosis in humans was proven some 30 years ago, its bioavailability has been the subject of numerous scientific studies [18]. Calcium carbonate, which is entirely composed of calcite, is typically used as the calcium supplement of choice worldwide, however, it is known that its bioavailability is very low, only around 20–30% [18]. Although the various calcium salts have the same chemical make-up, their structure differs. The orthorhombic structure of aragonite and the hexagonal structure of vaterite calcium salts in Aquamin dramatically increase the calcium surface area compared to the rhombohedral structure of calcite. Thus, stomach acid can come into greater contact with the calcium making it easy to dissolve into the body and providing greater efficacy. Additionally, calcium carbonate is not easily absorbed as the phosphorus binds tightly to the calcium.

This difference in bioavailability is one of the factors postulated to explain the positive Aquamin results observed in this study. In addition to calcium, Aquamin also contains 73 additional minerals many of which have a proven osteogenic potential through direct or indirect effects on bone cells or bone mineral and collagen. Magnesium is a major component of Aquamin, it is essential for all living cells, including osteoblasts and osteoclasts, as it is fundamental in adenosine triphosphate [19]. However, its role in bone is not confined to simply cell biology, a dietary magnesium restriction has been shown to promote osteoporosis [20]. Potassium is critical for calcium metabolism in many enzymes and potassium is also required for improved bone strength and density [21]. Copper plays a role in collagen crosslinking and fixing calcium within bones [22]. Some elements, such as strontium, are chemically similar to calcium. Strontium has an anabolic effect in bone, and this may have significant beneficial effects on bone balance [23]. The osteogenic role offered by these elements in combination with a readily available calcium source may have a synergistic effect. It is also recognised that calcium bioavailability is influenced and enhanced by the presence of other minerals [24]. Aquamin also contains a unique trace mineral profile gained from its marine source. Alone, these elements may be insignificant, but within a multi-mineral matrix they work synergistically and may give a powerful boost to the action of the calcium. As calcium supplementation alone has proven ineffective at preventing fracture or increasing bone mineral density [7], Aquamin's ability to prevent loss of trabecular bone structure, composition and strength is most probably a

result of the additional minerals present, either acting alone or synergistically with calcium.

Starting oral administration of Aquamin immediately following ovariectomy established Aquamin's capacity to prevent trabecular bone loss in the proximal tibia of rats associated with ovariectomy significantly better than calcium carbonate. Bone volume fraction was significantly altered by Aquamin. This change was brought about by a combination of changes in trabecular number, separation and thickness, all of which are incorporated into the software calculation of the trabecular bone volume fraction. The preservation of structure can clearly be seen in the microCT two-dimensional slices and the three-dimensional reconstructions.

Bone is a composite material consisting of a mineral phase (hydroxyapatite), collagen, non-collagenous proteins, lipids and water [25]. In the current study, there was a significant reduction in the mineral-matrix ratio measured in trabecular bone from the ovariectomy + calcium carbonate group at week 20 compared to the healthy controls. This is consistent with previous findings which found a significant reduction in the mineral-matrix ratio in normal vs. osteoporotic bone [17]. These data are supported by the significant reduction in hydroxyapatite measured in the trabecular bone from animals in this group. The FTIR mapping of the control samples was also indicative of a high mineral content. In contrast, the OVX + calcium carbonate showed reduced absorption indicating a reduced mineral level. Those animals which started Aquamin treatment immediately after ovariectomy were found to have a mineral-matrix ratio significantly greater than the OVX + calcium carbonate group indicating a preservation by Aquamin of the healthy composition. Again this is borne out in the hydroxyapatite data from microCT analysis where hydroxyapatite content was higher in the Aquamin-treated group than calcium carbonate group. FTIR maps of the Aquamin-treated group were more akin to the healthy control group signifying the preservation of mineral. The carbonate–phosphate ratio indicates the level of carbonate substitution in the hydroxyapatite crystal, a decrease in this ratio reflecting an increased phosphate content. However, no significant difference was seen in the carbonate-phosphate ratio in the current study.

In tandem with the significant changes in composition measured using FTIR and microCT were changes in the tissue level mechanical properties as measured using nanoindentation. The biomechanical parameters of trabecular bone from slices adjacent to those used in FTIR revealed unsurprising parallels. The Young's modulus of trabecular bone from ovariectomised animals receiving calcium in the form of calcium carbonate was significantly less than the healthy controls. This correlates with data from previous studies examining the tissue level mechanical properties of ovariectomised bone compared to healthy samples [16, 26]. Trabeculae from animals who received calcium through Aquamin had a significantly higher modulus and hardness than from the calcium carbonate-treated animals. The calcium carbonate-treated trabeculae had material strength values which were significantly less than healthy controls. In contrast, values from the Aquamin-treated groups were not significantly different from controls. While this effect has not been demonstrated previously, it is in keeping with the preservation of composition by Aquamin shown in the study.

