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Osteodietology

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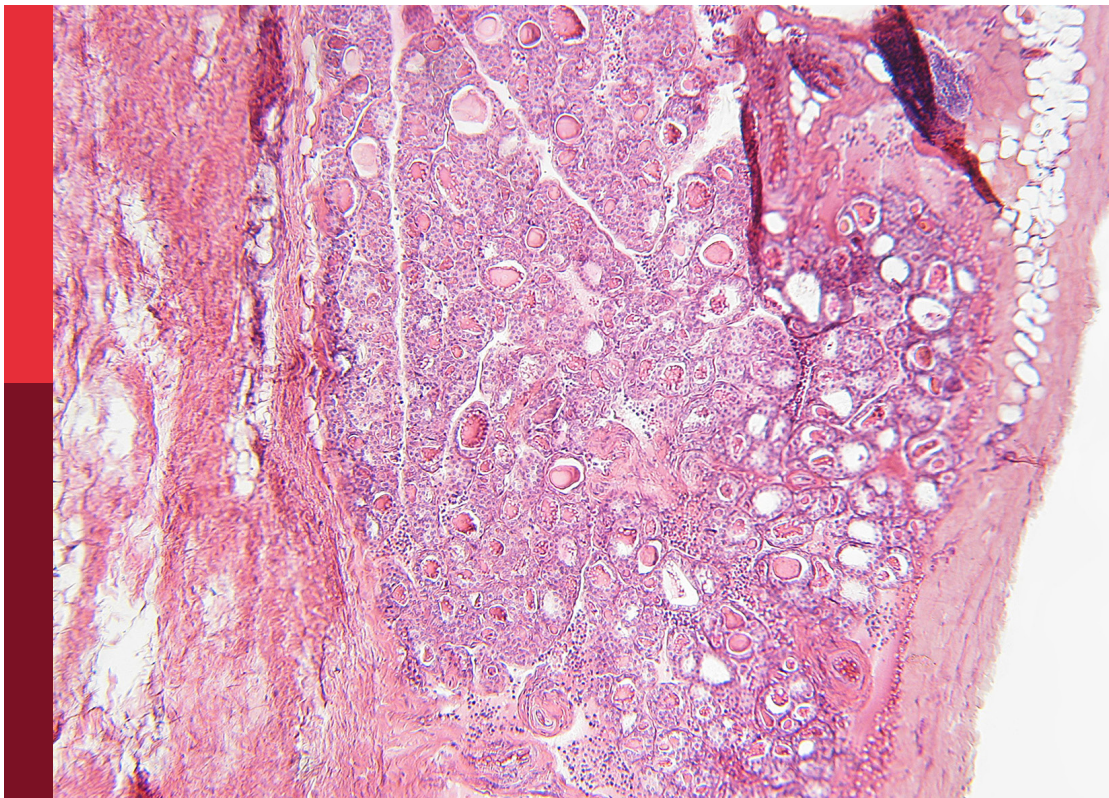

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Osteodietology

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REVIEW
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Flavonoids: Classification, Function, and Molecular Mechanisms Involved in Bone Remodelling

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Flavonoids are polyphenolic compounds spotted in various fruits, vegetables, barks, tea plants, and stems and many more natural commodities. They have a multitude of applications through their anti-inflammatory, anti-oxidative, anti-carcinogenic properties, along with the ability to assist in the stimulation of bone formation. Bone, a rigid connective body tissue made up of cells embedded in a mineralised matrix is maintained by an assemblage of pathways assisting osteoblastogenesis and osteoclastogenesis. These have a significant impact on a plethora of bone diseases. The homeostasis between osteoblast and osteoclast formation decides the integrity and structure of the bone. The flavonoids discussed here are quercetin, kaempferol, icariin, myricetin, naringin, daidzein, luteolin, genistein, hesperidin, apigenin and several other flavonoids. The effects these flavonoids have on the mitogen activated protein kinase (MAPK), nuclear factor kappa β (NF-kβ), Wnt/β-catenin and bone morphogenetic protein 2/SMAD (BMP2/SMAD) signalling pathways, and apoptotic pathways lead to impacts on bone remodelling. In addition, these polyphenols regulate angiogenesis, decrease the levels of inflammatory cytokines and play a crucial role in scavenging reactive oxygen species (ROS). Considering these important effects of flavonoids, they may be regarded as a promising agent in treating bone-related ailments in the future.

Keywords: flavonoids, bone, osteoblast, bone remodelling, osteoclast

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1 INTRODUCTION

Bone is a composite structure that handles a multitude of processes such as preservation of skeletal size, shape integrity, harbouring marrow and controlling mineral homeostasis. Modelling and remodelling form the basis of bone development and maintenance. These are processes that occur throughout the life. The cycle of bone formation and removal is coordinated all over the body but occur at various sites (1). The structure of a bone is the single most complicated organisation handling the calcium phosphorous metabolism in the human system. Numerous cells are involved in this system. Collagen, a triple helix combined with calcium and phosphorous, make up the basic components of the bone, reinforcing the material making up the human skeleton. There are two types of bones – cortical bones which are the solid ones and trabecular bones which have a soft and intricate structure (2). Bones, as we know, are essential for our posture, movement, protection and

housing of delicate organs and agility. The structural framework gives us genetic superiority over other species with respect to our ability to perform various tasks like swimming, walking, climbing and many more. *Homo erectus*, as the name suggests, was the first species to ever walk upright on the face of the earth. Since then, humanity has progressed to great lengths of development and evolution. This singularly portrays the importance of skeleton in the supremacy established by human beings. However, the mechanisms of bone formation and modification become very important. Sadly, the truth is that the mechanisms of bone remodelling aren't clearly laid out yet (3). This gives a great opportunity for researchers to study and understand deeply about the mechanisms in the near future. From time immemorial, study of history has always helped us to correct our mistakes and improve our knowledge of the concerned arena. In this case, several kinds of research on bone diseases in humans and animals have assisted in gaining knowledge on the mechanisms of bone remodelling cycle. The receptor activator of nuclear factor-kB (RANK)/RANK ligand/OPG and canonical Wingless-related integration site (Wnt) signalling are a part of the major signalling pathways. The bone remodelling cycle is regulated by paracrinal secretions such as growth factors, prostaglandins, cytokines and endocrinal secretions such as ergocalciferol, calcitonin, parathormone (PTH), glucocorticoids, thyroxine, estrogen and testosterone (4). Flavonoids, a group of naturally derived compounds with variable phenolic structures, are found in plant foods that are a part of our everyday lives. Flavonoids have many beneficial effects stemming from the significant presence of antioxidant activity, anti-resorptive effects and free radical scavenging capacities (5). They play a prime role in various sectors ranging from nutritional, pharmaceutical to medicinal and cosmetic applications. Research studies have shown that flavonoids assist in lowering the cardiovascular mortality rate and coronary heart disease (6). Flavonoids like Quercetin, Kaempferol, Genistein, Daidzein etc., show healing properties for osteoporosis, a leading cause for joint pain and loss of bone density by regulating osteoblast(OB) and osteoclast (OC) differentiation (7). Soy Isoflavones, in particular, show promising results with antiresorptive activity *via* osteoclast inhibition and promotion of osteoblast differentiation. This is because of their weak binding to the estrogen receptor and a higher affinity towards ERβ when compared to ERα, thus mimicking estrogen. Recent studies also indicate the role of soy isoflavones in activating signalling *via* bone morphogenetic proteins (BMP), thus exhibiting estrogen-independent properties (8). Asian foods have always been rich in flavonoid content, and this might be the probable cause of the increased lifespan of Asian individuals, as they assist in curing many fatal diseases like cancer, cardiovascular diseases and diabetes (8–10). Flavonoids have long been used in Chinese medicine to cure bone fractures, diabetes and many other morbidities (11–14). As many studies highlight, these phytochemicals have a plethora of functions and a huge potential for applications in various fields. Science is yet to divulge into the actualities of molecular mechanisms of flavonoids, and this paper attempts to devise a link between flavonoids and their potential to provide a cure for bone diseases like osteoporosis, inflammation of bone associated with rheumatoid arthritis and periodontal disease, and to give a lucid comprehension of primal flavonoids and the benefits they provide in the systemic metabolism of humans.

2 BONE REMODELLING

Bone is a complex dynamic structure under continuous remodelling characterised by the resorption of damaged or old bone by the osteoclasts, followed by its' replacement with the newly formed bone by the osteoblasts. A proper balance between bone resorption and formation is required to maintain a healthy skeleton (15). Bone remodelling tends to become absolutely necessary as it facilitates the primary bone to be replaced by the secondary bone which has higher mechanical strength, removes microfractures and ischemic fractures in bones and at last, assures a correct balance of Ca+/K+ (16). Bone remodelling requires the co-ordinated function of four types of cells namely, bone-lining cells, osteocytes, osteoclasts, and osteoblasts and involves four phases: activation phase, resorption phase, reverse phase and formation phase (17). Osteoclasts are cells sourced from the myeloid, distinctly marked by the presence of multiple nucleus and expression of tartrate-resistant acid phosphatase (TRAP) and the calcitonin receptor (18, 19). The cytokines Colony stimulating factor-1 (CSF-1) and receptor activator of nuclear factor – kappa B(NF-kB) ligand (RANKL) regulate the survival and differentiation of osteoclast precursor cells (1). After differentiation, osteoclasts form an association with the surface of bone through alpha-v beta integrin that transmits signals regulating the organization of the cytoskeleton. The signals thereby activate proto-oncogene tyrosine-protein kinase Src (c-Src), spleen tyrosine kinase (SYK), Guanine nucleotide exchange factor VAV3 Ras homologous GTPases (20). Microscopic trenches are formed on the bone trabeculae surface by secretion of hydrochloric acid and proteases, like cathepsin K (CTSK), into an extracellular lysosomal space to degrade the matrix and mineral parts of the bone (21). Several osteotropic factors such as Interleukin-11 (IL-11), IL-1, PTH and 1,25-$(OH)_2D_3$, indirectly enhance osteoclast formation by stimulation of RANKL on the surface of osteoblasts, followed by RANKL binding RANK on osteoclast precursors. This gives rise to the activation of downstream signalling pathways such as the NF-κB, Ak strain transforming (AKT) pathway, c-Jun N-terminal kinase (JNK) pathway, p38 mitogen activated protein kinase (MAPK), and extracellular signal regulated kinase (ERK) pathway (22–26). The other factors associated with RANK-activated signalling pathways like c-fos, c-src, TNF- Receptor associated Factor 6 (TRAF-6) and Nuclear factor of Activated T-cells (NFATc-1) also play an important role in regulation of osteoclastogenesis (27–29). The formation of osteoclasts and their subsequent activation is limited primarily by various factors, in particular osteoprotegerin (OPG) which plays an inhibitory role by acting

as a decoy receptor for RANKL. The homeostasis of RANKL/OPG is a major determinant for the integrity of bone (30).

Neural crest progenitor cells and mesodermal cells give rise to osteoblasts, leading to the differentiation of progenitors into proliferating preosteoblasts, osteoblasts and then into osteocytes. Runt-related transcription factor 2 (RUNX2) is essential for progenitor cell differentiation across the osteoblast lineage (31). During the proliferation of cells, RUNX2 regulates vascular endothelial growth factor (VEGF), osteocalcin (OCN), Receptor activator of nuclear factor kappa-B ligand (RANKL), dentin matrix protein 1 (DMP1) and sclerostin (32). osterix (OSX), insulin-like growth factor (IGF), Bone morphogenetic proteins (BMPs), fibroblast growth factor (FGF), endothelin-1 and PTH regulate differentiation of osteoblasts (33, 34). BMP and PTH are related to activating Wnt signalling pathways (35). The fully differentiated osteoblast is distinguished by coexpression of alkaline phosphatase and type I collagen, both crucial for production of bone matrix and the subsequent mineralization (36). Mature osteoblasts generate mineralization regulators such as osteonectin (ON), OCN, osteopontin (OPN) and RANKL required for osteoclast differentiation. During the end of their lifetime, osteoblasts change into either osteocytes embedded in mineralized matrix or lining cells wrapping the bone surfaces (37). Thus, the homeostasis between bone formation by osteoblasts and bone resorption by osteoclasts, tightly coupled and regulated by various pathways, transcription factors and secreted molecules decide the overall integrity and structure of the bone.

3 BONE DISEASES

When the cycle of bone remodelling gets disturbed, and the level of osteoclastogenesis exceeds the level of osteoblastogenesis, it weakens the bone resulting in conditions like osteoporosis, periodontitis and rheumatoid arthritis (21).

Osteoporosis is one of the leading causes of bone fractures. There are about nine million fracture incidences worldwide, resulting in a cost of $100 billion. Osteoporotic hip fractures have about 200 million occurrences, and this highlights the great danger that it poses. In first world countries like USA and Europe, even with top-notch medical facilities, 30% of women have osteoporosis, and 40% of post-menopausal women and 30% of men have a high chance of experiencing osteoporotic fracture (38). Sex steroid deficiencies post menopause alter the production of T-cell cytokines which in turn affect the production of RANKL/OPG by the cells of osteoblastic lineage leading to excessive differentiation of osteoclasts and hence excessive resorption. Moreover, pathological conditions involving inflammation increases osteoclastogenesis *via* the production of M-CSF, RANKL, PTHrP, cytokines and prostaglandins (39). An example of this is the overproduction of osteoclasts mediated by IL-6 being a cardinal pathophysiological change in sex-steroid induced osteoporosis (40). Another bone remodelling degenerative disease is periodontitis which involves alveolar bone loss (BL), gingival inflammation, clinical attachment loss (CAL), bleeding, exfoliation of the tooth and periodontal pocketing (41). The disease progression is characterised by excessive production of matrix metalloproteinases (MMPs), leukotrienes, M-CSF, inflammatory cytokines and mediators such as IL-6, IL-1β, TNF-α, prostaglandin E2 (PGE-2) by an over-reactive immune system. The cytokines IL-6 and IL-1β were identified to be the most potent cytokines contributing to bone resorption *via* activation of RANKL, thereby promoting osteoclast activity (42). Rheumatoid arthritis a chronic, systemic, inflammatory autoimmune disorder is characterised by symmetric, erosive synovitis and, in certain cases, extraarticular involvement (43). Bone erosion in RA is typified by the involvement of autoantibodies early in the disease as well as several inflammatory cytokines including TNF-α, IL-6, IL-1β and IL-17 which exert pro-osteoclastogenic effects *via* stimulation of production of RANKL and M-CSF (44–46).

When any disease is subjected to treatment, two parameters have to be primally considered: Selectivity and Therapeutic index (47). In addition to both of these prerequisites, convenience is also important while deciding treatment methods. Convenience refers to the preference of the patients to consume the drugs in particular routes than other routes. Though parenteral routes have many advantages, recipients traditionally prefer the oral route, as it is much less of a discomfort (48). Amongst the prerequisites mentioned above, therapeutic index and convenience are already satisfied as toxicity is almost zero and administration is through oral route. Though the third requirement, selectivity, is not adequate for flavonoids, this can be increased by changing the glucose content associated to give rise to glucoside compounds having higher selectivity, thus making flavonoids better and safe than any other medications present (49).

