Is there a role for vitamin C in preventing osteoporosis and fractures? A review of the potential underlying mechanisms and current epidemiological evidence

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Abstract

Osteoporosis and related fractures are a major global health issue, but there are few preventative strategies. Previously reported associations between higher intakes of fruits and vegetables and skeletal health have been suggested to be partly attributable to vitamin C. To date, there is some evidence for a potential role of vitamin C in osteoporosis and fracture prevention but an overall consensus of published studies has not yet been drawn. This review aims to provide a summary of the proposed underlying mechanisms of vitamin C on bone and reviews the current evidence in the literature, examining a potential link between vitamin C intake and status with osteoporosis and fractures. The Bradford Hill criteria were used to assess reported associations. Recent animal studies have provided insights into the involvement of vitamin C in osteoclastogenesis and osteoblastogenesis; and its role as a mediator of bone matrix deposition, affecting both the quantity and quality of bone collagen. Observational studies have provided some evidence for this in the general population showing positive associations between dietary vitamin C intake and supplements and higher bone mineral density or reduced fracture risk. However, previous intervention studies were not sufficiently well designed to evaluate these associations. Epidemiological data are particularly limited for vitamin C status and for fracture risk and good quality RCTs are needed to confirm previous epidemiological findings. This review also highlights that associations between vitamin C and bone health may be non-linear and further research is needed to ascertain optimal intakes for osteoporosis and fracture prevention.

Introduction

Osteoporosis is a "progressive systemic skeletal disease characterised by low bone mass and microarchitectural deterioration of bone tissue, with a consequent increase in bone fragility and susceptibility to fracture"[1]. The condition has been estimated to affect 75 million people in Europe, Japan and the United States[2]. Moreover, fragility fractures, the clinical manifestation of osteoporosis, are a major global health issue with an annual prevalence of 8.9 million fractures worldwide[3]. The elderly are the most at-risk population[4] and as the world's population aged 60 and 80 years plus is estimated to increase three and seven fold by 2100, respectively[5], osteoporosis and related fractures will become an increasingly bigger health burden.

Risk factors for the development of osteoporosis and fragility fractures include genetic and biological factors, although environmental factors, including diet, are of great interest for developing preventative strategies, as they are modifiable. To date, a wide range of nutrients, foods and food groups have been studied in relation to bone health, including fruits and vegetables with every increased serving or intakes of 1-4 portions per day, on at least three days per week, being positively associated with increased bone mass or a reduction in bone loss[6-9]. The mechanisms underlying these positive associations have not been fully elucidated but one such explanation is the potential buffering effect of the overall dietary acid load constituents in fruits and vegetables[10]. Moreover, epidemiological studies have suggested that these beneficial effects may also be due to micronutrients such as vitamin C which may have mechanisms independent of these buffering effects[11,12]. Vitamin C, an essential nutrient to humans found in citrus and soft fruits[13,14], has previously been linked to bone health, particularly bone structure. For example, in previous animal studies vitamin C deprivation resulted in a marked reduction in bone formation[15-17]; and superoxide-induced bone loss in mice was restored by oral administration of 1% vitamin C in drinking water, as evidenced by significant improvements in BMD, bone weight, bone strength and collagen cross-links[18]. In the last two decades, observational and intervention studies have investigated a potential role for vitamin C in osteoporosis and fracture prevention; however, an overall consensus of the results of published studies does not exist.

This article provides a review of the potential underlying mechanisms of vitamin C in bone metabolism. The current evidence in the literature investigating a potential role for vitamin C in the prevention of osteoporosis and related fractures will be discussed and avenues for future research highlighted. Databases, including MEDLINE (Ovid), PubMed and Google Scholar, were used to identify relevant observational and clinical studies published up to August 2013. As neither laboratory nor epidemiological studies can infer causality, criteria established by Sir Austin Bradford Hill in 1965 were used to assess whether vitamin C is causal in the prevention of osteoporosis and associated fractures[19]. The structure of the review will be discussed around these criteria.

Bradford Hill criteria

The Bradford Hill criteria (BHC) are a set of guidelines used to assess causality of hypotheses and associations from trial, laboratory and epidemiological research[19]. In brief, the nine criteria assess (1) biological plausibility, (2) coherence between laboratory and epidemiological studies, (3) temporality, (4) consistency, (5) strength, (6) analogy, (7) specificity, (8) dose-response effect, and (9) evidence from intervention studies. The criteria may not confirm the absence or presence of causality unconditionally, but are considered to be a useful tool for understanding associations between an exposure and a risk of disease.

Potential mechanisms of vitamin C in bone health

Scurvy, the clinical manifestation of vitamin C deficiency, is associated with wounds and fractures that fail to heal. The discovery of vitamin C in the early 20th century and subsequent animal studies lead to the suggestion that scurvy symptoms result from impaired collagen formation in vitamin C deficiency[20]. Collagen is an essential component of bone tissue; and more recently, many cell and animal studies reported that vitamin C may also mediate osteoclastogenesis and osteoblastogenesis[21-24], although the precise biological mechanisms have not been fully established yet.

Osteoclastogenesis

Vitamin C has been suggested to mediate osteoclast differentiation and possibly apoptosis[22,25] and findings have been relatively consistent. In cell cultures containing both osteoblasts and osteoclasts, vitamin C promoted osteoclastogenesis[26-28] and this was associated with an increase in RANKL expression[27]. In concordance with these findings, vitamin C deficiency resulted in a decrease in osteoclast differentiation[26,27]. However, in cultures containing only osteoclasts, stimulatory effects[29] as well as inhibitory effects[22,28,30] of vitamin C on osteoclast differentiation have been reported. Recent *in vitro* findings have helped explain these contradictory results by showing that vitamin C at a concentration of 50 µg/ml initially exhibited pro-oxidant activity resulting in an increase in the number, size and nucleation of osteoclasts, although vitamin C also initiated accelerated osteoclast death at later stages[25]. Deficiency studies are in agreement with most previous findings, indicating that vitamin C deficiency in animal models stimulated osteoclastogenesis via the up-regulation of the RANKL/RANK pathway[22,23]. Moreover, vitamin C deficient mice supplemented with vitamin C had a reduction in RANKL expression[23]. Although there is some consistency of previous cell and animal studies reporting on the effects of vitamin C on osteoclastogenesis, the current discrepancies require further investigation in humans to help decide if vitamin C may be involved in osteoclastogenesis via mediating the RANK/RANKL pathway.