The nanoindentation results also yielded some interesting data in terms of the cortical bone. Material strength assessment showed that both modulus and hardness in the Aquamin delay group were significantly greater than the healthy control group. MicroCT data indicated a sustained loss of trabecular bone volume following ovariectomy over the 20 week study. However, when the trabecular thickness was examined, it was found to decrease until week 8 at which point it began to increase again. These data support the theory of bone compensation whereby new bone is laid down on surviving trabeculae to compensate for the loss of other trabeculae [27, 28]. In addition to laying down new bone in trabeculae, new bone may also be laid down in the cortical shell [29]. However, as osteoporosis is characterised by thinning of the cortical shell this is probably not the case [30]. It is possible that secondary mineralisation is increased in the cortical bone. In conjunction with the availability of Aquamin, this could account for the higher tissue level mechanical values measured. It is not immediately clear what influence Aquamin in combination with ovariectomy had on cortical bone and further investigation of this is warranted.

This study has shown the ability of Aquamin to slow down the onset of bone loss in an animal model of osteoporosis. However, there is much that we are unsure of in terms of the mechanism of action of Aquamin. In addition to calcium, Aquamin contains 73 factors many of which have a proven positive influence on bone. A much larger study is required to determine their collective influence. To help with these assessments, it would be interesting to measure bone formation and resorption rates in further studies to ascertain whether Aquamin has an anabolic effect, an anti-resorptive effect or indeed both. This could be conducted by looking at serum biomarkers, through the use of fluorochrome markers to determine where bone formation is taking place or examining gene expression. It is also unclear whether the effects observed can be sustained or improved over a longer period of time. A longer term study would also advance our understanding of how Aquamin affects bone biology. An extended duration would also likely yield additional significant changes. Further study is also required to fully ascertain the

timeframes involved for a delayed treatment regime to be effective. Clinically, calcium is prescribed in combination with vitamin D. Therefore, an assessment of the combined influence of Aquamin and vitamin D would be merited.

Conclusion

Overall, this study has shown that in an animal model of osteoporosis. Aquamin results in less deterioration of trabecular bone than calcium carbonate. Trabecular architecture is significantly preserved when using Aquamin as compared to calcium carbonate. This is supported by a preservation of the mineral phase of bone which is mirrored by the improved material properties of the bone.

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Author's Contribution Orlaith Brennan, Denise O'Gorman and Fergal O'Brien were responsible for the study design. Orlaith Brennan prepared the first draft of the paper. Orlaith Brennan, Joseph Sweeney, Brian O'Meara, Amro Widaa, Franck Bonnier and Hugh Byrne all contributed to the experimental work. Orlaith Brennan and Joseph Sweeney were responsible for statistical analysis of the data. All authors revised the paper critically for intellectual content and approved the final version. All authors agree to be accountable for the work and to ensure that any questions relating to the accuracy and integrity of the paper are investigated and properly resolved.

Compliance with Ethical Standards

Conflicts of interest Dr. O'Gorman is an employee of Marigot Ltd. Prof O'Brien and Dr. Brennan have received funding from Marigot Ltd. to conduct research. Prof Byrne, Dr. Bonnier, Dr. Widaa, Mr. Sweeney and Mr. O'Meara have no disclosures.

Human and Animal Rights and Informed Consent All applicable international, national and institutional guidelines for the care and use of animals were followed. All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted.