4 NATURAL FLAVONOIDS

Flavonoids are bioactive compounds belonging to an important class of low molecular weight plant secondary metabolites having a polyphenolic structure. Flavonoids are widely found in fruits, vegetables, herbs, beverages, spices and oils. Hence, they are also known as dietary flavonoids (6, 50). Following terpenoids (30,000) and alkaloids (12,000), the third-largest group of natural products is represented by flavonoids, comprising nearly 10,000 compounds (51). All flavonoids contain 15 carbon atoms in their basic skeleton which are distributed as two six-membered rings and one three-carbon unit linked to them as C6-C3-C6 (51, 52). The 3-carbon unit bridging the phenyl groups usually cyclizes with oxygen to form a third ring. This core structure is called 2-phenylbenzopyranone (53). Flavonoids are most often associated with sugar in the conjugated form to be O-glycosides or C-glycosides. They can also exist as aglycones (54). The glycosides are normally attached to position 3 or 7, with the most common carbohydrates occupying those positions being D-glucose, L-rhamnose, gluco-rhamnose, galactose or arabinose (52). The other factors pertaining to the varied chemical nature of the flavonoids include patterns of hydroxylation, conjugation between aromatic rings, methoxy groups, and other substituents such as sulphates and prenyl groups (51, 55). Flavonoids have been

known to exhibit a broad spectrum of pharmacological and biochemical reactions associated with health promoting effects. Examples of such therapeutic properties are anti-inflammatory, hepatoprotective, anti-mutagenic, anti-oxidative, anti-neoplastic, anti-viral, anti-microbial, anti-helminthic, anti-allergic, anti-hormonal, anti-thrombotic, differentiation and apoptotic effects (6, 7, 56). Numerous *in-vitro* studies have shown flavonoids capacity in modulating the key cellular enzymes. Modulation of these enzymes, in turn, affect the important cellular pathways which regulate cell division and proliferation, inflammatory and immune responses, detoxification and platelet aggregation (57). Flavonoids act as potential metal-chelators and free radical scavengers. They neutralise free radicals by donating electrons from their conjugated double bonds and groups *via* resonance, thus acting as natural anti-oxidants (51, 56). Recent studies have discovered the connection between flavonoids and the regulation of bone metabolism. This property is being studied, to use flavonoids as a possible therapy in the future, for the treatment of osteoporosis (7).

5 CLASSIFICATION OF FLAVONOIDS

Flavonoids can be broadly categorised into three groups: the bioflavonoids, the iso-flavonoids (phytoestrogens) and the neo-flavonoids (white flavonoids) (50). The variations in the different classes and subclasses of flavonoids are attributed to factors such as the degree of unsaturation, the carbon of the C ring to which the B ring is attached, degree of hydroxylation, degree of oxidation, glycosylation pattern and other substitutions (51).

5.1 Iso-Flavonoids

In iso-flavonoids the B ring is attached to position 3 on the C-ring (6). Iso-flavonoids structurally resemble 17-beta estradiol and bind to oestrogen receptors. Hence, they are also known as phyto-oestrogens. Depending on the endocrine estrogenic levels, they can act as either agonists or antagonists (8, 58). Iso-flavonoids possess tremendous potential to fight various diseases including amelioration of osteoporosis and cardiovascular disease, prevention and treatment of hormone-related cancer, treatment of menopause symptoms and other age related diseases (59). The major sources of isoflavones are the leguminous plants belonging to the family Fabaceae/Leguminosae. Other sources include red clover, red wine, germs of alfalfa and linseed, with red clover containing the highest amount of phyto-estrogens (58, 60). Some examples of isoflavones are Genistein, daidzein, glycitein, biochanin A and formononetin (60).

5.2 Neo-Flavonoids

Flavonoids in which the B-ring is attached to position 4 of the C-ring are known as neo-flavonoids (NFs). The first neoflavone to be isolated was calophyllolide from Calophyllum inophyllum seeds (6). NFs have been categorised into two broad groups namely, the 4-phenylcoumarins (dalbergin group) and the diphenyl allyl compounds (latifolin group). They are distributed in a wide range of plants belonging to families Fabaceae, Clusicaea, Leguminosae, Rubiaceae, Passifloraceae, Thelypteridaceae and Polypodiaceae. The most abundantly found neo-flavone is Dalbergin isolated from various plants of the genus Dalbergia. NFs exhibit several therapeutic properties which include anti-allergic, anti-inflammatory, anti-osteoporotic, antimicrobial and anti-oxidant (61).

5.3 Bio-Flavonoids

Those class of flavonoids in which the B-ring is attached to position 2 of the c-ring are called as bio-flavonoids. They can be further subdivided into different subclasses depending on the structural features of the C-ring. These subclasses are flavones, flavonols, flavonones, flavan-3-ols/catechins, anthocyanidins and chalcones (6). Flavonols are the most common and largely occurring flavonoids in the plant kingdom. Examples of major dietetic flavonoids are quercetin, kaempferol, fisetin, isorhamnetin and myricetin, with quercetin being one of the most abundant flavonoids of the human diet (62). Flavones are majorly found in foods such as celery, lettuce and capsicum peppers (50). The main flavones of the human diet include apigenin and luteolin (62). Catechins, otherwise known as flavan-3-ols possess a hydroxyl group in C3 of C-ring (a dihydro-pyran heterocycle). Catechins, gallocatechin, epigallocatechin, epicatechin and gallate are a few compounds that fall under this category. A variety of fruits, vegetables and plant-based beverages contain abundant concentrations of catechin. Green tea is the main dietary source (63). Flavanones have a basic skeleton of 2-phenylbenzopiran-4-one. They play a vital role in regulating the metabolic pathways of other flavonoids (64). They are found in almost all citrus fruits and are responsible for their bitter taste. Hesperitin, naringin and eriodictyol are a few examples of this subclass (6). Anthocyanidins are another subclass of bio-flavonoids that are water-soluble and are found in the leaves, stems, roots, flowers and fruits of all higher plants. They are responsible for the red, purple and blue colour of certain fruits, which vary depending on the pH. Cyanidin, peonidin, pelargonidin, delphinidin, petunidin and malvidin are the most prevalent compounds (53). The last subclass, chalcones are open-chain flavonoids. They consist of two aromatic rings A and B joined by a 3-carbon a,b-unsaturated carbonyl group. Leguminosae, Asteraceae and Moraceae are the three families that contain the largest number of natural chalcones. Examples of chalcones include naringenin chalcone, isoliquiritigenin, phloretin, licodione, echinatin etc (65).

5.3.1 Quercetin

Quercetin is one of the most important and widely studied dietary bioflavonoids. It is ubiquitously found in fruits and vegetables (66). For several years in China, Quercetin and its derivatives have been used in the treatment of osteoporosis because of their natural anti-oxidant property (67). Quercetin regulates various pathways involved in maintaining bone homeostasis such as the RANK/RANKL/OPG System, MAPK signalling, apoptotic pathway, canonical Wnt/β Catenin signalling, BMP and transforming growth factor (TGF-β) signalling (**Figures 1A, B**). Further quercetin exhibits anti-oxidative, anti-inflammatory and angiogenic properties through which it maintains a balance between osteoblastogenesis and osteoclastogenesis (66). Prouillet et al., showed that, in MG-63 human osteoblasts, quercetin had a

stimulatory effect on alkaline phosphatase (ALP) activity in the range of 1-50 mM without any significant cytotoxic effects. Quercetin-induced ALP activation requires the ERK pathway and rapidly stimulates it, because inhibition of this pathway by the MEK inhibitor PD 98059 reduced the enhancing actions of quercetin. Moreover, the direct role of ER involved in the effect of quercetin was shown by the fact that ER antagonist ICI 182780 prevented quercetin-induced increase in ALP activity (68). While the previous study showed involvement of ER, another study on the effects of quercetin pretreatment on osteogenic differentiation and proliferation of Human Adipose Tissue Derived Stromal Cells (hADSC) indicated an ER-independent mechanism (69, 70). In mouse monocyte/macrophage cell line RAW264.7, quercetin and quercetin-3-O-glucoside (Q3G) were found to decrease the number of RANK-L-induced TRAP positive multi-nucleated osteoclast cells significantly in dose dependent manner. Treatment with quercetin suppressed the expression of osteoclast related genes such as the calcitonin receptor (CTR), CTSK, MMP-9 and NFATc1. NFATc1 is a master regulatory transcription factor of osteoclast differentiation regulated by RANK-L *via* activator protein-1 (AP-1) and NF-kB (71, 72). Actin-ring formation, which is important for bone resorption in osteoclast-like mononucleated cells (OCLs) was disrupted by quercetin. This suggests a possible role of quercetin in regulating the signal transducing molecules involved in actin-ring formation: p60 c-src tyrosine kinase, phosphoinositide-3-kinase (PI3K), GTP-binding proteins (GTP-BP) and protein kinase A (PKA) (73). IL-17 is an osteoclastogenic inflammatory cytokine promoting the production of other destructive cytokines such as the macrophage migration inhibitory factor (MIF), tumour necrosis factor-alpha (TNF-α) and RANK-L which in turn increase reactive oxygen species (ROS) and osteoclastic differentiation in rheumatoid arthritis (RA). IL-17-stimulated RA-fibroblasts-like synoviocytes (RA-FLS), when treated with quercetin decreased the production of RANK-L, TNF-α, IL-6 and IL-8. Quercetin decreased the IL-17-induced phosphorylation of mammalian target of rapamycin (mTOR), ERK and NF-kB in RA-FLS, whereas it increased the IL-17-induced phosphorylation of AMP-activated protein kinase (AMPK). Since AMPK is known to counteract and inhibit mTOR signalling, the effect of quercetin on AMPK activation suppresses mTOR and induces apoptosis in osteoclasts (74). MC3T3-E1 cells, treated with Lipopolysaccharide (LPS), a pro-inflammatory glycolipid suppressed the m-RNA and protein expression levels of ALP, RUNX2, OSX and OCN, thus inducing apoptosis and inhibiting the differentiation of osteoblasts *via* the JNK pathway. Quercetin reversed this condition by increasing the phosphorylation of ERK-1/2, which inhibited the induction of apoptosis by p38 MAPK and JNK. Further, quercetin upregulated the expression of anti-apoptotic proteins B-cell lymphoma-2 (BCL-2) and BCL-XL, while it downregulated the apoptotic proteins caspase-3, BCL-2 associated X apoptosis regulator (BAX) and cytochrome c (75). In osteoblasts isolated from foetal rat calvaria quercetin aglycone upregulated the m-RNA and protein levels of three anti-oxidant genes heme oxygenase-1 (HO-1), γ-glutamate cysteine ligase catalytic subunit (GCLC) and catalase. However, it did not upregulate Nuclear factor erythroid 2–related factor 2 (Nrf-2), the transcription factor of these three genes. Quercetin also downregulated the phosphorylated levels of ERK1/2 and NF-kB, which suggests an anti-inflammatory response associated with the activation of anti-oxidant genes (76). This is in contrast to the studies on MC3T3-L1 osteoblasts and MG-63 osteosarcoma cells (68, 77). Further studies are required to confirm the exact role of ERK1/2, NF-kB p65 and Nrf-2 in mediating the anti-oxidative responses (76). Zhou et al., investigated the effect of quercetin on angiogenesis and found that it increased the expression of angiogenic factors VEGF, angiopoietin 1 (ANG-1), basic fibroblast growth factor (bFGF) and TGF-b, ultimately leading to bone regeneration (66). Another pathway regulating bone homeostasis is the Wnt/b-catenin pathway. Pre-treatment of MC3T3-E1 cells with quercetin increased the protein levels of Wnt3 and β-catenin, which is responsible for osteoblast differentiation (75). One of the mechanisms by which TNF-α suppresses osteoblastogenesis is by inhibiting the activation of

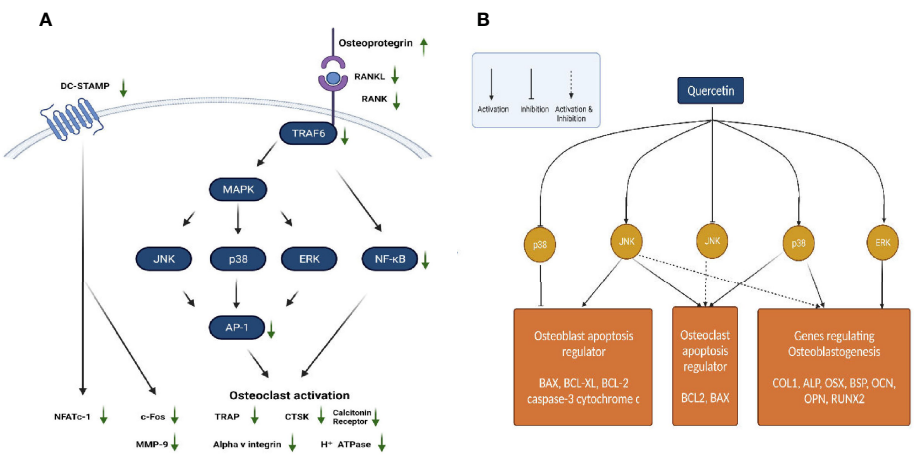

FIGURE 1 | **(A)** Regulation of RANK/RANKL/OPG system by quercetin. **(B)** Actions of quercetin on JNK, ERK and p38 MAPK pathways.

SMAD signal transduction by TGF-β and BMP-2. The effect of quercetin in this case, only added to the inhibitory effect of TNF-α, rather than suppressing it. Thus, the overall effect of quercetin on bone formation involves complex competing pathways which may depend on the dose and the concentrations of cytokines and growth factors prevailing in the micro-environment (72).