Osteoblastogenesis

Vitamin C may be involved in accentuating osteoblastogenesis. For example, a decrease in the number of osteoblasts and suppressed osteoblast differentiation has previously been observed in vitamin C deficient mice[23]. In concordance with these findings, an increase in the number of osteoblasts following vitamin C treatment has been reported from *in vitro* work[31]. Furthermore, studies using osteoblast-like cell cultures including human tissue have shown that osteoblast proliferation and differentiation was enhanced with the addition of vitamin C[21,24,31-33]. Concentrations of 50 µg/ml and 200 µg/ml vitamin C have previously been suggested as optimal and maximum concentrations for this effect[21,24].

Initially, work suggested that the effects of vitamin C on osteoblastogenesis may be through stimulating collagen synthesis[31,32], although more recent evidence suggests the underlying mechanisms are more complex. For example, vitamin C has been reported to mediate gene expression of a number of genes involved in pre-osteoblast cell activities including growth, metabolism, communication and death[34]. Furthermore, animal studies have shown that the expression of PPAR-γ may mediate osteoblast differentiation resulting in bone loss[35,36]. Recently, these findings have been investigated further and a link to vitamin C has been established. An *in vivo* study reported that PPAR-γ expression in osteoblasts was significantly up-regulated in vitamin C deficient mice and was accompanied by suppressed osteoblast differentiation; whereas treatment with vitamin C mediated PPAR-γ expression to almost normal levels[23]. To date, there is consistent experimental evidence for a beneficial role of vitamin C in osteoblastogenesis. Recent work suggesting that vitamin C may mediate PPAR-γ expression has provided more insight in to the mechanisms, and further experimental studies are needed to confirm these findings.

Bone collagen synthesis

Vitamin C is essential for collagen type I synthesis by osteoblasts. For example, early *in vitro* work reported that collagen synthesis increased more than four fold in the presence of ascorbate[37] and more recently, greater amounts of collagen were shown to be present at vitamin C concentrations of 200 µg/ml compared to 100 µg/ml and 25 µg/ml[24]. The underlying mechanisms for this are thought to relate to the role of vitamin C in stimulating collagen synthesis and as a cofactor of hydroxylation reactions within collagen fibres. For the former, vitamin C is an important initiator of collagen synthesis in osteoblasts[38], possibly via stimulating pro-collagen type I mRNA[39,40]; whereas for the latter, vitamin C is an essential activator of enzymes involved in the hydroxylation of proline and lysine residues within collagen fibres[41-44]. The hydroxylation reaction enables the formation of covalent bonds between the amino acid residues, increasing overall collagen strength. Early *in vitro*

and *in vivo* studies found that the lack of ascorbic acid resulted in the formation of underhydroxylated and unhydroxylated collagen[45-49], thus decreasing bone matrix stability and weakening bone structure. In contrast, the presence of vitamin C increased the hydroxylation of amino acid residues *in vitro*[50]. The hydroxylation of amino acid residues may occur while the collagen polypeptide chain is still being synthesised and attached to the ribosome[51,52]. However, more recent work suggested that this hydroxylation reaction takes place in the endoplasmic reticulum[53].

Experimental evidence for a role of vitamin C in bone collagen synthesis is well established. Vitamin C is important for the quality of collagen via its cofactor role in hydroxylation reactions in collagen fibres. Future studies should focus on the importance of vitamin C for the quantity of collagen synthesis via stimulating procollagen type I mRNA as there are currently only limited data on this potential link.

In summary, a range of mechanisms of vitamin C in maintaining bone health have been suggested in a number of experimental studies. Thus, there is some good evidence for the BHC of biological plausibility for vitamin C deficiency and osteoporosis. The evidence for a role of vitamin C in osteoblastogenesis and in quality aspects of bone collagen synthesis is consistent. In contrast, the links between vitamin C and osteoclastogenesis as well as quantity aspects of collagen synthesis are currently less well defined and require further investigation.

Measures of vitamin C intake and status

Vitamin C intake may be measured from dietary assessment methods such as food diaries and FFQs[54]. Food diaries assess habitual intake through a detailed description of foods and drinks consumed typically in the preceding three to seven days and FFQs make use of a food list with a frequency response section estimating intake usually from the previous 12 months. The mean vitamin C intake in the UK is 90 mg/d (calculated using food records) [55], reflecting sufficient intake according to the Reference Nutrient Intake (RNI) of 40 mg/d[13] and in comparison to the US recommendations of 90 mg/d and 75 mg/d for men and women, respectively[56]. The Lower Reference Nutrient Intake (LRNI) has been set in the UK at 10 mg/d and is based on the prevention and cure of scurvy[13]. Currently, there is no upper limit for vitamin C intake. However, very high intakes of 1000 mg/d and above, achieved through the use of supplements, may present with side effects including gastrointestinal discomfort and diarrhoea[57] and have previously been shown to increase the risk of renal stones[58].

The ability to accurately assess vitamin C intake varies between the different dietary methods, with the correlation coefficients between blood vitamin C concentrations and dietary intake being higher for food diaries, dietary recalls (both r 0.46; 95% CI 0.41, 0.52) and weighed records (r 0.39;

95% CI 0.25, 0.53) compared to the correlation coefficient between blood vitamin C concentrations and dietary intake estimated from FFQs (r 0.35; 95% CI 0.29, 0.40)[54]. Despite the ability to estimate vitamin C intake, the measurement of vitamin C status from blood may be more accurate than dietary intake assessments as it avoids human recall error and variations in individual bioavailability of the nutrient and accounts for factors that affect the vitamin C composition of food including length of storage of food items and cooking practises[59]. However, vitamin C in blood is influenced by a number of biological and lifestyle factors including age[60], sex[61,62], BMI[60], body fat distribution[63], smoking[64,65] and infection[66] which should be accounted for when evaluating its association with disease risk.