References

1. Johnell O, Kanis JA (2006) An estimate of the worldwide prevalence and disability associated with osteoporotic fractures. Osteoporos Int 17(12):1726–1733
2. Kanis JA, Johnell O (2005) Requirements for DXA for the management of osteoporosis in Europe. Osteoporos Int 16(3): 229–238
3. Reeve J, Meunier PJ, Parsons JA et al (1980) Anabolic effect of human parathyroid hormone fragment on trabecular bone in involutional osteoporosis: a multicentre trial. Br Med J 280(6228):1340–1344
4. Chapuy MC, Arlot ME, Duboeuf F, Brun J, Crouzet B, Arnaud S, Delmas PD, Meunier PJ (1992) Vitamin D3 and calcium to prevent hip fractures in the elderly women. N Engl J Med 327(23):1637–1642
5. Tang BM, Eslick GD, Nowson C, Smith C, Bensoussan A (2007) Use of calcium or calcium in combination with vitamin D supplementation to prevent fractures and bone loss in people aged 50 years and older: a meta-analysis. Lancet 370(9588):657–666
6. Institute of Medicine (2011) Dietary reference intakes for calcium and vitamin D. In: Ross AC, Taylor CL, Yaktine AL, Del Valle HB (eds). National Academies Press, Washington DC
7. Bolland MJ, Leung W, Tai V, Bastin S, Gamble GD, Grey A, Reid IR (2015) Calcium intake and risk of fracture: systematic review. Br Med J 351:h4580
8. Asmus HG, Braun J, Krause R, Brunkhorst R, Holzer H, Schulz W, Neumayer HH, Raggi P, Bommer J (2005) Two year comparison of sevelamer and calcium carbonate effects on cardiovascular calcification and bone density. Nephrol Dial Transplant 20(8):1653–1661
9. Bolland MJ, Barber PA, Doughty RN, Mason B, Horne A, Ames R, Gamble GD, Grey A, Reid IR (2008) Vascular events in healthy older women receiving calcium supplementation: randomised controlled trial. Br Med J 336(7638):262–266
10. Palacios C (2006) The role of nutrients in bone health, from A to Z. Crit Rev Food Sci Nutr 46(8):621–628
11. Brennan O, Stenson B, Widaa A, O'Gorman DM, O'Brien FJ (2015) Incorporation of the natural marine multi-mineral dietary supplement Aquamin enhances osteogenesis and improves the mechanical properties of a collagen-based bone graft substitute. J Mech Behav Biomed Mater 47:114–123
12. O'Gorman DM, Tierney CM, Brennan O, O'Brien FJ (2012) The marine-derived, multi-mineral formula, Aquamin, enhances mineralisation of osteoblast cells in vitro. Phytother Res 26(3):375–380
13. Widaa A, Brennan O, O'Gorman DM, O'Brien FJ (2014) The osteogenic potential of the marine-derived multi-mineral formula aquamin is enhanced by the presence of vitamin D. Phytother Res 28(5):678–684
14. Aslam MN, Kreider JM, Paruchuri T, Bhagavathula N, DaSilva M, Zernicke RF, Goldstein SA, Varani J (2010) A mineral-rich extract from the red marine algae *Lithothamnion calcareum* preserves bone structure and function in female mice on a Western-style diet. Calcif Tiss Int 86(4):313–324
15. Nielsen BD, Cate RE, O'Connor-Robison CI (2010) A marine mineral supplement alters markers of bone metabolism in yearling Arabian horses. J Equine Vet Sci 30(8):419–424
16. Brennan O, Kennedy OD, Lee TC, Rackard SM, O'Brien FJ (2009) Biomechanical properties across trabeculae from the proximal femur of normal and ovariectomised sheep. J Biomech 42(4):498–503
17. Boskey A, Pleshko Camacho N (2007) FT-IR imaging of native and tissue-engineered bone and cartilage. Biomaterials 28(15): 2465–2478
18. Meiron OE, Bar-David E, Aflalo ED, Shechter A, Stepensky D, Berman A, Sagi A (2011) Solubility and bioavailability of stabilized amorphous calcium carbonate. J Bone Miner Res 26(2): 364–372
19. Castiglioni S, Cazzaniga A, Albisetti W, Maier JA (2013) Magnesium and osteoporosis: current state of knowledge and future research directions. Nutrients 5(8):3022–3033
20. Rude RK, Singer FR, Gruber HE (2009) Skeletal and hormonal effects of magnesium deficiency. J Am Coll Nutr 28(2):131–141
21. Jehle S, Zanetti A, Muser J, Hulter HN, Krapf R (2006) Partial neutralization of the acidogenic Western diet with potassium citrate increases bone mass in postmenopausal women with osteopenia. J Am Soc Nephrol 17(11):3213–3222

22. Opsahl W, Zeronian H, Ellison M, Lewis D, Rucker RB, Riggins RS (1982) Role of copper in collagen cross-linking and its influence on selected mechanical properties of chick bone and tendon. J Nutr 112(4):708–716
23. Marie PJ, Ammann P, Boivin G, Rey C (2001) Mechanisms of action and therapeutic potential of strontium in bone. Calcif Tissue Int 69(3):121–129
24. Fairweather-Tait S, Hurrell RF (1996) Bioavailability of minerals and trace elements. Nutr Res Rev 9(1):295–324
25. Boskey A, Mendelsohn R (2005) Infrared analysis of bone in health and disease. J Biomed Opt 10(3):031102
26. Brennan O, Kennedy OD, Lee TC, Rackard SM, O'Brien FJ, McNamara LM (2011) The effects of estrogen deficiency and bisphosphonate treatment on tissue mineralisation and stiffness in an ovine model of osteoporosis. J Biomech 44(3): 386–390
27. Brouwers JE, van Rietbergen B, Huiskes R, Ito K (2009) Effects of PTH treatment on tibial bone of ovariectomized rats assessed by in vivo micro-CT. Osteoporos Int 20(11):1823–1835
28. Sheng ZF, Dai RC, Wu XP, Fang LN, Fan HJ, Liao EY (2007) Regionally specific compensation for bone loss in the tibial trabeculae of estrogen-deficient rats. Acta Radiol 48(5):531–539
29. Seeman E (2003) Reduced bone formation and increased bone resorption: rational targets for the treatment of osteoporosis. Osteoporos Int 14(Suppl 3):S2–S8
30. Cummings SR, Cosman F, Jamal SA (2002) Osteoporosis: an evidence-based guide to prevention and management. American College of Physicians, Philadelphia