5.3.2 Kaempferol

Kaempferol and its derivatives are natural bioflavonoids enriched in fruits and vegetables and are used as nutraceuticals. Kaempferol possesses various medicinal properties some of which are directly associated with bone-sparing effects (78). Both adipocytes and osteoblasts are differentiated from multipotential mesenchymal stem cells in bone marrow. During the process of ageing, there is a reciprocal increase in adipogenesis and decrease in osteogenesis in the bone marrow, which has to be inhibited and reversed to treat bone diseases such as osteoporosis. The *in vitro* studies of Ritu et al., showed that kaempferol inhibited the differentiation of bone marrow mesenchymal stem cells(BMSCs) to adipocytes, whereas it stimulated increased osteoblast differentiation (79). This is supported by the fact that kaempferol downregulated the LPS-induced expression of lipid-anabolism genes (sterol regulatory element binding protein-1c [SREBP-1c], fatty acid synthase [FAS] and peroxisome proliferated activated receptor-gamma [PPAR-g]) in BMSCs (**Figure 2**). On the contrary, it promoted the expression of genes involved in lipid catabolism (carnityl palmitoyl transferase [CPT-1], PPAR-α and acetyl CoA carboxylase [ACC]), thus preventing adipogenesis (80). Kaempferol treatment of BMSCs increased the expression of important downstream regulatory proteins in the mTOR pathway, which suggests its involvement in the differentiation of osteoblasts. The role of mTOR in osteogenesis was validated by Zhao et al., where treatment BMSCs with a specific inhibitor of mTOR called rapa, resulted in decreased levels of osteogenic activity. However, several other studies exhibit controversies over the role of mTOR in bone formation (81). In mouse calvarial osteoblast cell line MC3T3, kaempferol inhibited the TNF a-induced signalling in osteoblasts and thereby reduced the secretion of osteoclastogenic cytokines interleukin-6 (IL-6) and monocyte chemoattractant protein-1 (MCP-1). It also blocked the TNFa-induced nuclear translocation of NF-kB, a transcriptional regulator of MCP-1. Further, kaempferol antagonised the RANKL induced differentiation of RAW264.7 cells to osteoclasts by inhibiting c-Fos expression, an immediate early oncogene, which is indispensable for osteoclastogenesis (82). In LPS treated BMSCs, kaempferol reversed the downregulation of expression of chondrogenic markers SRY-Box Transcription Factor 9 (SOX-9), COL-2 and Aggrecan and strongly elevated their levels. Besides, kaempferol caused a significant decrease in the levels of matrix metallo-proteinase-3 (MMP-3), MMP-13, ADAM metallopeptidase with thrombospondin Type 1 Motif-4 (ADAMTS-4), ADAMTS-5. The inflation of pro-inflammatory cytokines IL-6, IL-1β, inducible nitric oxide synthase (iNOS) and TNF-α induced by lipopolysaccharide (LPS) was reduced by kaempferol, while it increased the level of anti-inflammatory cytokine IL-10 (80, 83). The LPS-induced activation of NF-kB was also inhibited by kaempferol, as was shown by the reduced nuclear staining of p-65 (80). Treatment with kaempferol of ATDC5 cells, led to a marked increase in the mRNA levels of genes encoding COL-2 and COL-10, which are markers of fully differentiated chondrocytes. Also, kaempferol induced the activation of ERK and p38 MAP kinase pathway. Further, it promoted the expression of BMP-2 and BMP-4, thereby suggesting that stimulation of chondrogenesis occurs *via* BMP-2 signalling pathway in ATDC-5 cells (84). Treatment with 8-prenyl kaempferol, a prenyl flavonoid on MC3T3-E1 cell line, regulated osteoblast differentiation *via* BMP-2 signalling pathway, which subsequently triggered SMAD1/5/8. This led to the activation of the transcription factor RUNX2 which promoted bone mineralization by regulating the expression of COL-1, OPN and ON (85). Kaempferol induced luciferase activity in rat primary osteoblasts transfected with pERE-Luc and also triggered phosphorylation of ER-a, which suggested that kaempferol acts *via* ER activation. This was confirmed when pre-treatment with ICI 182,780 completely blocked the kaempferol-induced pERE-Luc activity. Additionally, kaempferol upregulated ALP activities and the transcription of several bone differentiation marker genes such as the COL1A1, ON, OCN, RUNX2 and OSX (86). A study by prouillet et al., on MG-63 human osteoblastic cell strain demonstrated that kaempferol induced increase in ALP activation involves the ERK pathway. This was shown by incubating the cells with PD 98059, an inhibitor of ERK pathway, which reduced the stimulatory effects of kaempferol on ALP. ICI 182780, a pure anti-estrogen, inhibited ERK activation and reduced the levels of ALP in kaempferol treated cells which shows that kaempferol activates ERK pathway *via* the ERs (69, 85). MAP kinase activation *via* a non-genomic action of ER can lead to downstream modulation of the transcription factor AP-1 which has been predicted to have a binding site on the promoter of the ALP gene. This transcription factor can act as a possible link between rapid ERK activation and increased ALP activity (69). Pretreatment with kaempferol of MC3T3-E1 cells exhibited a marked reduction in antimitochondrial antibody (AMA) induced-cell damage by preventing mitochondrial membrane potential dissipation, complex IV inactivation, $[Ca^{2+}]_i$ elevation, and ROS production. Kaempferol induced the activation of AKT, PI3K and cAMP response binding element protein (CREB) inhibited by AMA, which are known to be involved in osteoblast-like cell proliferation and differentiation (87). RANK-L induced differentiation of RAW 264.7 cells to osteoclasts was shown to be inhibited by kaempferol by suppressing the expression of osteoclastogenic factors TRAF6, NFAT-c1, and c-Fos. Osteoclastogenesis was also suppressed by inhibiting autophagy related factors beclin-1 and sequestosome 1 (p62/SQSTM1) (83, 88). Furthermore, in dexamethasone-induced rat calvarial osteoblasts, kaempferol decreased osteoblast apoptosis by inducing expression of the anti-apoptotic gene BCL-2 and suppressing BAX, a pro-apoptotic gene (78).

5.3.3 Icarin

Icariin is the main active prenylated flavonol glycoside isolated from the herb Epimedium pubescens. It has been widely used for several centuries in Chinese herbal medicine and is known to possess "bone strengthening" properties (89, 90). Naturally isolated icariin is becoming an interesting alternative in the

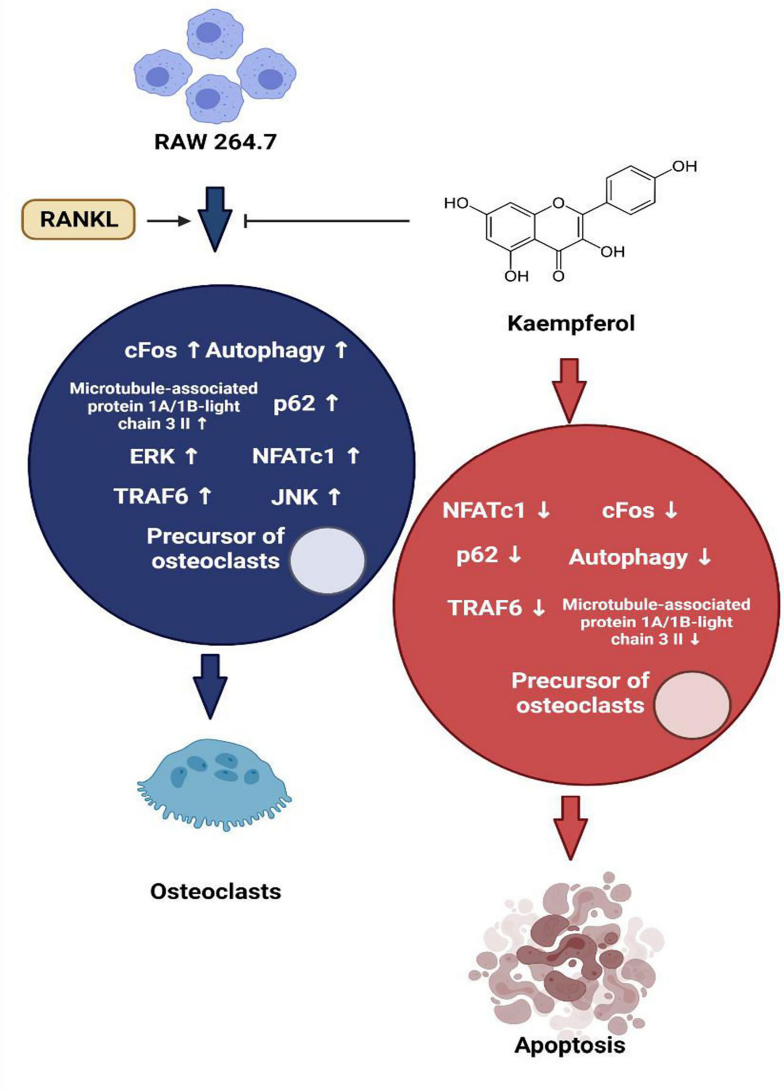

FIGURE 2 | Regulation of autophagy and apoptosis of osteoclasts by kaempferol *via* degradation of p62/SQSTM1.

prevention and treatment of bone diseases (90). It has been known to enhance osteoblastic differentiation and proliferation, inhibit bone resorption, and induce apoptosis of osteoclasts (89). Pre-osteoblastic MC3T3-E1 cells treated with icariin upregulated the levels of osteogenic markers RUNX2, OCN, BSP and ALP in a dose dependant manner. Besides, Icariin was effective in upregulating RUNX2, BSP and OCN levels in mouse primary osteoblasts as well. mRNA expression levels of inhibitor of DNA binding-I (Id-1), a transcriptional target of BMP/SMAD signalling was increased in MC3T3-E1 cells when treated with icariin, whereas expression of RUNX2 m-RNA was upregulated in both MC3T3- E1 cells and POBs. This suggests the involvement of BMPs and RUNX2 signalling in osteogenesis induced by icariin (91, 92). In adult female osteoblast-like cells, icariin caused a significant increase in ALP activity and nitric oxide (NO) levels followed by increased proliferation and mineralisation of osteoblasts (**Figure 3A**). NO is known to exhibit inhibitory effects on bone resorption by suppressing osteoclasts activity and precursor recruitment connected to iNOS activity. Moreover, icariin treatment increased BMP-2/SMAD protein expression as well. Both NO and BMP-2/SMAD activate the transcription of RUNX2 gene, thereby regulating bone homeostasis. Icariin also attenuated caspase-3 activity in the osteoblast-like cells on the 28th day of treatment with icariin, thereby exhibiting its anti-apoptosis effect (93). Sheng et al. demonstrated that treatment with icariin upregulated OCN synthesis, ALP activity, calcium deposition and collagen synthesis in BMSCs, thus promoting osteogenic differentiation. Additionally, icariin increased the expression levels of marker genes and proteins namely RUNX2, OSX, BMP-2 and IGF in

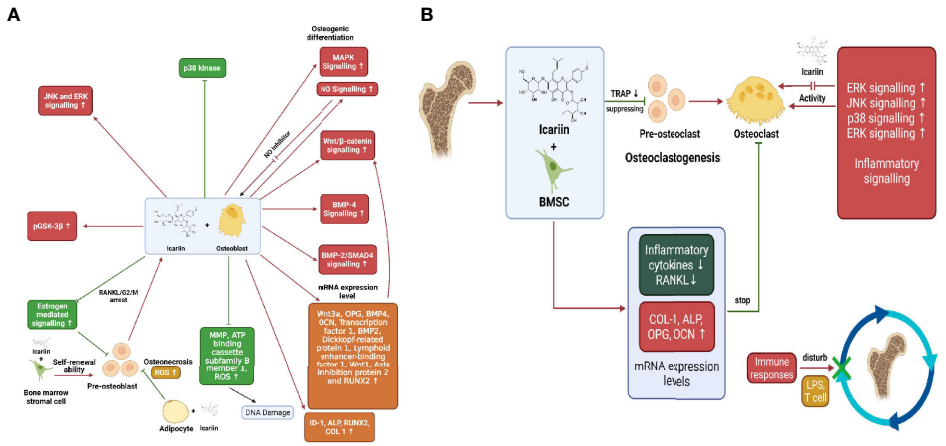

FIGURE 3 | **(A)** Osteogenic effects exerted by icariin through promotion of osteoblastogenesis and inhibition of adipocyte differentiation from pre-osteoblasts. **(B)** Inhibition of bone resorption by icariin *via* inhibition of osteoclast-related genes and pathways.

osteogenic cultures. Ming et al. and Huang et al. found that icariin inhibited osteoclastogenesis induced by RANKL and M-CSF in mouse bone marrow culture and inhibited bone resorption by stimulating apoptosis of mature osteoclasts (89). In the study of Wu et al., on BMSCs, it was found that ERK, p38 and JNK signalling pathways were all phosphorylated indicating their participation in osteoblast proliferation, differentiation and mineralisation. Blocking these three pathways significantly inhibited ALP activity and expression of COL1, OPN and OCN. Besides, icariin treatment has also been reported to instigate osteogenic differentiation of BMSCs through the activation of PI3K–AKT–eNOS–NO–sGC–cGMP–PKG signalling pathway (94). Icariin caused significant inhibition of NF-kB activation in RANKL-induced RAW264.7 cells by degradation of nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha (IkB-a) (**Figure 3B**). RANKL-induced expression of downstream regulatory factors c-Fos and NFATc1 were decreased after treatment with icariin, which in turn reduced the levels of target osteoclastogenic proteins such as CTSK and TRAP (95). Treatment with icariin on bone mesenchymal stem cells upregulated the expression of osteogenic genes RUNX2, ALP, and COL1 and decreased the expression levels of adipogenic genes —PPARγ, fatty acid binding protein-4 (Fabp4), and adipsin, thus inhibiting the differentiation of BMSCs into adipocytes. Icariin promoted the phosphorylation of Glycogen synthase kinase-3β (GSK-3b) and elevated the levels of active b-catenin in the nucleus of BMSCs. Inhibition of the Wnt signalling pathway brought down the phosphorylation of GSK-3b, caused degradation of b-catenin and upregulated the expression of adipogenic genes, thus confirming the intervention of the Wnt pathway in the differentiation of BMMSCs (92, 96). Recently it was found that cyclin D1, a mitogenic signal sensor that pushed cells from G0 phase into the proliferative cycle, was significantly increased in icariin treated BMSCs (92). Iron overload and accumulation in post-menopausal women and elderly men has been found to be linked to bone metabolism abnormalities like osteopenia, osteomalacia and osteoporosis. Treatment with icariin reversed the iron-overload-induced elevation of ROS and mitochondrial dysfunction caused by the collapse of mitochondrial membrane potential. Thus, icariin attenuated the increase in osteoclasts differentiation and promoted osteoblasts proliferation and differentiation in iron-overloaded MC3T3-E1 osteoblasts (97). Icariin inhibited hypoxia induced apoptosis in neonatal rat calvarial osteoblasts. It reduced the expression levels of caspase-3 and upregulated the mRNA expression levels of BCL-2, thereby inhibiting apoptosis. Also, icariin supplementation diminished the intracellular malondialdehyde (MDA) levels and ROS production, while increasing the activity of SOD, anti-oxidant enzyme to ameliorate the hypoxia induced stress (98). In the LPS-induced osteoclastogenesis model, icariin treatment reduced the LPS-induced activities of osteoclast differentiation marker protein TRAP and regulator of bone resorption- acid phosphatase (ACP). Moreover, icariin suppressed the LPS-induced RANKL expression, whereas it elevated the LPS-inhibited expression of OPG, an osteogenic marker. In addition, icariin could inhibit the synthesis of osteoclastogenic pro-inflammatory cytokines such as IL-6 and TNF-a. Alongside this, icariin reduced prosteoglandin-E2 (PGE-2) production by obstructing synthesis of cyclo-oxygenase -2 (COX-2), therefore inhibiting bone resorption (99). Considering the limitations of animal models in determining the therapeutic efficacy and pharmacological properties of icariin and derivatives, further verification using mammalian models, primates and human clinical trials is required (90).

5.3.4 Myricetin

Myricetin belongs to a subclass of bio-flavonoids called flavonols. It is majorly found in berries, fruits, vegetables, medicinal herbs and tea plants (100, 101). Myricetin is known to possess antioxidant, anti-inflammatory, antimicrobial, anti-viral, antioxidative, anti-tumorigenic and antiallergic properties. Recent studies also provide evidence for myricetin exhibiting

osteo-protective properties and inhibiting osteo-clastogenesis (102, 103). Huang et al., demonstrated that myricetin treatment suppressed RANKL-induced differentiation of mouse macrophage RAW264.7 cells into characteristic TRAP-positive multinucleated osteoclast-like cells (OCL). The hypothesis that impairment of osteoclast differentiation would also result in the inhibition of osteoclast bone resorption was confirmed by bone resorption assay, which showed complete bone resorption activity at myricetin concentrations ≥50 mm (100). In the Titanium particle-induced mouse calvarial osteolysis model, myricetin disrupted the RANKL-induced F-actin ring formation, a characteristic feature of mature osteoclasts responsible for bone resorption. It also decreased the RANKL-induced expression of osteoclastogenic markers TRAP, CTR, CTSK, V-ATPase-d2, c-Fos, and NFATc1 (**Figure 4**). Further, Myricetin inhibited the production of pro-inflammatory cytokines TNF-a and IL-1b, thereby suppressing the NF-kB pathway and MAPK pathways (p38, JNK1/2, and ERK1/2) responsible for osteoclast formation and bone resorption (104). Ying et al., showed that treatment with myricetin elevated the serum OCN and ALP levels in rats with streptozotocin-induced diabetic osteoporosis. The levels of serum anti-oxidants SOD and catalase were also increased in response to addition of myricetin (105). In human chondrocytes, myricetin reduced the levels of IL-1b stimulated inflammatory mediators and cytokines such as PEG-2, COX-2, iNOS, IL-6 and TNF-a as well as the elevated levels of MMPs, thereby inhibiting extracellular matrix (ECM) degradation and promoting generation of COL-2. Regulation of these mediators was associated with the repression of NF-kB pathway by the activation of Nrf2/HO-1 with a possible mediation of the PI3K/AKT pathway (106). Pre-treatment of human gingival fibroblasts with myricetin suppressed the LPS-induced expression of MMP-1, MMP-2 and MMP-8. RANKL-stimulated RAW264.7 cells when pre-treated with myricetin, exhibited reduced phosphorylation of p38 and ERK pathways, inhibited phosphorylation of c-Src and impeded the degradation of IkB-a. Moreover myricetin showed inhibitory effects on the m-RNA expression of osteoclastogenic markers such as TRAP, c-FOS, CTSK and NFATc-1 (107). Myricetin exhibits protective effects against 2-deoxy-D-ribose induced oxidative damage in MC3T3-E1 cells by decreasing the levels of protein carbonyl, advanced oxidation protein products, and MDA. Besides it elevated the levels of ALP activity, collagen content, calcium deposition, OCN and OPG in the presence of 2-deoxy-D-ribose (108). In human bone marrow stromal cells (hBMSCs), myricetin upregulated the levels of m-RNA expressions of osteogenic markers OCN, COL-1, ALP and RUNX2. Apart from that, myricetin triggered the Wnt/b-catenin pathway and