Dietary intake and plasma concentrations of vitamin C, when plotted against each other, show a sigmoidal relationship[67,68]. Average vitamin C intakes (60-100 mg/d) reflect plasma levels of around 40-60 µM/l. Higher intakes result in a progressive flattening of the curve and very high intakes of 400 mg/d and above appear to saturate vitamin C in plasma at concentrations of 70-85 µmol/l, leading to the excretion of the vitamin[68]. The mean plasma vitamin C concentration of the general UK population is 53 µmol/L[69]. Vitamin C status may be categorised as severely deficient at plasma levels below 11 µmol/L indicating biochemical depletion; and 1% of men and 2% of women in the UK are classified as such[69].

Current evidence on vitamin C, osteoporosis and fracture prevention

There is evidence from epidemiological studies for a potential role of vitamin C in maintaining different aspects of bone health, although the results have varied between studies. In the next section, randomised controlled trials (RCTs) as the best indicator of causality will be discussed first and this will be followed by observational studies in hierarchical order of decreasing ability to determine causality. All types of studies will be evaluated against the BHC.

Intervention studies

RCTs are the only studies that can definitively infer causality and determine factors influencing disease, making them the gold standard in limiting selection bias and confounding. To our knowledge, there is only one such published RCT with a double-blind design that has examined the effects of vitamin C supplementation on indicators of bone health (Table 1). The study involving 30 men and women compared bone density of one group taking a placebo with that of two groups receiving 400 IU of vitamin E daily and either 500 mg/d or 1000 mg/d of vitamin C for 12 months[70]. The group with the highest vitamin C intake had significantly less hip bone loss compared to the placebo group (effect sizes and P-values not shown), although no such observations were made at the lumbar spine. However, this study did not investigate the effects of vitamin C independently and the inclusion

criteria allowed for smokers and for participants with controlled chronic disease which may have biased the study outcomes. Thus, it remains unclear to what extent vitamin C was involved in preventing bone loss in this study.

Two intervention studies used a combination of an exercise programme and supplementation with vitamin C and E[71,72]. The first study was a randomised placebo-controlled pilot study in 34 women who followed an intervention of 60 minutes of resistant training three times per week and daily supplementation with vitamin C (1000 mg/d) and E (600 mg/d) for six months. Women were randomised into four treatment groups of placebo, vitamins, exercise and placebo, or exercise and vitamins[72]. BMD of the lumbar spine but not the femoral neck decreased significantly by 1% in the placebo group over six months (BMD pre: 1.01 ± 0.17 g/cm^2; BMD post: 1.00 ± 0.16 g/cm^2; P<0.05) and was maintained in the other groups. No additive effects of the exercise intervention and the vitamin supplementation were found. However, the results may have been biased by changes in dietary habits as a reduction in vitamin C intake over the course of the study period was reported for the vitamin intervention group. Moreover, the study did not report on blinding in the protocol. The second study, a two month intervention in 13 men and women, included an hour of aerobic exercise three times per week and the daily use of vitamin C (500 mg/d) and vitamin E (100 mg/d) supplements for all subjects[71]. Although markers of calcium homeostasis improved significantly (effect sizes not reported), the bone formation marker BSALP decreased unexpectedly by 14.5% (P-value not reported). However, this study lacked a control group, was undertaken in only 13 individuals, and since it was a mixed intervention, the effects of vitamin C could not be distinguished. Moreover, both studies were of short duration of only two to six months, although changes in BMD are more likely to be observed after a longer duration of treatment.

In summary, evidence from current trials investigating potential preventative effects of vitamin C in osteoporosis remains equivocal, even though the doses were greater than with diet alone. There are limitations regarding study design, inclusion and exclusion criteria, limited duration of treatment, small sample sizes and dietary intake that was not controlled for. Moreover, published intervention studies have used vitamin supplements containing vitamin E in addition to vitamin C and have included exercise programmes during treatment. Future trials should consider having more participants, stricter inclusion and exclusion criteria and interventions consisting of vitamin C supplementation only. The BHC of evidence from intervention studies is therefore not met.

Prospective and longitudinal studies

Prospective cohort studies may be used to investigate the aetiology of a disease as the exposure is measured prior to the condition occurring, making studies less prone to recall bias than case-control studies. They may thus also be used to evaluate the BHC of temporality. Furthermore, as cases and

controls are drawn from the same population, there is less selection bias. To date, only one prospective and two longitudinal studies have investigated potential vitamin C and bone associations (Table 2). One study of 944 men and women from the UK with a mean age of 72 years reported significantly less total hip BMD loss of up to 54% for higher dietary intakes of vitamin C (99-363 mg/d) compared to lower intakes (7-57 mg/d)[73]. Another study using a US cohort of 606 subjects with a mean age of 75 years reported that lumbar spine and trochanter BMD loss, but not femoral neck and radial shaft BMD loss, decreased significantly across tertiles of dietary vitamin C intake in men but not in women[74]. However, as highlighted above, the findings were not consistent across these two studies with results varying mainly for gender and bone site. Potential explanations for this might be that the first study used 7-day food diaries and did not adjust for important confounders including age, gender and smoking[73], in contrast to the second study which used a semiquantitive FFQ and measured BMD via two different types of bone scans (i.e. DPA at baseline and DXA at follow-up)[74]. However, DXA scans have been shown to produce lower results than DPA scans[75], hence the effect size in this study may be more modest than the true result.

A potential role for vitamin C in fracture prevention has only been investigated in one previous prospective study of 918 US men and women with a mean age of 75 years. There was a risk reduction in hip fracture of 44% for supplemental vitamin C intake (mean: 260 mg/d compared to 0 mg/d) and of 69% for total (dietary and supplemental) vitamin C intake (mean: 313 mg/d compared to 94 mg/d) after 15-17 years of follow-up (RR and 95% CI not reported), although no significant risk reductions were found at other fracture sites[76]. As this study was comparatively small, further large prospective cohort studies of older men and women with long follow-up, which investigate fractures as the clinical endpoint of osteoporosis, are needed.

In summary, there are only limited data from three prospective and longitudinal studies investigating potential associations between vitamin C and bone health. Although these prospective studies meet the BHC of temporality, it is difficult to assess the strength of the associations and the potential for a dose-response relationship as not all studies reported effect sizes. Moreover, issues regarding analogy, inferring the absence of another confounder related to the predictor variable, and consistency were present. A greater number of prospective and longitudinal studies and more concordant adjustment for confounding factors may help establish more consistent findings of the relationship between vitamin C intake and osteoporosis and associated fractures. Moreover, the lack of evidence for a relation between vitamin C status and bone health needs to be investigated further as the only study investigating this did not adjust for age, gender and smoking[73].

Case-control studies

Case-control studies, summarised in Table 3, are used to examine specific exposures as potential risk factors of a disease in people with and without the condition. Recall bias, where case subjects tend to have a better recollection of specific exposures than the controls, and selection bias, resulting from both outcomes being pre-defined, are common issues of these studies. To date, three case-control studies have consistently shown that osteoporosis and fracture patients had lower serum vitamin C concentrations (cases: 17-37 μmol/L; controls: 23-54 μmol/L) and lower plasma vitamin C concentrations (cases: 30 μmol/L; controls: 55 μmol/L) than controls[77-79]. Only one study reported differently, but the authors inferred that their findings reflected most recent changes in food intake[80].

In contrast to vitamin C status measures, findings for potential differences in dietary vitamin C intakes between cases and controls are less consistent[79,80]. Differences in measures of dietary intake and relatively small sample sizes may explain some of these inconsistent findings. However, associations with osteoporosis and fracture risk were reported when population intakes were stratified into quartiles of dietary vitamin C intake. For example, one case-control study showed a marginally significant fracture risk reduction for participants in the second quartile of vitamin C intake compared to the first (OR 0.39, 95% CI 0.15, 1.00; vitamin C intake range: 204-247 mg/d compared to ≤203 mg/d)[79]. This was not significant for higher vitamin C intakes, possibly due to the high vitamin C intake of the study population (mean: 200 mg/d). Moreover, another case-control study reported that those in the third quartile of vitamin C intake had a significantly reduced risk of osteoporosis referent to the lowest quartile (OR 0.29, 95% CI 0.09, 0.96; vitamin C intake range: 137-176 mg/d compared to ≤92 mg/d)[81]. Recall bias in this study was low due to the diagnosis of osteoporosis at screening and the subsequent reporting of current vitamin C intake.

In conclusion, published case-control studies of osteoporosis and fracture patients have reported consistently lower blood vitamin C concentrations but not dietary intake of vitamin C. Thus, the BHC of consistency is currently not fulfilled. Although reported effect sizes appear to be large, this evidence is currently limited to only two studies. More case-control studies are needed to help clarify the discrepancies in vitamin C intake between osteoporosis and fracture patients and matched controls currently reported in the literature.

Cross-sectional studies

Cross-sectional studies are used to report the prevalence of a disease in a defined population at a specific point in time. Whether the exposure predated the disease or not cannot be determined. Previous cross-sectional studies are summarised in Table 4. Positive associations indicated that higher dietary vitamin C intake was associated with 3-5% higher BMD[6] and every 100 mg/d increment in vitamin C intake was associated with 0.01-0.02 g/cm^2 higher BMD[11,12], although there is currently limited understanding of this clinical relevance. Moreover, users of vitamin C supplements (mean

[range] = 745 mg/d [70-5000 mg/d]) had 4% higher BMD and users of supplement doses of ≥1000 mg/d had 14% higher BMD than non-users[82]. Although positive associations between dietary vitamin C intake and supplements and bone density have previously been reported, findings have been inconsistent[8,9,74,83-85]. The use of different dietary assessment methods as means of measuring vitamin C intake and differences in the adjustment for confounding factors may explain some of these discrepancies. Dietary methods have included semiquantitative FFQs with 97–126 food items[6,8,11,74,84,86,87], three to seven-day food diaries[9,88] and 24-hour recalls[12,83]. Moreover, total (dietary and supplemental) vitamin C intake has not been linked with BMD in women[83,84,88]; and both positive and negative associations have been reported in men[74], although the latter findings may have been biased by the population's smoking behaviour. Dietary intakes of vitamin C have previously been shown to be significantly lower in smokers than non-smokers[65] and serum vitamin C levels are lower in smokers independent of dietary intakes[64,65]. Hence, the exclusion of smokers to the study may have led to more consistent findings.

Potential associations between vitamin C from the diet or in serum and fracture risk have currently been examined in only one cross-sectional study of more than 13000 men and women aged 20-90 years[12]. Findings were non-significant, although men with mean dietary vitamin C intakes of 200 mg/d reported fewer fractures than men with higher or lower intakes. One may be critical about the large age range of the study population. As osteoporosis and associated fractures are known to be more prevalent in the elderly population[4], the inclusion of very young participants may be an explanation for the non-significant findings.

Cross-sectional data on vitamin C and markers of bone homeostasis are sparse with only two studies investigating potential associations. One study found that higher intakes of vitamin C were associated with lower excretion of deoxypyridinoline *(no effect size shown)*, indicating reduced bone resorption[8]. Similarly, the other study reported a significant association between the duration of vitamin C supplement use and markers of bone resorption, with serum CTX concentrations being 0.022 pg/mL lower for every 1-year supplement use increment[85].

Although there are data from a number of cross-sectional studies investigating vitamin C and bone health associations, the BHC of consistency, analogy and temporality were not fulfilled. The effect sizes of present cross-sectional studies are comparable to those previously reported for other dietary factors including potassium, although many studies did not report effect sizes. The limited number of Bradford Hill criteria currently fulfilled by cross-sectional studies may indicate that the reported associations between vitamin C intake and osteoporosis and fractures are less reliable evidence than relationships reported by prospective cohort studies and RCTs.

In summary, support for studies, which have investigated the potential underlying mechanisms between vitamin C and osteoporosis prevention, has come from a variety of epidemiological studies, although differences in study populations, dietary exposure, outcome measures and use of confounding factors in statistical analyses may have resulted in inconsistent findings. Current observational data are particularly limited for men as most studies have consisted of only women and for biological markers of vitamin C status which may be less subjective to recall bias and factors influencing the vitamin C content of food[89]. More observational studies in the general population are needed to address these limitations.