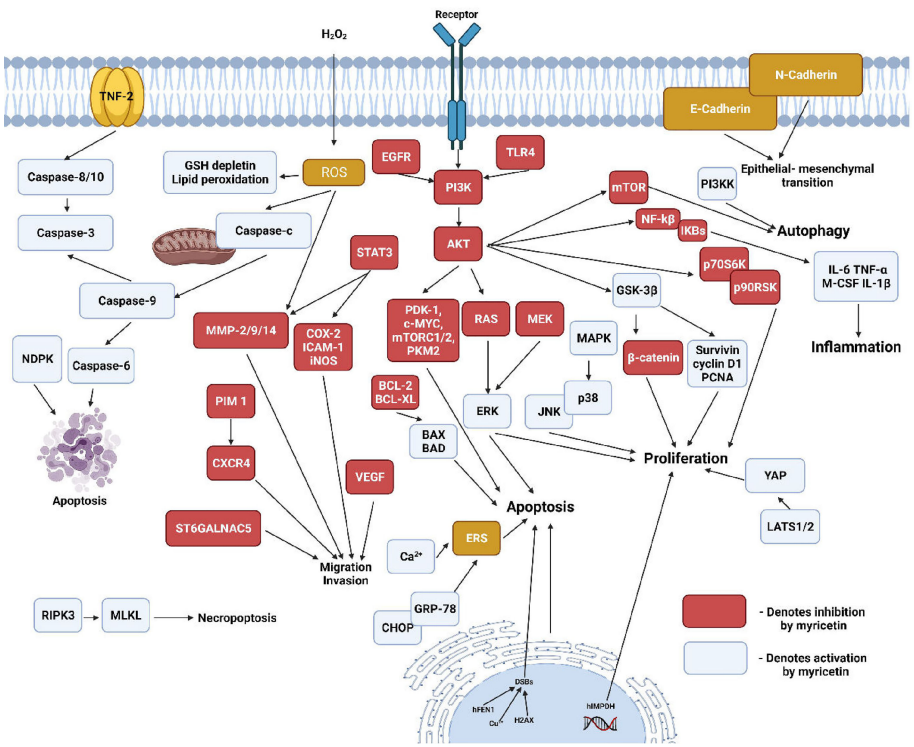

FIGURE 4 | Regulation of cellular pathways by myricetin. (Nucleoside diphosphate kinase-NDPK, Receptor-interacting serine/threonine-protein kinase 3-RIPK3, Mixed lineage kinase domain-like-MLKL, C-X-C chemokine receptor type 4- CXCR4, ST6 N-Acetylgalactosaminide Alpha-2,6-Sialyltransferase 5- ST6GALNAC5, Phosphoinositide-dependent kinase 1-PDK 1, Pyruvate kinase M2-PMK2, Toll-like receptor 4- TLR4, CCAAT/enhancer binding protein homologous protein-CHOP, Glucose-regulated protein 78-GRP-78, Endoplasmic reticulum stress-ERS, Proliferating cell nuclear antigen-PCNA, Yes-associated protein-YAP-1, Large tumour suppressor kinase-1/2- LATS1/2, p90 ribosomal S6 kinase-p90RSK, Ribosomal protein S6 kinase beta-1-p70S6K).

upregulated the expression of several downstream genes such as T-cell factor-1(TCF-1) and lymphoid enhancer factor-1 (LEF-1) (109). Hsu et al., showed that treatment with myricetin on the conditionally immortalized human fetal osteoblastic cell line (hFOB) and the human osteosarcoma cell line MG-63, caused a significant upregulation of BMP-2, which in turn increased the phosphorylated levels of SMAD 1/5/8 and p38, one of the MAPK pathways (110). The effects of myricetin on Dexamethasone(DEX) treated MC3T3 cells revealed that, it ameliorated the DEX-induced inhibition of bone formation markers namely RUNX2, BSP, OPN, OCN, COL1A1 and ALP. Besides myricetin promoted matrix mineralisation *via* the ERK signalling pathway and downregulated TRAP activity and C-terminal telopeptide of type I collagen (CTx) in DEX treated cells (111). Pre-treatment with myricetin on MG-63 cells, reduced the synergistic effect of IL-1b and TNF-a on anti-Fos immunoglobulin-M (IgM) mediated apoptosis of osteoblasts, thereby attenuating the activation of apoptotic proteins caspase-8 and caspase-3, and upregulating the levels of the anti-apoptotic protein FLICE inhibitory protein (FLIP) (112). Overall, myricetin has proven to exhibit osteogenic properties and further studies are required to use it as a therapeutic agent against bone diseases.

5.3.5 Naringin

Naringin, a polymethoxylated flavonoid glycoside, is an active ingredient of citrus fruits and Chinese herbal medicine. It possesses several pharmacological effects, including bone-protective properties. Zhu et al. demonstrated that naringin exhibits anti-osteoporosis property in a fashion similar to estrogen, by binding to the estrogen receptors. This might replace estrogen-replacement therapy in clinical use (113, 114). In the study of Li et al., naringin promoted the osteogenic proliferation and differentiation of BMSCs and also exhibited a 5-7 day delay between the start of naringin treatment and the burst of ALP expression. This suggested a delayed differentiation pattern of the BMSCs in response to naringin treatment (114). In human amniotic fluid stem cells (hAFSCs), naringin was shown to upregulate ALP activity and calcium deposition in a dose dependent manner. Naringin significantly promoted the expression of osteogenic marker genes including ALP, OPN and COL-1 as well as the osteoclastogenesis-inhibition marker gene OPG, thus enhancing the osteogenic differentiation of hAFSCs (**Figure 5**). This differentiation was shown to be regulated *via* the BMP and Wnt/b-catenin pathways involving BMP-4, RUNX2, b-catenin and cyclin D1 (115). Further, naringin induces the apoptosis of osteoclasts *via* inhibition of activation of the death receptor pathway (Fas, TNF) or mitochondrial apoptosis pathway. In the study conducted by Li et al., it was confirmed that naringin could downregulate the mRNA expression levels of the pro-apoptotic marker gene BCL-2 and downregulate the expression levels of the anti-apoptotic marker gene BAX (116). Treatment of RAW627.4 cells with naringin abrogated RANKL induced formation of TRAP positive osteoclast cells. Additionally, naringin attenuated the gene expression levels of osteogenic markers such as CTSK, CTR and TRAP as well as osteoclastogenic fusion genes including dendritic cell-specific transmembrane protein (DC-STAMP), and V-ATPase d2 (d2). Further, naringin suppresses the RANKL induced activation of NF-kB *via* inhibition of degradation of IkB and suppresses the activation of ERK pathway as well (117). Recent studies have shown naringin being an HMG-CoA reductase inhibitor, might possibly promote BMP-2 expression and induce bone formation, suggesting the possible involvement of mevalonate pathway. In co-cultures of osteoblasts and bone marrow cells, naringin suppressed the IL-1 induced osteoclastogenesis (118). Naringin may also possess the ability to downregulate the expression of PPARγ in BMSCs, thus reducing adipogenesis and promoting bone formation. In addition, naringin inhibited the mRNA expression of osteoclastogenic markers including RANK, TRAP, MMP-9 and NFATc1, whereas it upregulated c-Fos expression in RAW627.4 cells (119). Li et al., showed that increased levels of SOD, catalase and MDA in dexamethasone (DEX)-treated-inflammatory bowel disease (IBD) rats were significantly reduced by the intervention of naringin (120). The experiments of Wu et al., revealed that naringin induced osteoblast proliferation, differentiation and maturation in cultured osteoblasts. Besides, in MC3T3-E1 osteoblastic cells, the stimulatory effects of naringin on the expression of BMP-2 was found to involve the activations of PI3K, AKT, c-Fos/c-Jun and AP-1 pathways. Furthermore, it was found that the osteo-protective effects of naringin on UMR-106 cells were attributed to its positive effect on the Wnt/b-catenin pathway *via* AMPK and AKT signalling (121, 122). Kanno et al., demonstrated that naringin inhibited the LPS-induced production of NO and the expression of inflammatory gene products such as TNF-a, IL-6, iNOS, COX-2 and the transcriptional activity of NF-kB. Suppression of these pro-inflammatory cytokines which are the positive regulators of osteoclastogenesis *via* the inhibition of NF-kB might result in the inhibition of osteoclastogenesis and bone resorption (123). Further, naringin promoted angiogenesis and neovascularization during fracture callus formation in murine osteoporotic models, likely by regulating the expression of VEGF in osteocytes (119). Naringin's diverse effects on bone indicate its potential in the treatment and prevention of many common orthopaedic conditions. Naringin strongly reduces osteoclastogenesis, inflammation, and adipogenesis and promotes osteoblastic differentiation from progenitor cells for the maintenance and preservation of both cartilage and bone. However additional research is required to assess the ways in which the pharmacokinetic properties of naringin can be improved, in order to optimize its therapeutic effects.

5.3.6 Daidzein

Daidzein is a phytoestrogen belonging to the iso-flavonoid group and abundantly found in soy products. Considering the fact that daidzein can bind to estrogen receptors a and b and have estrogenic effects, they can be used as an alternative to estrogen replacement therapy (124). Osteoblast cell cultures treated with exhibited enhanced osteoblast viability and induced their differentiation from osteoprogenitors to terminally differentiated osteoblasts. Moreover, daidzein increased the ALP activity, OCN synthesis and the mRNA expression levels of BMP-2 in primary osteoblast cell cultures (125). Exposure of porcine osteoblasts to daidzein

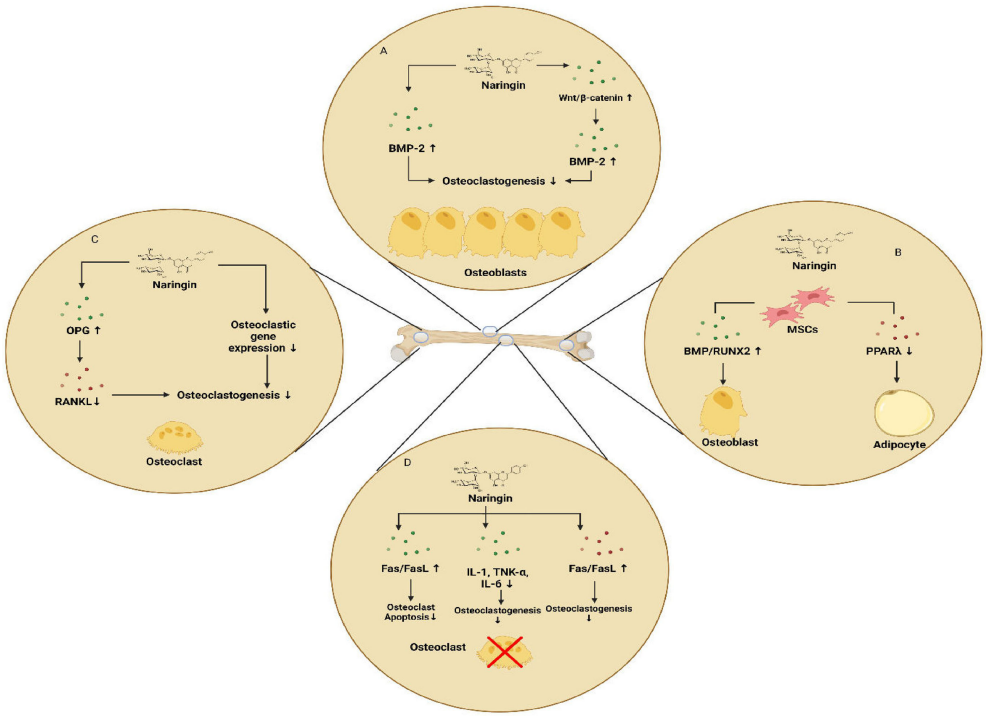

FIGURE 5 | Effects of naringin on bone **(A)** naringin-induced upregulation of osteoblastogenesis *via* regulation of BMP-2 and Wnt/β-catenin pathways **(B)** upregulation of osteoblastogenesis and downregulation of adipogenesis **(C)** inhibition of osteoclastogenesis and osteolysis by naringin mediated by the inhibition of RANK/RANKL interaction **(D)** inhibition of bone resorption by inducing apoptosis of osteoclasts and reducing inflammatory cytokines that induce osteoclast formation.

increased the nuclear levels of the osteogenic transcription factor RUNX2 that was blocked by ICI 182,780. Daidzein also caused a heightened secretion of OPG in the medium of porcine control OB, while it decreased the membrane content of RANKL (126). Picherit et al. demonstrated that in ovariectomised rat model of postmenopausal osteoporosis, oral administration of daidzein arrested both cancellous and cortical bone loss or only cortical bone loss, while manifesting no estrogenic activity on the uterus. Therefore, this proves that daidzein has no adverse effects on the uterus (127). Treatment with daidzein on ovariectomized mice significantly reduced the production of ROS and TNF-a by activated T-cells, both of which are involved in the stimulation of osteoclastogenesis (128). In osteoblast like MG-63 cells, administration of daidzein caused a remarkable elevation in the levels of ALP and COL-1 and also protected against cisplatin induced apoptosis *via* an ER-dependent MEK/ERK and PI3K/AKT activation (129). Daidzein promoted osteoblast proliferation and differentiation *via* the BMP pathway, which upregulated the phosphorylated levels of SMAD 1/5/8. This in turn, led to an increase in the expression of osteogenic marker genes, including ALP, RUN-X2, COL-1 and OSX (130). Furthermore, daidzein demonstrated anti-osteoclastic activity in RAW264.7 cells by downregulating the expression levels of TNF-a induced c-Fos and NFAT-c1 (both of which are important regulators of osteoclast differentiation) in an ER dependent manner. In addition, daidzein inhibited nuclear translocation of NFAT-c1 and also reduced the levels of NF-kB and DC-STAMP levels (131). However, high levels of daidzein cannot not always be beneficial. A correct balance is always required for optimum activity. A study by Dang et al., using mouse bone marrow cells and mouse osteoprogenitor KS483 cells has shown that at concentrations below 20 μM, they inhibit osteogenesis and at concentrations higher than 30 μM, it stimulates adipogenesis (124). This proves that a proper amount of daidzein should be taken, and high or lower levels may not tend to be beneficial to the human body.