Moderate versus high vitamin C intakes

Results from three observational studies have indicated that significant associations with bone health were surprisingly stronger for moderate rather than higher vitamin C intakes[6,79,81]. For example, vitamin C intake was significantly associated with higher bone density or a reduction in fracture risk for the second quartile[79] or for the third quartile[6,81] of vitamin C intake rather than the highest intake levels. Similar cross-sectional observations have been reported for associations with serum vitamin C levels[12]. This may suggest that vitamin C may be related to bone density in a bell-shaped dose-response fashion with intakes below and above the optimum not being beneficial. The potential underlying mechanisms for this may relate to the properties of vitamin C rather than bone tissue itself. It has previously been suggested that vitamin C may not only have antioxidant properties, but may also exhibit pro-oxidant traits at higher concentrations, as supplementation of men and women with 500 mg of vitamin C per day was shown to promote oxidative DNA damage[90] which may also be relevant to osteoporosis. Moreover, there is evidence from *in vitro* studies of a vitamin C dose-dependent suppression of bone cell growth and differentiation as well as collagen type I synthesis[21,24,91]. For example, vitamin C concentrations of 50 µg/ml were optimal for stimulation of human osteoblast-like cell lines and collagen type I synthesis, whereas higher levels resulted in the inhibition of cell differentiation[21]. Another experimental study investigating bovine osteoblast-like cell proliferation observed similar effects, although vitamin C concentrations of 200 µg/ml were found to be most effective[24]. As suggested by the authors, the use of different cell types may be an explanation for the inconsistencies in optimal vitamin C concentrations in these cell culture studies. The potential bell-shaped dose-response relationship between vitamin C and indicators of bone health may also further explain the lack of positive results reported in the intervention studies discussed above which included high supplement doses of 500–1000 mg/d. The potentially detrimental effects of higher vitamin C concentrations on the skeleton need to be investigated further; and this is a crucial step towards establishing optimal vitamin C intake levels.

Discussion and conclusions

Evaluating the current evidence for a potential role of vitamin C in osteoporosis and fracture prevention according to the Bradford Hill criteria (BHC) in the absence of RCTs provides some clarity regarding causality[19]. The BHC of specificity, inferring that a cause leads to a single effect, cannot be met as biological functions of vitamin C are versatile. However, there is emerging experimental evidence for a potential role of vitamin C in bone health, thus fulfilling the BHC of biological plausibility. The mechanisms include the involvement of vitamin C in osteoclastogenesis via RANKL expression, osteoblastogenesis via PPAR-γ expression[22,23] and collagen synthesis via stimulation of pro-collagen mRNA expression and the hydroxylation of collagen fibres[38-40]. A number of observational studies support these findings, thus the BHC of coherence between laboratory and epidemiological studies is met. However, differences in study populations, different methods of measuring dietary exposure, outcome measures and use of confounding factors in these observational studies may have resulted in inconsistent findings. Consequently, the BHC of consistency and analogy are currently not fulfilled. Addressing these limitations in future epidemiological studies may help establish more consistent results.

Most observational studies published to date were of a cross-sectional nature. Thus, the BHC of temporality, inferring that the exposure preceded the disease outcome, was not met, and more cohort studies in the general population are needed to overcome this problem. Moreover, evaluating the BHC of the strength of the association based on the evidence currently available in the literature leads to equivocal conclusions as a large number of studies did not report effect sizes of their findings. Future studies should report effect sizes to help understand the overall clinical relevance of vitamin C for the prevention of osteoporosis and fractures.

The present review has highlighted that potential associations between vitamin C and bone health may not follow an expected dose-response curve due the vitamin exhibiting antioxidant properties at lower and pro-oxidant traits at higher concentrations. Potentially detrimental effects on the skeleton from higher vitamin C concentrations need to be investigated further, as this may be an issue with vitamin C supplementation, and understanding this is a crucial step towards establishing optimal vitamin C intake levels for the general population.

The final BHC of evidence from intervention studies is currently not fulfilled; although the conventional hierarchy of the validity of study designs may be less applicable to nutritional research, as cross-sectional studies tend to capture long-term dietary intake more so than intervention studies. Nevertheless, published intervention studies were not designed to evaluate the independent effects of vitamin C supplementation on potential improvements in bone health as interventions included additional supplementation with vitamin E and exercise programmes. Overall, the data are limited as only one double-blind RCT and two intervention studies have investigated this and dietary intake was

not controlled for. Moreover, further issues regarding study design, inclusion and exclusion criteria, duration of treatment and sample size were present. To our knowledge, published RCTs investigating the potential link between vitamin C and bone that use a supplement containing vitamin C only are still lacking and are urgently needed.

In conclusion, over the last few decades, *in vitro* and *in vivo* studies have provided insights and knowledge as to how vitamin C may influence the mechanisms that benefit the skeleton; and observational studies have provided some evidence for a potential role of vitamin C in osteoporosis and fracture prevention. However, data are limited as good quality studies are scarce and more investigations, particularly well-designed RCTs, are urgently needed to address the limitations outlined in this review.

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Conflicts of interest

There are no conflicts of interest.

Authorship

A. A. W. was the principal investigator and initiated the study. H. F. conducted the literature review and drafted the manuscript. A. A. W. and A. R. H. directed the preparation of the manuscript and A. A. W., A. R. H and A. J. contributed significantly to the drafting and critical reviewing of the article. All authors read and approved the final manuscript.

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Table 1. Summary of intervention studies investigating the effects of vitamin C on bone mineral density and markers of bone turnover.