5.3.7 Luteolin

Luteolin is a flavonoid found in many herbal extracts and has been a part of the traditional culture in Asian countries through medicines and supplements. Exposure of mouse bone marrow derived macrophages (BMMs) to luteolin inhibited osteoclast differentiation induced by RANKL and also downregulated the expression of osteoclast related genes such as NFATc1, c-Src, DC-STAMP, MMP-9, CTSK and TRAP. Moreover, luteolin suppressed bone resorption in a dose-dependent manner in mature osteoclasts incubated with RANKL and M-CSF (132). In RAW264.7 cells, luteolin inhibited the formation of mature TRAP-positive osteoclasts induced by RANKL *via* the suppression of activating transcription factor (ATF2) downstream of p38 MAPK and NFATc1, thus inhibiting bone resorption. This was accompanied by the disruption of actin rings of the osteoclasts (133). The effects of luteolin on the prevention of bone loss in experimental periodontitis

in Wistar rats were assessed and it was found that treatment with luteolin remarkably decreased the alveolar bone loss by attenuating osteoclastogenic activity and production of osteoclastogenic markers including MMP-9 and RANKL. Besides, it upregulated osteoblastic activity *via* the increased expression of osteogenic markers such as tissue inhibitor of metalloproteinase (TIMP-1), BMP-2, and OPG expressions (134). Nash et al. demonstrated that Luteolin-treated mouse osteoblasts exhibited elevated ALP activity and collagen formation *via* interactions with estrogen receptors (135). Luteolin treatment of MC3T3-E1 osteoblasts abrogated the 3-morpholinosydnonimine (SIN-1)-induced production of oxidative stress markers which included NO, PGE2, TNF-a and IL-6, thus preventing osteoclastogenesis and bone resorption in diseases linked with the overproduction of inflammatory mediators such as arthritis (136). In cultured human periodontal ligament cells (HPDLCs), administration of 1μmol of luteolin strongly enhanced cell viability, ALP activity and increased calcified nodules content. Additionally luteolin significantly upregulated the mRNA and protein expression levels of osteoblast specific markers such as ALP, BMP2, OSX and OCN and the relative expression levels of β-catenin and cyclin D1 (137). Yang et al., demonstrated that in murine calvarial osteoblasts administration of luteolin suppressed the IL-1b-induced expressions of MMP-9 and MMP-13 *via* a possible inhibition of the ERK pathway, thus preventing excessive degradation of bone matrix (138). Luteolin dose dependently suppressed the mRNA and protein expression levels of pro-inflammatory cytokines and mediators including TNF-a, IL-6, COX-2 and iNOS in LPS-stimulated mouse alveolar macrophage MH-S and peripheral macrophage RAW 264.7 cell lines *via* inhibition of phosphorylated NF-kB and AP-1 mediated through blockage of Akt and IkB kinase (IKK) phosphorylation. Further, luteolin inhibited the production of ROS as well (139). In a study by Abasi et al., it was found that luteolin at lower concentrations conferred protection against high-glucose-induced cell death compared to its cytotoxic effects at high doses. Thus, in order to utilise the protective cations of luteolin, it is safest to avoid consuming high doses of luteolin in food supplements (140).

5.3.8 Genistein

Genistein, a phytoestrogen, is a non-steroidal compound, that shows structural similarity to estradiol-17β. This enables genistein to bind to sex hormone binding proteins and estrogen receptors, thus exhibiting anti-estrogenic and estrogenic properties, the former being done by competing with estradiol with estrogen receptors (141, 142). Anderson et al. discovered a tendency in OVX rats treated with genistein to maintain a better bone mass when compared to the untreated control rats and conjugated estrogen-treated rats, with the low-dose genistein treated groups exhibiting the highest numerical effect on bone retention. Several studies have implied that at low doses genistein acts through estrogen receptors, thus rendering bone-preserving effects. However, it has also been shown that genistein at high doses might induce multiple cellular effects and may not necessarily cause estrogen receptor activation. Thus, further studies are required to determine the effects of non-pharmacological doses of genistein (143). In a study conducted by Li et al. on Sprague Dawley rats, it was found that genistein at both high and low doses, caused a remarkable increase in the BMD, bone volume and also resulted in denser subchondral trabecular bone *in vivo*. At low doses, genistein upregulated the mRNA expression levels of osteogenic markers including ALP, OCN, OPG, ERα and ERβ, whereas it downregulated the osteoclastogenic marker RANKL both *in vivo* and invitro. High dose genistein decreased the mRNA levels of bone homeostasis related markers such as ALP, OCN, OPG, RANKL and ERα, while it increased ERβ expression levels invitro and *in vivo*, thus not only inhibiting bone resorption but also bone formation at higher doses- (144). Fanti et al., demonstrated that treatment with genistein of OVX rats lead to an approximate 50% percent reduction in distal femur cancellous bone loss and loss of whole tibia BMD. Highest genistein dose (25 mg/g/day) resulted in larger uterine size compared to the intermediate dose which provided maximum bone-sparing effects but lesser uterine size, thus suggesting a possible non-estrogen mediated mechanism of genistein such as direct interaction with cellular enzymes including *via* direct interaction with cellular enzymes as diverse as 5-LOX, COX, cyclic AMP phosphodiesterase, protein kinases, DNA topoisomerase II and 11b-hydroxysteroid dehydrogenase (**Figure 6**). Moreover, genistein treatment suppressed the elevated levels of pro-inflammatory cytokine TNF-a, an inhibitor of osteogenesis (145). In MC3T3 pre-osteoblastic cells, treatment with genistein altered the expression levels of genes associated with cell proliferation, cell migration, cell differentiation, and inflammatory responses. Successive knockdown analyses showed that two upregulated genes (Ereg and Efcab2) and three downregulated genes (Hrc, Gli1, and Iftm5) play crucial roles in the differentiation of osteoblasts *via* increasing the expressions of osteoblast-associated markers such as RUNX2, ALP and BMP-2 (146). Administration of genistein to human bone marrow stromal cells suppressed its differentiation into adipocytes by inhibiting the mRNA levels of PPARg and CCAAT/enhancer binding proteins (C/EBPs), while it enhanced osteoblastogenesis, thus preventing bone loss associated with excessive adipogenesis (147). Besides, genistein was also found to increase the expression levels of b-catenin and reduced the levels of IL-6 in Sprague Dawley rats (148). Liao et al. have shown that genistein promotes osteoblastic differentiation by the activation of p38 MAPK-RUNX2 pathway. Moreover, several other studies have revealed a possible cross talk between this pathway and other pathways mediated by BMP and protein kinase C (PKC) (149). Genistein also has shown to induce osteoblast proliferation and differentiation from BMSCs through the involvement of ER–NO–cGMP pathway (150). The expressions of two main osteoclastogenic markers c-Fos and NFATc1, were found to be inhibited by genistein. Furthermore, genistein inhibited RANKL-induced degradation of IkB and nuclear translocation of NF-kB and also suppressed the expressions of IL-1 and CTSK mediated by tyrosine kinase-NF-kB pathway. These effects led to the inhibition of differentiation of osteoclasts and subsequent bone resorption (89).

5.3.9 Hesperidin

Hesperidin, also called Hesperetin-7-O-glucuronide is a flavonoid abundantly found in citrus fruits and belongs to the flavonoid

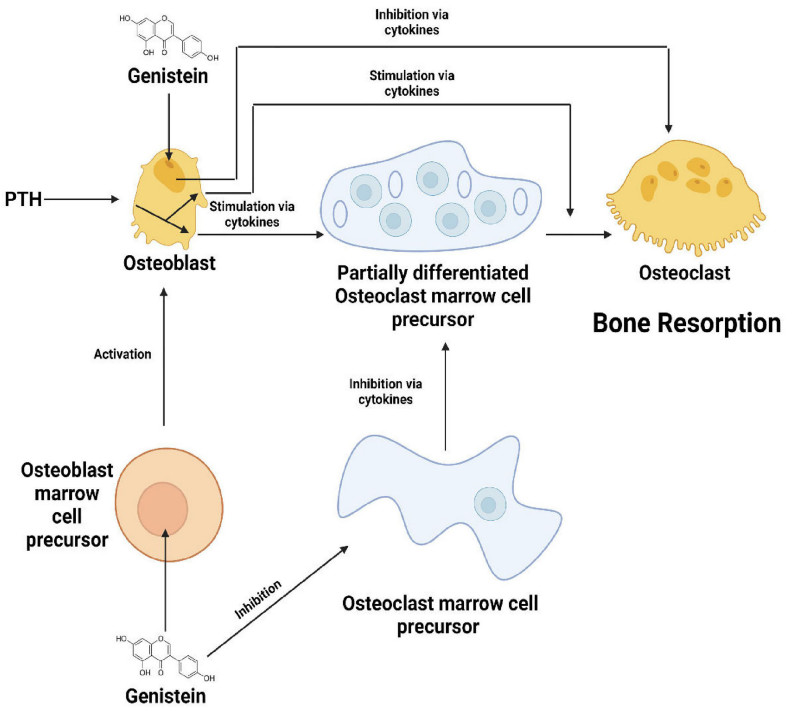

FIGURE 6 | Effects of genistein on osteoblasts, osteoclasts and their precursor cells.

subgroup called flavonones. Hesperidin is a glycoside flavonoid which when absorbed gets hydrolysed into the aglycone form by gut microbiota and undergoes further metabolic changes-1 (151). Several studies have reported hesperidin to act as a potential bioactive compound in maintaining bone health in OVX rat models (152). In primary osteoblasts obtained from wistar rats, hesperidin, was found to upregulate the mRNA levels of ALP and OCN *via* upregulation of RUNX2 and OSX, the two important transcription factors in relation to osteoblasts, which are a part of the MAPK and BMP signalling pathways (**Figure 7**). Phosphorylation of SMAD1/5/8 complex also seemed to be increased, thus suggesting the participation of the BMP pathway through activation of SMAD1/5/8. Moreover, noggin, a protein secreted by osteoblasts and known to hinder the BMP pathway was found to be downregulated by hesperidin (151). Besides, treatment with hesperidin showed slight modulation in the levels of c-Jun and c-Fos, which form a part of the transcription factor AP-1 responsible for the activation of osteoblast-related genes. This indicates the possible intervention of hesperidin through the MAPK signalling pathways (152). In periodontal ligament stem cells (PDLSCs), administration of hesperitin, increased the mRNA level of the osteogenic transcription factor Fos-related antigen-1 (FRA-1) and also the protein levels of OPN and COL-1A. Under conditions of high glucose, the ROS produced by PDLSCs were scavenged by hesperitin. Furthermore, hesperitin also stimulated the activation of Wnt/b-catenin pathway mediated by the activation of PI3K/AKT signalling (153). A study by Kim et al., demonstrated a possible antiresorptive effect of hesperitin through the inhibition of four pathways namely NIK/IKK, ERK, p38, and JNK, which in turn suppressed the NF-kB signalling responsible for osteoclastogenesis and also showed effects on the redox regulating transcription factors Trx/Ref-1 (154). Additionally, exposure to hesperidin of male gonad-intact senescent rats, attenuated the production of the pro-inflammatory cytokine IL-6 (155). Although the exact mechanism of action of hesperidin hasn't been elucidated, the above-mentioned pathways have been discovered as of yet to be regulated by hesperidin.

5.3.10 Apigenin

Apigenin is a flavonoid belonging to the subgroup flavone and is widely present in several fruits and vegetables such as olives, apples and parsley. Although only minimum information is present on the role of apigenin in bone metabolism, a few studies indicate the role of apigenin in preventing bone loss (156). Pre-treatment of H2O2 induced MC3T3-E1 cells with apigenin, caused an upregulation of anti-oxidant enzymes SOD1, SOD2 and glutathione peroxidase (GPx), thus counteracting the ROS produced. Further, apigenin remarkably increased the expression levels of genes responsible for osteoblast differentiation such as ALP, OPN, OPG, BSP, OSX, OCN and BMPs (BMP2, BMP4 and BMP7). Other anti-oxidant properties of apigenin include activating H2O2-induced reduced expression of AKT2, PI3K and ERK, all of which are key-regulators of pathways involved in survival, thus inhibiting apoptosis

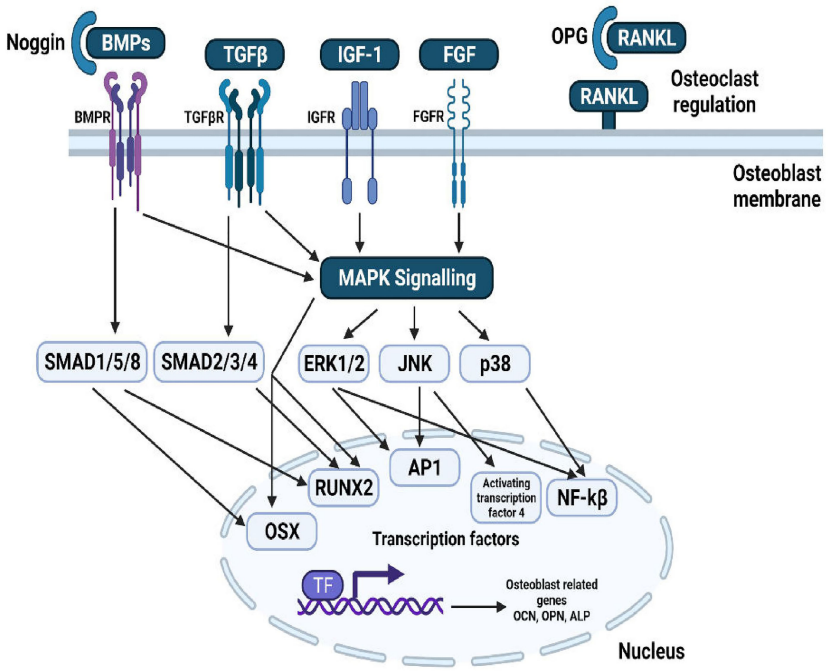

FIGURE 7 | Effect of hesperidin on signalling pathway regulating differentiation of osteoblasts.

osteoblasts (**Figure 8**). These findings suggest the role of apigenin in the treatment of bone diseases associated with oxidative stress (157). Apigenin treatment of TNF-a-induced MC3T3-E1 osteoblasts, reduced its production of IL-6 and NO involved in bone resorption, suggesting apigenin's intervention in treating bone disorders such as osteoporosis characterised by excessive production of inflammatory cytokines (158). In a study by Lee et al., it was demonstrated that apigenin supressed the activity of collagenase in RA and also showed that apigenin inhibited LPS-induced production of NO and COX-2 by RAW 264.7 macrophage cells. In addition, apigenin significantly attenuated the TNFa-induced adhesion of monocytes to human umbilical vein endothelial cell (HUVEC) monolayer and TNF-a-stimulated elevation of vascular cellular adhesion molecule-1 (VCAM-1), intracellular adhesion molecule-1 (ICAM-1), and E-selectin-mRNA, all of which are involved in RA (159). Treating LPS-induced macrophages with apigenin, profoundly suppressed the production of IL-6, IL-1β, and TNF-α *via* regulating various signalling pathways. Apigenin suppressed LPS-induced production of IL-1β by disrupting caspase-1 activation *via* hampering the inflammasome assembly. Also, apigenin arrested the LPS-stimulated production of IL-6 and IL-1β by decreasing the mRNA stability through inhibition of ERK1/2 activation. Additionally, apigenin inhibited the activation of NF-kB *via* induced by TNF-α and IL-1β thus providing evidence to use apigenin for potentially treating inflammatory bone diseases (160). Furthermore, Zhang et al., have shown the involvement of JNK and p38 MAPK signalling pathways in stimulating osteoblast differentiation *via* upregulation of osteoblast-specific genes (161). In conclusion, the pathways discussed above provide evidence to use apigenin as a possible intervention in the treatment of bone-related diseases. **Figure 9** depicts an overview of the flavonoids that regulate molecular mechanism in bone remodelling.