Study	Subjects	Duration; study design	Age (yrs)	Primary outcome	Intervention	Results*	Comments
Maimoun[71] 2008 France	*n* 13 (4 men, 9 women)	2 months; /	69 - 79	BSALP, OC and CTX	No groups. All participants received the following treatment: 60 min of aerobic exercise 3 times/wk, vit. C (500 mg/d) & vit. E (100 mg/d)	M	BSALP concentration decreased sig. by 14.5% (*P=Data not reported*).
Chuin[72] 2009 Canada/France	*n* 34 (women)	6 months; randomised, controlled pilot study	61 - 73	FN and LS BMD	4 groups. Placebo group (*n* 7): placebo (lactose); Vit. group (*n* 8): ascorbic acid (1,000 mg/d) & α-tocopherol (600 mg/d); Exercise & placebo group (*n* 11): 60 min of resistance training 3 times/wk & placebo (lactose); Exercise & vit. group (*n* 8): 60 min of resistance training 3 times/wk & ascorbic acid (1,000 mg/d) & α-tocopherol (600 mg/d)	M	LS BMD decreased sig. by 1% in the placebo group (BMD pre: 1.01 ± 0.17 g/cm^2; BMD post: 1.00 ± 0.16 g/cm^2; $P<0.05$) but remained stable in the three intervention groups.
Ruiz-Ramos[70] 2010 Mexico	*n* 90 (25 men, 65 women)	12 months; double-blind RCT	68	TH and LS BMD	3 groups: Placebo group (*n* 30): placebo *(no details)*; Low vit. group (*n* 30): ascorbic acid (500 mg/d) & α-tocopherol (400 IU/d); High vit. group (*n* 30): ascorbic acid (1000 mg/d) & α-tocopherol (400 IU/d)	M	The high vit. group lost sig. less TH bone compared to the placebo group *(Details not reported)*.

BSALP, alkaline phosphatase; OC, osteocalcin; CTX, collagen type 1 cross-linked C-telopeptide; vit., vitamin; sig., significant(ly); FN, femoral neck; LS, lumbar spine; BMD, bone mineral density; RCT, randomised placebo-controlled trial; TH, total hip.

** Results were significant (S), non-significant (NS) or of mixed nature (M).*

Table 2. Prospective and longitudinal studies assessing associations between vitamin C intake or status and bone mineral density or fracture risk.

Study	Follow-up	Subjects	Age (yrs)	Dietary assessment	Vit. C intake (mg/d)*	Outcome measures and analyses	Results†	Comments
Kaptoge[73] 2003 UK	2-5 yrs	*n* 944 (470 men; 474 women)	72 (67-79)	7dD	Median (range) dietary intake: Tertile 1 = 73 (7-57) Tertile 2 = 78 (58-98) Tertile 3 = 132 (99-363) *Data for plasma levels not shown.*	2-5 year change in TH BMD stratified by tertiles of either dietary vit. C intake or plasma vit. C levels	Diet: M Plasma: NS	Women in tertile 2 and 3 of dietary vit. C intake had approx. 52% and 54% less TH BMD loss, respectively (*P*=0.015 and *P*=0.010; *P*-trend=0.016).
Sahni[74] 2008 US	4 yrs	*n* 606 (213 men; 393 women)	75	FFQ	Mean (SD) dietary intake: Men = 141 (73) Women = 158 (83) Mean (SD) suppl. intake: Men = 82 (235) Women = 95 (248) Group 1 = 0 Group 2 < 90 / 75‡ Group 3 ≥ 90 / 75‡ Mean (SD) total intake: Men = 223 (259) Women = 253 (267) *Intake data for tertiles not shown.*	4-year change in LS, FN, T and RS BMD stratified by tertiles of dietary or total vit. C intake or categories of suppl. vit. C intake and either calcium intake, vit. E intake, smoking or oestrogen use	Diet: M Suppl.: NS	LS and T BMD loss was sig. less with higher dietary vit. C intakes in men (*P*-trend≤0.05). FN and T BMD loss was sig. less for higher total vit. C intake among men with low calcium intakes and with low total vit. E intakes (*P*-trend≤0.03). A 102% reduction in T BMD loss between extreme tertiles of total vit. C intake among men with low calcium intakes (*P*<0.05).
Sahni[76] 2009 US	15-17 yrs	*n* 918 (39.1% men; 60.9% women)	75	FFQ	Median (range) dietary intake: Tertile 1 = 86 Tertile 2 = 133 Tertile 3 = 208 Suppl. intake: Tertile 1 = 0 Tertile 2 < 75 Tertile 3 ≥ 75 Median (range) total intake: Tertile 1 = 94 / 95§ Tertile 2 = *Data not shown* Tertile 3 = 313 / 308§	Risk of hip fracture or non-vertebral fracture stratified by tertiles of dietary, suppl. or total vit. C intake in the combined sample of men and women	Diet: NS Suppl.: M Total: M	A reduction in hip fracture of 69% between extreme tertiles of suppl. vit. C intake (*P*=0.007; *P*-trend=0.02) and of 44% for total vit. C intake (*P*=0.04; *P*-trend=0.04).

Vit., vitamin; 7dD, 7-day food diary; TH, total hip; BMD, bone mineral density; approx., approximately; FFQ, food frequency questionnaire; suppl., supplement(al); LS, lumbar spine; FN, femoral neck; T, trochanter; RS, radial shaft; sig., significant(ly).

** Total intake is the sum of dietary intake and intake from supplements.*

† Results were significant (S), non-significant (NS) or of mixed nature (M).

‡ Data shown for men / women.

§ Data shown for hip / non-vertebral fracture analyses.

Table 3. Case-control studies assessing vitamin C intake or status in osteoporosis and fracture patients in comparison to controls.

Study	Subjects	Age (yrs)	Dietary assessment	Mean or range vit. C intake or blood conc.	Outcome measure(s) and analyses	Results*	Comments
Falch[77] 1998 Norway	*n* 40 hip fracture cases; 102 controls (men and women)	83	N/A	Serum conc.: CA = 37 µmol/L, CO = 50 µmol/L Serum conc. in 20 age-matched case-control pairs: CA = 34 µmol/L, CO = 54 µmol/L	Serum vit. C conc. in cases and controls or in 20 case-control pairs matched for age	Serum: S	Serum vit. C conc. were significantly lower in cases than in controls (P<0.01).
Lumbers[80] 2001 UK	*n* 75 hip fracture cases; 50 controls (women)	80 (61-103)	three 24hR	Dietary intake: CA = 60.7 mg/d, CO = 55.2 mg/d Plasma conc.: CA = 42.7 µmol/L, CO = 20.8 µmol/L	Vit. C intakes or plasma conc. in cases and controls	Intake: NS Plasma: S	Plasma conc. were significantly higher in cases than in controls (P<0.001).
Maggio[78] 2003 Italy	*n* 75 osteoporosis cases; 75 controls (women)	60+	N/A	Plasma conc.: CA = 30.0 µmol/L, CO = 55.5 µmol/L	Plasma vit. C conc. in cases and controls	Plasma: S	Cases had sig. lower plasma vit. C conc. than controls (P<0.001).
Martinez-Ramirez[79] 2007 Spain	*n* 167 fracture cases; 167 controls (20% men; 80% women)	65+	FFQ	Intake: CA = 268 mg/d, CO = 275 mg/d Quartile 1 ≤ 203 mg/d Quartile 2 = 204-247 mg/d Quartile 3 = 248-334 mg/d Quartile 4 > 334 mg/d Serum conc.: CA = 17.6 µmol/L, CO = 23.3 µmol/L Quartile 1 ≤ 8.4 µmol/L Quartile 2 = 8.5-19.6 µmol/L Quartile 3 = 19.7-34.1 µmol/L Quartile 4 > 34.1 µmol/L	Vit. C intakes or serum conc. in cases and controls and in association with fracture risk	Intake: M Serum: S	A marginal sig. fracture risk reduction for quartile 2 *versus* 1 of vit. C intake (OR 0.39, 95% CI 0.15, 1.00; P-trend=0.87). Mean serum conc. were sig. lower in cases than in controls (P=0.012). A sig. reduction in fracture risk for quartile 4 *versus* 1 of serum conc. (OR 0.31, 95% CI 0.11, 0.87; P-trend=0.03).
Park[81] 2011 South Korea	*n* 72 osteoporosis cases; 72 controls (women)	50-70	FFQ	Dietary intake: Quartile 1 ≤ 91.5 mg/d Quartile 2 = 91.5-136.9 mg/d Quartile 3 = 136.9-176.3 mg/d Quartile 4 > 176.3 mg/d	Dietary vit. C intake & risk of osteoporosis	Intake: S	A sig. reduction in the risk of osteoporosis for quartile 3 *versus* 1 of dietary vit. C intake (OR 0.29, 95% CI 0.09, 0.96; P-trend=0.24).

Vit., vitamin; conc., concentration(s); N/A, not applicable; CA, cases; CO, controls; 24hR, 24-hour dietary recall; FFQ, food frequency questionnaire.
**Results were significant (S), non-significant (NS) or of mixed nature (M).*

Table 4. Cross-sectional studies assessing associations between vitamin C intake or status and bone mineral density, markers of bone turnover or fracture risk.

Study	Subjects	Age (yrs)	Dietary assessment	Mean (SD); range vit. C intake* or blood conc.	Outcome measures and analyses	Results†	Comments
Sowers[83] 1985 US	*n* 324 (women)	67 (55-80)	24hR	Total intake: Low calcium group = 211 (351) mg/d Low calcium group = 268 (309) mg/d	Association between MR BMD and vit. C intake	Total: NS	Vit. C intake was only marginally associated with MR BMD (*Effect size not shown*; *P*=0.051).
Leveille[86] 1997 US	*n* 1892 (women)	72 (55-64)	FFQ	Dietary intake = 113 (52); 12-399 mg/d Suppl. intake = 294 (447); 0-2500 mg/d Duration of suppl. use: Group 1 = non-user Group 2 = 1-5 yrs Group 3 =5-10 yrs Group 4 ≥ 10 yrs Total intake = 407 (454); 13-2560 mg/d	FN BMD stratified by vit. C intake or FN BMD stratified by duration of vit. C suppl. use and either age groups (55-64yrs, 65-74yrs and 75+) or oestrogen use	Diet: NS Suppl: M Total: NS	Approx. 6.7% and 3.2% higher FN BMD for longest supplement users compared to non-users in women aged 55-64yrs (*P*=0.02; *P*-trend=0.01) and in women who had never taken oestrogen (*P*=0.02; *P*-trend=0.02), respectively.
New[6] 1997 UK	*n* 994 (women)	47 (44-50)	FFQ	Dietary intake = 126 (96); 16-1164 mg/d *Intake data for quartiles not shown.*	LS, FN, T and WT BMD stratified by quartiles of dietary vit. C intake	Diet: S	Dietary vit. C intake correlated sig. with LS BMD (r^2 0.10; *P*<0.001). Approx. 4.5% higher LS BMD (*P*<0.002), 3% higher FN BMD (*P*<0.01) and higher T and WT BMD *(Effect sizes not shown*; *P*<0.02*)* for quartile 3 *versus* 1 of dietary vit. C intake.
Hall[11] 1998 US	*n* 775 (women)	56 (45-64)	FFQ	Dietary intake = 140 (76) mg/d *Note: dietary calcium intake:* *Low (n 199) < 500 mg/d* *High (n 574) > 500 mg/d*	LS, FN and TH BMD stratified by 100mg/d increments of dietary vit. C intake with and without additional stratification by low and high dietary calcium intake	Diet: M	FN and TH BMD were 0.017 g/cm^2 higher for each 100 mg/d increase in dietary vit. C intake (*P*=0.002 and *P*=0.005). For every 100 mg/d increment in dietary vit. C intake, LS, FN and TH BMD increased sig. by 0.0199 g/cm^2 (*P*=0.024), 0.0190 g/cm^2 (*P*=0.002) and 0.0172 g/cm^2 (*P*=0.010), respectively, in those with high calcium intakes.
New[8] 2000 UK	*n* 62 (women)	47 (45-54)	FFQ	Dietary intake = 103 (66); 24-453 mg/d *Intake data for quartiles not shown.*	LS, FN, T, WT and forearm BMD and PYD, DPD and OC stratified by quartiles of dietary vit. C intake	Diet: M	Sig. lower mean DPD excretion across quartiles of dietary vit. C intake (*Effect size not shown*; *P*-trend<0.02).
Morton[82] 2001 US	*n* 994 (women)	72 (50-98)	N/A	Suppl. intake: Non-users = 0 mg/d Users = 745 mg/d; 70-5000 mg/d Group 1 = 0 mg/d (non-users) Group 2 ≤ 500 mg/d Group 3 ≥ 1000 mg/d	LS, FN, TH, MR and UR BMD stratified by use of vit. C suppl. with and without additional stratification by	Suppl.: M	4.1% higher FN BMD for suppl. users compared to non-users (*P*=0.02). For current users of oestrogen, calcium and vit. C suppl., BMD was higher by approx. 6% at the TH (*P*=0.05), 9% at the FN (*P*=0.0001) and 12% at the UR (*P*=0.02) compared to non-vit. C users. Approx. 14%