5.3.11 Other Flavonoid

Puerarin, a natural isoflavone isolated from the Chinese herb Pueraria lobata, exhibits osteogenic effects similar to 17-β-estradiol, suggesting a therapeutic role in the treatment of osteoporosis in the future. Puerarin treatment on rat osteoblasts increased the levels of ALP and stimulated osteoblastic proliferation *via* a possible mediation of the PI3K/Akt pathway (162). Puerarin alleviated pathological bone graft defects and apoptosis of BMSCs and increased their proliferation and differentiation. Further, it decreased the levels of proinflammatory cytokines and promoted the levels of anti-inflammatory cytokines, thus ameliorating bone loss *via* inflammation (163). In human osteoblasts (hOBs), treatment with puerarin was shown to inhibit serum-free-induced apoptosis by upregulating the expression of BCL-2 and downregulating the expression of BAX through the activation ERK-1/2 signalling pathway (164). Besides puerarin is well accepted as an autophagy regulator and osteoclastogenesis inhibitor, with the exact role of autophagy in puerarin-regulated osteoclastogenesis still being unclear (165). In RANKL-induced BMMs, osteoclastogenesis was alleviated with puerarin treatment, which inhibited the expression of

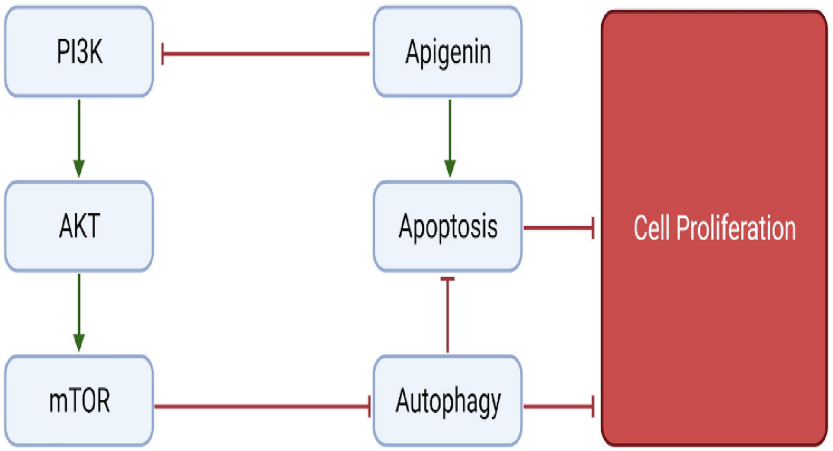

FIGURE 8 | Regulation of autophagy and apoptotic pathways by apigenin.

osteoclastogenic genes and the TRAF6/ROS-dependent MAPK/NF-κB signalling pathway (166). Petunidin, a compound belonging to the flavonoid family anthocyanin has been shown to be a promising natural agent in inhibiting osteoclastogenesis and promoting bone formation. Treating RAW264.7 cells with petunidin significantly inhibited osteoclastogenesis by suppressing the mRNA expression of osteoclastogenic markers c-Fos, NFATc1, MMP9, CTSK, and DC-STAMP. Petunidin stimulated the gene expression of osteogenic markers BMP-2 and OCN, whereas it inhibited mRNA expression of MMP-2, MMP-9, MMP-13 and the proteolytic activities of MMP-9 and MMP-13 in MC3T3-E1 cells (167). The isoflavonoid formononetin has been suggested to be a natural selective estrogen receptor modulator (SERM), and exhibit estrogenic activity on bone cells, thus inhibiting the development of osteoporosis in post-menopausal women (168). A study by Singh et al. revealed that treatment with formononetin on overiectomised (OVx) osteopenic mice repaired the cortical bone defect and promoted bone regeneration accompanied by elevated expression of osteogenic markers BMP-2, RUNX2 and OCN (169). Formononetin treatment on C2C12 progenitor cells remarkably enhanced ALP activity, calcium deposition, and the expression of osteogenesis specific markers including ALP, RUNX2, OCN and BMP isoforms. It was also demonstrated that osteogenic differentiation in these cells treated with formononetin was enhanced by p38 MAPK dependent SMAD 1/5/8 signalling pathways (170). In BMMs, treatment with formononetin regulated OPG and RANKL expression levels, and inhibited RANKL induced TNF-α, IL-1β, IL-6, MCP-1 and macrophage inflammatory protein-1α (MIP-1α). These were accompanied by a reduction in RANKL induced

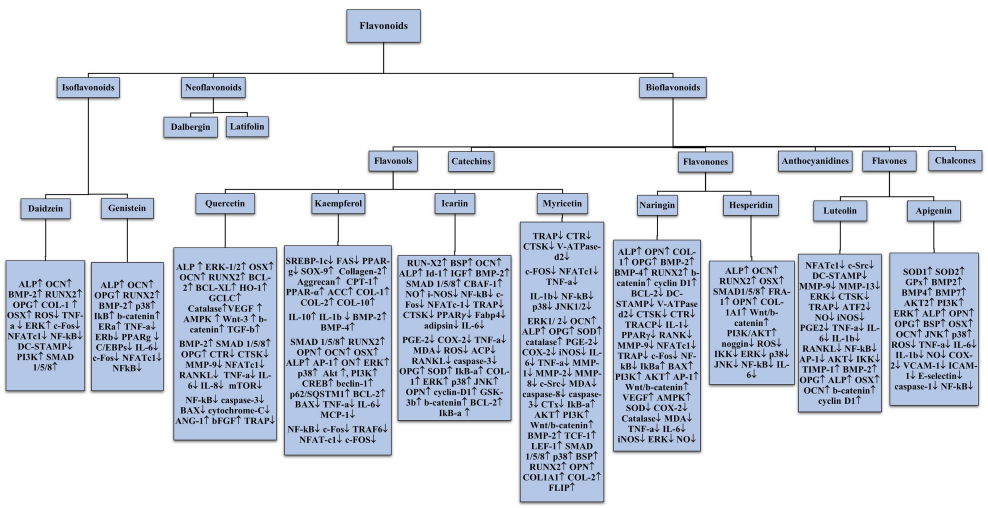

FIGURE 9 | Overall schematic depiction of flavonoids regulating molecules in bone remodelling.

activation of the NF-κB p65 subunit, degradation of IκBα, activation of AKT, ERK, JNK and p38 MAPK (171). In addition, formononetin inhibited classic osteoclastogenic markers significantly. Furthermore, it possesses angiogenic properties required for bone fracture healing and upregulates expression of VEGF and VEGF-R2 (172). Naringenin a dihydro flavonoid compound abundantly found in fruits such as orange, pomelo and drynaria has shown to possess osteogenic effects. In BMSCs, treatment with naringenin upregulated the gene and protein expression levels of ALP, RUNX2, C-X-C chemokine receptor type 4 (CXCR4) and stromal cell-derived factor 1 (SDF-1) *via* the SDF-1/CXCR4 signalling pathway (173). Calycosin, an isoflavonoid phytoestrogen, significantly suppressed osteoclast formation from BMMs and inhibited the expression of osteoclastogenic markers, including CTSK, TRAP and MMP-9. Moreover, calycosin attenuated the expression levels of NFATc1 and c-Fos *via* inhibition of activation of NF-kB and MAPKs thereby preventing bone resorption (174). Curcuma longa, a member of the family Zingiberaceae commonly referred to as turmeric, contains an important flavonoid named curcumin. A study by Folwarczna et al. exhibited that curcumin reduced serum estradiol and mineralization, and increased bone formation and histomorphometric properties of the bone (175–178). Curcumin was found to downregulate the Wnt/β-catenin pathway, AKT pathway, BCL-2, NF-kB, COX-2 and activated GSK-3β, thus preventing oxidative stress and inflammatory responses induced by these pathways (177, 179–187). A study by Notoya et al., utilising rat calvarial osteoblast-like cells, showed that curcumin inhibited the proliferation of osteoblasts without induction of apoptosis. This occurred due to the expression of p21 protein, which resulted in the arrest of cell cycle (188). In another study by Yamaguchi et al. with an analogue of curcumin UBS109, it was found to increase SMAD activity, BMP-induced SMAD activation and TGF-β-induced SMAD activation. It was also found to inhibit TNF-α-induced SMAD suppression. This might be crucial to enhance the differentiation of osteoblasts (189). Also, curcumin slightly inhibited the enhancement of RANKL by IL-1α in human bone marrow stromal cells (190). Epigallocatechin-3-gallate is a flavonoid found abundantly in green tea. Epigallocatechin (EGCG) was found to have promote differentiation of osteoblasts in murine BMSCs. In a study by Lin et al., EGCG showed upregulation of osteogenic-related genes including osteocalcin, RUNX2, OCN, ALP and BMP2, resulting in increased mineralization in a cultured mesenchymal stem cell line derived from bone marrow (191). The expression of RUNX2 and OSX, which are important for mesenchymal stromal cells to differentiate to osteoblasts, was increased by EGCG, and thereby resulted in increased osteogenesis (192). Oleuropein, a flavonoid found in green olives and the olive tree, has recently been deeply researched for its multiple health benefits (193). In a study by Santiago-Mora et al. using the periodontitis model in rats, Oleuropein downregulated the genes linked with adipogenesis such as lipoprotein lipase and PPAR-γ and upregulated the factors promoting osteogenesis such as OCN, RUNX2 and ALP and eventually enhanced osteoblastic differentiation (194). Moreover, oleuropein reduced JNK, p38 MAPK and ERK1/2, and prevented the translocation of NF-KB from cytosol to nucleus which is important for the activation of NF-KB (195). Castejon et al. also demonstrated a downregulation of MAPK and NF-KB pathway, reduced MMP-3, COX-2, TNF-α, MMP-1 and IL-6 levels in IL-1β-induced synovial fibroblast cells by oleuropein (196, 197). OCN and BMP4 are augmented by this flavonoid, and TRAP osteoclasts are inhibited (194, 198). BMP4 is linked with high OPG production, and this leads to a higher rate of osteoblastogenesis (199).

6 CONCLUSION AND FUTURE PERSPECTIVE

Bone-related disorders as such are a growing problem in aging populations especially post-menopausal women experiencing acute estrogen deficiency. The long-term progression of these diseases give rise to serious consequences such as fractures which create significant negative impacts including reduced quality of life, sustained disability and a growing economic burden due to their high medical costs. The current treatment options consisting of antiresorptive agents (such as bisphosphonates, hormone-replacement therapy, selective oestrogen-receptor modulators and anti-RANKL antibodies) and/or anabolic agents (such as intermittent low doses of teriparatide and antisclerostin antibodies) are not free from adverse effects that limit their use (66). This is where flavonoids come into role. These naturally derived phytochemicals possessing potent bone conserving properties beyond calcium and vitamin D exhibit fewer or no side effects compared to conventional therapies. A number of flavonoids are being evaluated for their properties beyond their chemical anti-oxidant capacity, such as anti-inflammatory effects. By regulating cell signalling pathways that influence osteoblast and osteoclast differentiation, these bioactive compounds have been reported to promote bone formation and inhibit bone resorption. However, there is no single mechanism that can elucidate the actions of flavonoids, rather it is a combination of a myriad of pathways. Despite the presence of several gaps, attempts are being made to develop a unifying model to integrate the identified molecular targets and signalling pathways and show how flavonoids from different plant sources might affect them (8). Only a small number of studies on flavonoids have been extrapolated to human clinical trials. In a double-blind placebo randomised controlled study by Hassan et al., on type 2 diabetes mellitus patients, the effects of quercetin administration on biomarkers of bone mineralisation were investigated. It was found that patients who received an oral supplementation of quercetin at 500mg/day for a period of 3 months exhibited increased levels of serum OCN, Vitamin D and calcium compared to their pre-treatment levels (66). A similar study carried out for a combined dosage administration of icariin, genistein and daidzein for 24 months in postmenopausal women showed reduced bone loss and improved BMD in the lumbar spine and femoral neck (90). Furthermore, ongoing studies suggest the possibility of incorporation of flavonoids in bone scaffolds and grafts to ensure local administration and sustained release of flavonoids which can aid in quicker bone healing. This strategy

has been considered to overcome the shortcomings concerned with bioavailability, stability and other biopharmaceutical properties of flavonoids so that a desired concentration can be maintained at the target site (200). Despite having such tremendous implications on bone health, only a limited number of studies on flavonoids have been extended beyond animal models. In order to translate these animal data to dietary interventions in humans, we also require comparative data of the various sources of flavonoids. Therefore, proper identification of the flavonoids' sources, bioactive ingredients and their effective doses remains crucial to undertake and invest in future clinical trials (8). However, the study of interactions of flavonoids with various cellular pathways and their potential to aid in the prevention or repair of bone defects possesses tremendous scope and is definitely a rich area for future research.

AUTHOR CONTRIBUTIONS

PR, RJ, SS, and SV collected literature and drafted the manuscript. AD provided technical help. SV secured funding, designed the work, and approved the final submitted manuscript. All authors contributed to the article and approved the submitted version.

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REFERENCES

1. Raggatt LJ, Partridge NC. Cellular and Molecular Mechanisms of Bone Remodeling. *J Biol Chem* (2010) 285:25103–8. doi: 10.1074/jbc.R109.041087
2. Vimalraj S. Alkaline Phosphatase: Structure, Expression and Its Function in Bone Mineralization. *Gene* (2020) 754:144855. doi: 10.1016/j.gene.2020.144855
3. Kular J, Tickner J, Chim SM, Xu J. An Overview of the Regulation of Bone Remodelling at the Cellular Level. *Clin Biochem* (2012) 45:863–73. doi: 10.1016/j.clinbiochem.2012.03.021
4. Kenkre JS, Bassett JHD. The Bone Remodelling Cycle. *Ann Clin Biochem* (2018) 55:308–27. doi: 10.1177/0004563218759371
5. Kumar S, Pandey AK. Chemistry and Biological Activities of Flavonoids: An Overview. *Sci World J* (2013) 2013:162750. doi: 10.1155/2013/162750
6. Panche AN, Diwan AD, Chandra SR. Flavonoids: An Overview. *J Nutr Sci* (2016) 5:e47. doi: 10.1017/jns.2016.41
7. Bellavia D, Dimarco E, Costa V, Carina V, De Luca A, Raimondi L, et al. Flavonoids in Bone Erosive Diseases: Perspectives in Osteoporosis Treatment. *Trends Endocrinol Metab* (2021) 32:76–94. doi: 10.1016/j.tem.2020.11.007
8. Weaver CM, Alekel DL, Ward WE, Ronis MJ. Flavonoid Intake and Bone Health. *J Nutr Gerontol Geriatr* (2012) 31:239–53. doi: 10.1080/21551197.2012.698220
9. Vimalraj S, Saravanan S, Subramanian R. Rutin-Zn(II) Complex Promotes Bone Formation - A Concise Assessment In Human Dental Pulp Stem Cells and Zebra. *Chem Biol Interact* (2021) 349:109674. doi: 10.1016/j.cbi.2021.109674
10. Hasan Waheed Janabi A, Ali Kamboh A, Saeed M, Xiaoyu L, BiBi J, Majeed F, et al. Spectroscopic Investigation on the Interaction of DNA With Superparamagnetic Iron Oxide Nanoparticles Doped With Chromene *via* Dopamine as Cross Linker. *Iran J Basic Med Sci* (2020) 23:140–53. doi: 10.22038/IJBMS.2019.35125.8353
11. Mukwaya E, Xu F, Wong MS, Zhang Y. Chinese Herbal Medicine for Bone Health. *Pharm Biol* (2014) 52:1223–8. doi: 10.3109/13880209.2014.884606
12. Li J, Bai L, Li X, He L, Zheng Y, Lu H, et al. Antidiabetic Potential of Flavonoids From Traditional Chinese Medicine: A Review. *Am J Chin Med* (2019) 47:933–57. doi: 10.1142/S0192415X19500496
13. Liu HX, Lin WH, Wang XL, Yang JS. Flavonoids From Preparation of Traditional Chinese Medicines Named Sini-Tang. *J Asian Natural Prod Res* (2005) 7:139–43. doi: 10.1080/1028602042000204063
14. Shi Z, Li T, Liu Y, Cai T, Yao W, Jiang J, et al. Hepatoprotective and Anti-Oxidative Effects of Total Flavonoids From Qu Zhi Qiao (Fruit of Citrus Paradisi Cv.Changshanhuyou) on Nonalcoholic Steatohepatitis *In Vivo* and *In Vitro* Through Nrf2-ARE Signaling Pathway. *Front Pharmacol* (2020) 11. doi: 10.3389/fphar.2020.00483
15. Feng X, McDonald JM. Disorders of Bone Remodeling. *Annu Rev Pathol* (2011) 6:121. doi: 10.1146/annurev-pathol-011110-130203
16. Rucci N. Molecular Biology of Bone Remodelling. *Clin Cases Miner Bone Metab* (2008) 5(1):49–56.
17. Parra-Torres AY, Valdés-Flores M, Orozco L, Velázquez-Cruz R. Molecular Aspects of Bone Remodeling. In: *Topics in Osteoporosis* (2013) Mexico: InTechOpen. doi: 10.5772/54905
18. Hayman AR. Tartrate-Resistant Acid Phosphatase (TRAP) and the Osteoclast/Immune Cell Dichotomy. *Autoimmunity* (2008) 41:218–23. doi: 10.1080/08916930701694667
19. Hsiao C-YY, Chen T-HH, Chu T-HH, Ting Y-NN, Tsai P-JJ, Shyu J-FF. Calcitonin Induces Bone Formation by Increasing Expression of Wnt10b in Osteoclasts in Ovariectomy-Induced Osteoporotic Rats. *Front Endocrinol* (2020) 11:613. doi: 10.3389/fendo.2020.00613
20. Teitelbaum SL. Osteoclasts: What do They do and How do They do It? *Am J Pathol* (2007) 170:427–35. doi: 10.2353/ajpath.2007.060834
21. Boyce B, Yao Z, Xing L. Osteoclasts Have Multiple Roles in Bone in Addition to Bone Resorption. *Crit Rev Eukaryot Gene Expr* (2009) 19:171–80. doi: 10.1615/CritRevEukarGeneExpr.v19.i3.10
22. Hofbauer LC, Heufelder AE. Role of Receptor Activator of Nuclear Factor-κb Ligand and Osteoprotegerin in Bone Cell Biology. *J Mol Med* (2001) 79:243–53. doi: 10.1007/s001090100226
23. Horwood NJ, Elliott J, Martin TJ, Gillespie MT. Osteotropic Agents Regulate the Expression of Osteoclast Differentiation Factor and Osteoprotegerin in Osteoblastic Stromal Cells. *Endocrinology* (1998) 139:4743–6. doi: 10.1210/endo.139.11.6433
24. Kim N, Odgren PR, Kim DK, Marks SC, Choi Y. Diverse Roles of the Tumor Necrosis Factor Family Member TRANCE in Skeletal Physiology Revealed by TRANCE Deficiency and Partial Rescue by a Lymphocyte-Expressed TRANCE Transgene. *Proc Natl Acad Sci U S A* (2000) 97:10905–10. doi: 10.1073/pnas.200294797
25. Lee SE, Woo KM, Kim SY, Kim HM, Kwack K, Lee ZH, et al. The Phosphatidylinositol 3-Kinase, P38, and Extracellular Signal-Regulated Kinase Pathways are Involved in Osteoclast Differentiation. *Bone* (2002) 30:71–7. doi: 10.1016/S8756-3282(01)00657-3
26. Nakchbandi IA, Grey A, Masiukiewicz U, Mitnick M, Insogna K. Cytokines in Primary Hyperparathyroidism. *Parathyroids* (2001) 81:411–21. doi: 10.1016/B978-012098651-4/50027-4
27. Boyce BF, Yoneda T, Lowe C, Soriano P, Mundy GR. Requirement of Pp60c-Src Expression for Osteoclasts to Form Ruffled Borders and Resorb Bone in Mice. *J Clin Invest* (1992) 90:1622–7. doi: 10.1172/JCI116032
28. Grigoriadis AE, Wang ZQ, Cecchini MG, Hofstetter W, Felix R, Fleisch HA, et al. C-Fos: A Key Regulator of Osteoclast-Macrophage Lineage Determination and Bone Remodeling. *Science* (1994) 266:443–8. doi: 10.1126/science.7939685
29. Roodman GD, Windle JJ. Paget Disease of Bone. *J Clin Invest* (2005) 115:200–8. doi: 10.1172/JCI24281
30. Takayanagi H. Mechanistic Insight Into Osteoclast Differentiation in Osteoimmunology. *J Mol Med (Berlin Germany)* (2005) 83:170–9. doi: 10.1007/s00109-004-0612-6

31. Komori T, Yagi H, Nomura S, Yamaguchi A, Sasaki K, Deguchi K, et al. Targeted Disruption of Cbfa1 Results in a Complete Lack of Bone Formation Owing to Maturational Arrest of Osteoblasts. *Cell* (1997) 89:755–64. doi: 10.1016/S0092-8674(00)80258-5
32. Lian JB, Stein GS, Javed A, van Wijnen AJ, Stein JL, Montecino M, et al. Networks and Hubs for the Transcriptional Control of Osteoblastogenesis. *Rev Endocr Metab Disord* (2006) 7:1–16. doi: 10.1007/s11154-006-9001-5
33. Nakashima K, Zhou X, Kunkel G, Zhang Z, Deng JM, Behringer RR, et al. The Novel Zinc Finger-Containing Transcription Factor Osterix is Required for Osteoblast Differentiation and Bone Formation. *Cell* (2002) 108:17–29. doi: 10.1016/S0092-8674(01)00622-5
34. Qin L, Qiu P, Wang L, Li X, Swarthout JT, Soteropoulos P, et al. Gene Expression Profiles and Transcription Factors Involved in Parathyroid Hormone Signaling in Osteoblasts Revealed by Microarray and Bioinformatics. *J Biol Chem* (2003) 278:19723–31. doi: 10.1074/jbc.M212226200
35. Westendorf JJ, Kahler RA, Schroeder TM. Wnt Signaling in Osteoblasts and Bone Diseases. *Gene* (2004) 341:19–39. doi: 10.1016/j.gene.2004.06.044
36. Murshed M, Harmey D, Millán JL, McKee MD, Karsenty G. Unique Coexpression in Osteoblasts of Broadly Expressed Genes Accounts for the Spatial Restriction of ECM Mineralization to Bone. *Genes Dev* (2005) 19:1093–104. doi: 10.1101/gad.1276205
37. Eriksen EF. Cellular Mechanisms of Bone Remodeling. *Rev Endocr Metab Disord* (2010) 11:219–27. doi: 10.1007/s11154-010-9153-1
38. Sozen T, Ozisik L, Calik Basaran N. An Overview and Management of Osteoporosis. *Eur J Rheumatol* (2017) 4:46–56. doi: 10.5152/eurjrheum.2016.048
39. Raisz LG. Pathogenesis of Osteoporosis: Concepts, Conflicts, and Prospects. *J Clin Invest* (2005) 115:3318–25. doi: 10.1172/JCI27071
40. Manolagas SC. Cellular and Molecular Mechanisms of Osteoporosis. *Aging Clin Exp Res 1998* (2013) 10(3):182–90. doi: 10.1007/BF03339652
41. Flemmig TF. Periodontitis. *Ann Periodontol* (1999) 4:32–8. doi: 10.1902/annals.1999.4.1.32
42. Qasim SSB, Al-Otaibi D, Al-Jasser R, Gul SS, Zafar MS. An Evidence-Based Update on the Molecular Mechanisms Underlying Periodontal Diseases. *Int J Mol Sci* (2020) 21. doi: 10.3390/ijms21113829
43. Harris ED. Rheumatoid Arthritis. Pathophysiology and Implications for Therapy. *N Engl J Med* (1990) 322:1277–89. doi: 10.1056/NEJM199005033221805
44. Guo Q, Wang Y, Xu D, Nossent J, Pavlos NJ, Xu J. Rheumatoid Arthritis: Pathological Mechanisms and Modern Pharmacologic Therapies. *Bone Res* (2018) 6:1–14. doi: 10.1038/s41413-018-0016-9
45. Fang Q, Zhou C, Nandakumar KS. Molecular and Cellular Pathways Contributing to Joint Damage in Rheumatoid Arthritis. *Mediators Inflamm* (2020) 2020:3830212. doi: 10.1155/2020/3830212
46. Firestein GS. Evolving Concepts of Rheumatoid Arthritis. *Nature* (2003) 423:356–61. doi: 10.1038/nature01661
47. Rodan GA, Martin TJ. Therapeutic Approaches to Bone Diseases. *Science* (2000) 289:1508–14. doi: 10.1126/science.289.5484.1508
48. Zhang J, Xie Z, Zhang N, Zhong J. Nanosuspension Drug Delivery System: Preparation, Characterization, Postproduction Processing, Dosage Form, and Application. In: *Nanostructures for Drug Delivery*. Shanghai: Elsevier Inc. (2017). p. 413–43. doi: 10.1016/B978-0-323-46143-6.00013-0
49. Werner SR, Morgan JA. Controlling Selectivity and Enhancing Yield of Flavonoid Glycosides in Recombinant Yeast. *Bioprocess Biosyst Eng* (2010) 33:863–71. doi: 10.1007/s00449-010-0409-7
50. Welch AA, Hardcastle AC. The Effects of Flavonoids on Bone. *Curr Osteoporos Rep* (2014) 12:205–10. doi: 10.1007/s11914-014-0212-5
51. Santos E, Maia B, Ferriani A, Teixeira S. *Flavonoids: Classification, Biosynthesis and Chemical Ecology*. Brazil: InTechOpen (2017). doi: 10.5772/67861
52. Tapas A, Sakarkar DM, Kakde R. Flavonoids as Nutraceuticals: A Review. *Trop J Pharm Res* (2008) 7(3):1089–99. doi: 10.4314/tjpr.v7i3.14693
53. Corradini E, Foglia P, Giansanti P, Gubbiotti R, Samperi R, Laganà A. Flavonoids: Chemical Properties and Analytical Methodologies of Identification and Quantitation in Foods and Plants. *Natural Prod Res* (2011) 25:469–95. doi: 10.1080/14786419.2010.482054
54. Silva MP, Alves JSF, Siqueira EM, de Souza Neto MA, Abreu LS, Tavares JF, et al. Isolation and Identification of the Five Novel Flavonoids from Genipa americana Leaves. *Molecules* (2018) 23(10):2521. doi: 10.3390/molecules23102521
55. Havsteen BH. The Biochemistry and Medical Significance of the Flavonoids. *Pharmacol Ther* (2002) 96:67–202. doi: 10.1016/S0163-7258(02)00298-X
56. Kandaswami C, Middleton EJ. Free Radical Scavenging and Antioxidant Activity of Plant Flavonoids. *Adv Exp Med Biol* (1994) 366:351–76. doi: 10.1007/978-1-4615-1833-4_25
57. Hollman PC, Katan MB. Dietary Flavonoids: Intake, Health Effects and Bioavailability. *Food Chem Toxicol* (1999) 37:937–42. doi: 10.1016/S0278-6915(99)00079-4
58. Ko K-P. Isoflavones: Chemistry, Analysis, Functions and Effects on Health and Cancer. *Asian Pac J Cancer Prev* (2014) 15:7001–10. doi: 10.7314/APJCP.2014.15.17.7001
59. Sharma V, Ramawat KG. *Isoflavonoids BT - Natural Products: Phytochemistry, Botany and Metabolism of Alkaloids, Phenolics and Terpenes*. KG Ramawat and J-M Mérillon, editors. Berlin Heidelberg: Springer (2013) p. 1849–65. doi: 10.1007/978-3-642-22144-6_61
60. Křížová L, Dadáková K, Kašparovská J, Kašparovský T. Isoflavones. *Molecules* (2019) 24:1076–104. doi: 10.3390/molecules24061076
61. Kumar P, Ahamad T, Mishra DP, Khan MF. *Plant Neoflavonoids: Chemical Structures and Biological Functions BT - Plant-Derived Bioactives: Chemistry and Mode of Action*. MK Swamy, editor. The Gateway, Singapore: Springer Singapore (2020) p. 35–57. doi: 10.1007/978-981-15-2361-8_3
62. Kawser Hossain M, Abdal Dayem A, Han J, Yin Y, Kim K, Kumar Saha S, et al. Molecular Mechanisms of the Anti-Obesity and Anti-Diabetic Properties of Flavonoids. *Int J Mol Sci* (2016) 17:569. doi: 10.3390/ijms17040569
63. Bernatoniene J, Kopustinskiene DM. The Role of Catechins in Cellular Responses to Oxidative Stress. *Molecules* (2018) 23:965. doi: 10.3390/molecules23040965
64. Ruiz-Cruz S. *Flavonoids: Important Biocompounds in Food*. S Chaparro-Hernández, editor. Sonora, México: IntechOpen (2017). Ch. 16. doi: 10.5772/67864
65. Rozmer Z, Perjési P. Naturally Occurring Chalcones and Their Biological Activities. *Phytochem Rev* (2016) 15:87–120. doi: 10.1007/s11101-014-9387-8
66. Wong SK, Chin KY, Ima-Nirwana S. Quercetin as an Agent for Protecting the Bone: A Review of the Current Evidence. *Int J Mol Sci* (2020) 21:1–37. doi: 10.3390/ijms21176448
67. Braun KF, Ehnert S, Freude T, Egaña JT, Schenck TL, Buchholz A, et al. Quercetin Protects Primary Human Osteoblasts Exposed to Cigarette Smoke Through Activation of the Antioxidative Enzymes HO-1 and SOD-1. *TheScientificWorldJournal* (2011) 11:2348–57. doi: 10.1100/2011/471426
68. Wong RWK, Rabie ABM. Effect of Quercetin on Bone Formation. *J Orthop Res* (2008) 26:1061–6. doi: 10.1002/jor.20638
69. Prouillet C, Mazière JC, Mazière C, Wattel A, Brazier M, Kamel S. Stimulatory Effect of Naturally Occurring Flavonols Quercetin and Kaempferol on Alkaline Phosphatase Activity in MG-63 Human Osteoblasts Through ERK and Estrogen Receptor Pathway. *Biochem Pharmacol* (2004) 67:1307–13. doi: 10.1016/j.bcp.2003.11.009
70. Kim YJ, Bae YC, Suh KT, Jung JS. Quercetin, a Flavonoid, Inhibits Proliferation and Increases Osteogenic Differentiation in Human Adipose Stromal Cells. *Biochem Pharmacol* (2006) 72:1268–78. doi: 10.1016/j.bcp.2006.08.021
71. Tsuji M, Yamamoto H, Sato T, Mizuha Y, Kawai Y, Taketani Y, et al. Dietary Quercetin Inhibits Bone Loss Without Effect on the Uterus in Ovariectomized Mice. *J Bone Miner Metab* (2009) 27:673–81. doi: 10.1007/s00774-009-0088-0
72. Yamaguchi M, Weitzmann MN. Quercetin, a Potent Suppressor of NF-κB and Smad Activation in Osteoblasts. *Int J Mol Med* (2011) 28:521–5. doi: 10.3892/ijmm.2011.749
73. Woo J-T, Nakagawa H, Notoya M, Yonezawa T, Udagawa N, Lee I-S, et al. Quercetin Suppresses Bone Resorption by Inhibiting the Differentiation and Activation of Osteoclasts. *Biol Pharm Bull* (2004) 27:504–9. doi: 10.1248/bpb.27.504
74. Kim HR, Kim BM, Won JY, Lee KA, Ko HM, Kang YS, et al. Quercetin, a Plant Polyphenol, Has Potential for the Prevention of Bone Destruction in Rheumatoid Arthritis. *J Med Food* (2019) 22:152–61. doi: 10.1089/jmf.2018.4259