					oestrogen use or by oestrogen and calcium use; and BMD stratified by dose of vit. C suppl.		higher UR BMD for women with the highest vit. C suppl. dose compared to non-users (P<0.05; P-trend<0.04).
Simon[12] 2001 US	*n* 13080 (6137 men; 6943 women); (*n* 11849 for BMD analyses)	(20-90)	24hR	Men: Dietary intake = 102 (104) mg/d Serum conc. = 38.0 (23.8) µmol/L Pre-menopausal women: Dietary intake = 81 (83) mg/d Serum conc. = 43.7 (25.6) µmol/L Post-menopausal women: Dietary intake = 88 (80) mg/d Serum conc. = 50.5 (27.8) µmol/L	TH BMD or self-reported fractures stratified by 100 mg/d increments in dietary vit. C intake or by SD increments in serum ascorbic acid conc.	Diet: M Serum: M	In men, TH BMD was highest at serum ascorbic acid conc. between about 28.4-56.8 µmol/L and self-reported fractures were least common at dietary vit. C intakes of about 200 mg/d; whereas higher and lower conc. were associated with lower TH BMD (P<0.05) and a higher self-reported fracture prevalence (P=0.01). In pre-menopausal women, TH BMD was 0.01 g/cm^2 higher for every 100 mg/d increase in dietary vit. C intake (P=0.002).
Ilich[88] 2003 US	*n* 136 (women)	69 (57-88)	3dD	Dietary intake = 128 (70); 23-402 mg/d	Dietary vit. C intake as a predictor of WB BMD and BMC and of TH, FN, WT, T, RS, UR and hand BMD	Diet: S	Dietary vit. C intake was a predictor of BMD of more than 1% for TH (P=0.012), T (P=0.047) and RS (P=0.027) BMD and a marginally sig. predictor of WT BMD (P=0.052).
Wolf[84] 2005 US	*n* 11068 (women)	63 (50-79)	FFQ	Dietary intake = 84 (49) mg/d Total intake = 170 (182) mg/d	WB, LS, TH, FN and T BMD stratified by dietary or total vit. C intake with or without additional stratification by either calcium intake, smoking or HRT use	Diet: NS Total: NS	A sig. positive interaction effect between HRT use and total vit. C intake for WB (P=0.045), LS (P=0.03), TH (P=0.029) and FN (P=0.004) BMD.
Pasco[85] 2006 Australia	*n* 533 (women)	56-82	N/A	Duration of suppl. use (vit. C + E): Group 1 = 0 yrs (non-user) Group 2 < 5 yrs Group 3 ≥ 5 yrs	WB BMD, serum CTX and BSALP stratified by use or duration of vit. C and E suppl.	Suppl.: M	The duration of vit. C and E suppl. use (≥5 years) was associated with sig. lower CTX conc. compared to non-suppl. users (P<0.05). CTX conc. were 0.022 pg/mL lower for each year of vit. suppl. use (P=0.05).
Prynne[9] 2006 UK	*n* 257 (111 boys; 101 girls); *n* 67 (older women)	17 (16-18); 68 (60-83)	7dD	Dietary intake: Boys = 96 mg/d Girls = 95 mg/d Older women = *Data not shown.*	WB, LS, TH, FN and T BMD stratified by vit. C intake	Diet: M	In boys, each 100% change in vit. C intake was associated with a 3-5% change in BMD at all sites (P<0.05).

Sahni[74] 2008 US	*n* 874 (334 men; 540 women)	75	FFQ	Dietary intake: Men = 141 (73) mg/d Women = 158 (83) mg/d Suppl. intake: Men = 82 (235) mg/d Women = 95 (248) mg/d Group 1 = 0 mg/d Group 2 < 90 / 75 mg/d‡ Group 3 ≥ 90 / 75 mg/d‡ Total intake: Men = 223 (259) mg/d Women = 253 (267) mg/d *Intake data for tertiles not shown.*	LS, FN, T and RS BMD stratified by tertiles of dietary or total vit. C intake or categories of suppl. vit. C intake and either calcium intake, vit. E intake, smoking or oestrogen use	Diet: NS Suppl.: M Total: M	In men, total vit. C intake was positively associated with FN BMD among never-smokers (*P*-trend=0.04). In current smokers, total and suppl. vit. C intake were negatively associated with T BMD (*P*-trends=0.01).
Sugiura[87] 2011 Japan	*n* 293 (women)	60	FFQ	Dietary intake = 170 (161-179) mg/d§ Tertile 1 = 47-139 mg/d| Tertile 2 = 140-214 mg/d| Tertile 3 = 215-625 mg/d|	Risk of low radial BMD stratified by tertiles of dietary vit. C intake	Diet: S	Sig. lower risk of low radial BMD for tertile 3 vs 1 of dietary vit. C intake (OR 0.25, 95% CI 0.07, 0.82; *P*-trend=0.01).

Vit., vitamin; conc., concentration(s); 24hR, 24-hour dietary recall; MR, mid radius; BMD, bone mineral density; FFQ, food frequency questionnaire; suppl., supplement(al); FN, femoral neck; approx., approximately; LS, lumbar spine; T, trochanter; WT, Ward's triangle; sig., significant; TH, total hip; PYD, pyridinoline; DPD, deoxypyridinoline; OC, osteocalcin; N/A, not applicable; UR, ultradistal radius; 3dD, 3-day food diary; WB, whole body; RS, radial shaft; CTX, collagen type 1 cross-linked C-telopeptide; BSALP, bone-specific alkaline phosphatase; 7dD, 7-day food diary.

** Total intake is the sum of dietary intake and intake from supplements.*

† Results were significant (S), non-significant (NS) or of mixed nature (M).

‡ Data shown for men / women.

§ Geometric mean (95% CI).

| Intake range.