75. Guo C, Yang RJ, Jang K, Zhou XL, Liu YZ. Protective Effects of Pretreatment With Quercetin Against Lipopolysaccharide-Induced Apoptosis and the Inhibition of Osteoblast Differentiation *via* the MAPK and Wnt/β-Catenin Pathways in MC3T3-E1 Cells. *Cell Physiol Biochem* (2017) 43:1547–61. doi: 10.1159/000481978
76. Luo Z, Liang W, Luo Z, Ge S, Li M, Du J, et al. Oral Administration of Quercetin Inhibits Bone Loss in Rat Model of Diabetic Osteopenia. *Eur J Pharmacol* (2011) 670:317–24. doi: 10.1016/j.ejphar.2011.08.014
77. Messer JG, Hopkins RG, Kipp DE. Quercetin Metabolites Up-Regulate the Antioxidant Response in Osteoblasts Isolated From Fetal Rat Calvaria. *J Cell Biochem* (2015) 116:1857–66. doi: 10.1002/jcb.25141
78. Wong SK, Chin KY, Ima-Nirwana S. The Osteoprotective Effects of Kaempferol: The Evidence From *In Vivo* and *In Vitro* Studies. *Drug Des Dev Ther* (2019) 13:3497–514. doi: 10.2147/DDDT.S227738
79. Trivedi R, Kumar S, Kumar A, Siddiqui JA, Swarnkar G, Gupta V, et al. Kaempferol has Osteogenic Effect in Ovariectomized Adult Sprague-Dawley Rats. *Mol Cell Endocrinol* (2008) 289:85–93. doi: 10.1016/j.mce.2008.02.027
80. Zhu J, Tang H, Zhang Z, Zhang Y, Qiu C, Zhang L, et al. Kaempferol Slows Intervertebral Disc Degeneration by Modifying LPS-Induced Osteogenesis/Adipogenesis Imbalance and Inflammation Response in BMSCs. *Int Immunopharmacol* (2017) 43:236–42. doi: 10.1016/j.intimp.2016.12.020
81. Zhao J, Wu J, Xu B, Yuan Z, Leng Y, Min J, et al. Kaempferol Promotes Bone Formation in Part *via* the mTOR Signaling Pathway. *Mol Med Rep* (2019) 20:5197–207. doi: 10.3892/mmr.2019.10747
82. Pang JL, Ricupero DA, Huang S, Fatma N, Singh DP, Romero JR, et al. Differential Activity of Kaempferol and Quercetin in Attenuating Tumor Necrosis Factor Receptor Family Signaling in Bone Cells. *Biochem Pharmacol* (2006) 71:818–26. doi: 10.1016/j.bcp.2005.12.023
83. Imran M, Rauf A, Shah ZA, Saeed F, Imran A, Arshad MU, et al. Chemo-Preventive and Therapeutic Effect of the Dietary Flavonoid Kaempferol: A Comprehensive Review. *Phytother Res* (2019) 33:263–75. doi: 10.1002/ptr.6227
84. Nepal M, Li L, Cho HK, Park JK, Soh Y. Kaempferol Induces Chondrogenesis in ATDC5 Cells Through Activation of ERK/BMP-2 Signaling Pathway. *Food Chem Toxicol* (2013) 62:238–45. doi: 10.1016/j.fct.2013.08.034
85. Chiou WF, Lee CH, Liao JF, Chen CC. 8-Prenylkaempferol Accelerates Osteoblast Maturation Through Bone Morphogenetic Protein-2/P38 Pathway to Activate Runx2 Transcription. *Life Sci* (2011) 88:335–42. doi: 10.1016/j.lfs.2010.12.009
86. Guo AJ, Choi RC, Zheng KY, Chen VP, Dong TT, Wang ZT, et al. Kaempferol as a Flavonoid Induces Osteoblastic Differentiation *via* Estrogen Receptor Signaling. *Chin Med* (2012) 7:1–7. doi: 10.1186/1749-8546-7-10
87. Choi EM. Kaempferol Protects MC3T3-E1 Cells Through Antioxidant Effect and Regulation of Mitochondrial Function. *Food Chem Toxicol* (2011) 49:1800–5. doi: 10.1016/j.fct.2011.04.031
88. Kim CJ, et al. The Effects of Kaempferol-Inhibited Autophagy on Osteoclast Formation. *Int J Mol Sci* (2018) 19:1–13. doi: 10.3390/ijms19010125
89. Ming LG, Chen KM, Xian CJ. Functions and Action Mechanisms of Flavonoids Genistein and Icariin in Regulating Bone Remodeling. *J Cell Physiol* (2013) 228:513–21. doi: 10.1002/jcp.24158
90. Wang Z, Wang D, Yang D, Zhen W, Zhang J, Peng S. The Effect of Icariin on Bone Metabolism and its Potential Clinical Application. *Osteoporos Int* (2018) 29:535–44. doi: 10.1007/s00198-017-4255-1
91. Zhao J, Ohba S, Shinkai M, Chung U, Nagamune T. Icariin Induces Osteogenic Differentiation *In Vitro* in a BMP- and Runx2-Dependent Manner. *Biochem Biophys Res Commun* (2008) 369:444–8. doi: 10.1016/j.bbrc.2008.02.054
92. Fu S, Yang L, Hong H, Zhang R. Wnt/β-Catenin Signaling is Involved in the Icariin Induced Proliferation of Bone Marrow Mesenchymal Stem Cells. *J Tradit Chin Med* (2016) 36:360–8. doi: 10.1016/S0254-6272(16)30050-4
93. Hsieh TP, Sheu SY, Sun JS, Chen MH, Liu MH. Icariin Isolated From Epimedium Pubescens Regulates Osteoblasts Anabolism Through BMP-2, SMAD4, and Cbfa1 Expression. *Phytomedicine* (2010) 17:414–23. doi: 10.1016/j.phymed.2009.08.007
94. Wu Y, Xia L, Zhou Y, Xu Y, Jiang X. Icariin Induces Osteogenic Differentiation of Bone Mesenchymal Stem Cells in a MAPK-Dependent Manner. *Cell Prolif* (2015) 48:375–84. doi: 10.1111/cpr.12185
95. Xu Q, Chen G, Liu X, Dai M, Zhang B. Icariin Inhibits RANKL-Induced Osteoclastogenesis *via* Modulation of the NF-κB and MAPK Signaling Pathways. *Biochem Biophys Res Commun* (2019) 508:902–6. doi: 10.1016/j.bbrc.2018.11.201
96. Huang J, Bao Y, Xiang W, Jing X, Guo J, Yao X, et al. Icariin Regulates the Bidirectional Differentiation of Bone Marrow Mesenchymal Stem Cells Through Canonical Wnt Signaling Pathway. *Evid Based Complement Alternat Med* (2017) 2017:1–12. doi: 10.1155/2017/8085325
97. Jing X, Du T, Chen K, Guo J, Xiang W, Yao X, et al. Icariin Protects Against Iron Overload-Induced Bone Loss *via* Suppressing Oxidative Stress. *J Cell Physiol* (2019) 234:10123–37. doi: 10.1002/jcp.27678
98. Ma HP, Ma XN, Ge BF, Zhen P, Zhou J, Gao YH, et al. Icariin Attenuates Hypoxia-Induced Oxidative Stress and Apoptosis in Osteoblasts and Preserves Their Osteogenic Differentiation Potential *In Vitro*. *Cell Prolif* (2014) 47:527–39. doi: 10.1111/cpr.12147
99. Hsieh TP, Sheu SY, Sun JS, Chen MH. Icariin Inhibits Osteoclast Differentiation and Bone Resorption by Suppression of MAPKs/NF-κB Regulated HIF-1α and PGE2 Synthesis. *Phytomedicine* (2011) 18:176–85. doi: 10.1016/j.phymed.2010.04.003
100. Huang J, Wu C, Tian B, Zhou X, Ma N, Qian Y. Myricetin Prevents Alveolar Bone Loss in an Experimental Ovariectomized Mouse Model of Periodontitis. *Int J Mol Sci* (2016) 17:1–13. doi: 10.3390/ijms17030422
101. Gupta G, Siddiqui MA, Khan MM, Ajmal M, Ahsan R, Rahaman MA, et al. Current Pharmacological Trends on Myricetin. *Drug Res* (2020) 70:448–54. doi: 10.1055/a-1224-3625
102. Fu YX, Wang YH, Tong XS, Gong Z, Sun XM, Yuan JC, et al. EDACO, a Derivative of Myricetin, Inhibits the Differentiation of Gaoyou Duck Embryonic Osteoclasts *In Vitro*. *Br Poult Sci* (2019) 60:169–75. doi: 10.1080/00071668.2018.1564239
103. Gulsoy Z, Yucel Z, Guven B, Balli U. Investigation of Oxidative Stress in Experimental Periodontitis Treated With Myricetin. *Ann Med Res* (2020) 27:3272. doi: 10.5455/annalsmedres.2020.02.124
104. Wu C, Wang W, Tian B, Liu X, Qu X, Zhai Z, et al. Myricetin Prevents Titanium Particle-Induced Osteolysis *In Vivo* and Inhibits RANKL-Induced Osteoclastogenesis *In Vitro*. *Biochem Pharmacol* (2015) 93:59–71. doi: 10.1016/j.bcp.2014.10.019
105. Ying X, Chen X, Wang T, Zheng W, Chen L, Xu Y. Possible Osteoprotective Effects of Myricetin in STZ Induced Diabetic Osteoporosis in Rats. *Eur J Pharmacol* (2020) 866:172805. doi: 10.1016/j.ejphar.2019.172805
106. Pan X, Chen T, Zhang Z, Chen X, Chen C, Chen L, et al. Activation of Nrf2/HO-1 Signal With Myricetin for Attenuating ECM Degradation in Human Chondrocytes and Ameliorating the Murine Osteoarthritis. *Int Immunopharmacol* (2019) 75:105742. doi: 10.1016/j.intimp.2019.105742
107. Ko SY. Myricetin Suppresses LPS-Induced MMP Expression in Human Gingival Fibroblasts and Inhibits Osteoclastogenesis by Downregulating NFATc1 in RANKL-Induced RAW 264.7 Cells. *Arch Oral Biol* (2012) 57:1623–32. doi: 10.1016/j.archoralbio.2012.06.012
108. Lee KH, Choi EM. Myricetin, a Naturally Occurring Flavonoid, Prevents 2-Deoxy-D-Ribose Induced Dysfunction and Oxidative Damage in Osteoblastic MC3T3-E1 Cells. *Eur J Pharmacol* (2008) 591:1–6. doi: 10.1016/j.ejphar.2008.06.004
109. Ying X, Chen X, Feng Y, Xu HZ, Chen H, Yu K, et al. Myricetin Enhances Osteogenic Differentiation Through the Activation of Canonical Wnt/β-Catenin Signaling in Human Bone Marrow Stromal Cells. *Eur J Pharmacol* (2014) 738:22–30. doi: 10.1016/j.ejphar.2014.04.049
110. Hsu YL, Chang JK, Tsai CH, Chien TTC, Kuo PL. Myricetin Induces Human Osteoblast Differentiation Through Bone Morphogenetic Protein-2/P38 Mitogen-Activated Protein Kinase Pathway. *Biochem Pharmacol* (2007) 73:504–14. doi: 10.1016/j.bcp.2006.10.020
111. Fan S, Gao X, Chen P, Li X. Myricetin Ameliorates Glucocorticoid-Induced Osteoporosis Through the ERK Signaling Pathway. *Life Sci* (2018) 207:205–11. doi: 10.1016/j.lfs.2018.06.006
112. Kuo PL. Myricetin Inhibits the Induction of Anti-Fas IgM-, Tumor Necrosis Factor-α- and Interleukin-1 β-Mediated Apoptosis by Fas Pathway Inhibition in Human Osteoblastic Cell Line MG-63. *Life Sci* (2005) 77:2964–76. doi: 10.1016/j.lfs.2005.05.026
113. Zhu Z, Xie W, Li Y, Zhu Z, Zhang W. Effect of Naringin Treatment on Postmenopausal Osteoporosis in Ovariectomized Rats: A Meta-Analysis and

Systematic Review. *Evid Based Complement Alternat Med* (2021) 2021:1–8. doi: 10.1155/2021/6016874

114. Li N, Jiang Y, Wooley PH, Xu Z, Yang SY. Naringin Promotes Osteoblast Differentiation and Effectively Reverses Ovariectomy-Associated Osteoporosis. *J Orthop Sci* (2013) 18:478–85. doi: 10.1007/s00776-013-0362-9
115. Liu M, Li Y, Yang S-T. Effects of Naringin on the Proliferation and Osteogenic Differentiation of Human Amniotic Fluid-Derived Stem Cells. *J Tissue Eng Regen Med* (2017) 11:276–84. doi: 10.1002/term.1911
116. Li F, Sun X, Ma J, Ma X, Zhao B, Zhang Y, et al. Naringin Prevents Ovariectomy-Induced Osteoporosis and Promotes Osteoclasts Apoptosis Through the Mitochondria-Mediated Apoptosis Pathway. *Biochem Biophys Res Commun* (2014) 452:629–35. doi: 10.1016/j.bbrc.2014.08.117
117. Ang ESM, Yang X, Chen H, Liu Q, Zheng MH, Xu J. Naringin Abrogates Osteoclastogenesis and Bone Resorption *via* the Inhibition of RANKL-Induced NF-κB and ERK Activation. *FEBS Lett* (2011) 585:2755–62. doi: 10.1016/j.febslet.2011.07.046
118. Hirata M, Matsumoto C, Takita M, Miyaura C, Inada M. Naringin Suppresses Osteoclast Formation and Enhances Bone Mass in Mice. *J Health Sci* (2009) 55:463–7. doi: 10.1248/jhs.55.463
119. Yu KE, Alder KD, Morris MT, Munger AM, Lee I, Cahill SV, et al. Re-Appraising the Potential of Naringin for Natural, Novel Orthopedic Biotherapies. *Ther Adv Musculoskelet Dis* (2020) 12:1–21. doi: 10.1177/1759720X20966135
120. Li C, Zhang J, Lv F, Ge X, Li G. Naringin Protects Against Bone Loss in Steroid-Treated Inflammatory Bowel Disease in a Rat Model. *Arch Biochem Biophys* (2018) 650:22–9. doi: 10.1016/j.abb.2018.05.011
121. Wang D, Ma W, Wang F, Dong J, Wang D, Sun B, et al. Stimulation of Wnt/β-Catenin Signaling to Improve Bone Development by Naringin *via* Interacting With AMPK and Akt. *Cell Physiol Biochem* (2015) 36:1563–76. doi: 10.1159/000430319
122. Wu J-B, Fong YC, Tsai HY, Chen YF, Tsuzuki M, Tang CH. Naringin-Induced Bone Morphogenetic Protein-2 Expression *via* PI3K, Akt, C-Fos/c-Jun and AP-1 Pathway in Osteoblasts. *Eur J Pharmacol* (2008) 588:333–41. doi: 10.1016/j.ejphar.2008.04.030
123. Kanno SI, Shouji A, Tomizawa A, Hiura T, Osanai Y, Ujibe M, et al. Inhibitory Effect of Naringin on Lipopolysaccharide (LPS)-Induced Endotoxin Shock in Mice and Nitric Oxide Production in RAW 264.7 Macrophages. *Life Sci* (2006) 78:673–81. doi: 10.1016/j.lfs.2005.04.051
124. Dang ZC, Löwik CWGM. The Balance Between Concurrent Activation of ERs and PPARs Determines Daidzein-Induced Osteogenesis and Adipogenesis. *J Bone Miner Res* (2004) 19:853–61. doi: 10.1359/jbmr.040120
125. Jia TL, Wang HZ, Xie LP, Wang XY, Zhang RQ. Daidzein Enhances Osteoblast Growth That may be Mediated by Increased Bone Morphogenetic Protein (BMP) Production. *Biochem Pharmacol* (2003) 65:709–15. doi: 10.1016/S0006-2952(02)01585-X
126. de Wilde A, Lieberherr M, Colin C, Pointillart A. A Low Dose of Daidzein Acts as an Erβ-Selective Agonist in Trabecular Osteoblasts of Young Female Piglets. *J Cell Physiol* (2004) 200:253–62. doi: 10.1002/jcp.20008
127. Picherit C, Coxam V, Bennetau-Pelissero C, Kati-Coulibaly S, Davicco MJ, Lebecque P, et al. Daidzein is More Efficient Than Genistein in Preventing Ovariectomy- Induced Bone Loss in Rats. *J Nutr* (2000) 130:1675–81. doi: 10.1093/jn/130.7.1675
128. Tyagi AM, Srivastava K, Sharan K, Yadav D, Maurya R, Singh D. Daidzein Prevents the Increase in CD4+CD28null T Cells and B Lymphopoesis in Ovariectomized Mice: A Key Mechanism for Anti-Osteoclastogenic Effect. *PloS One* (2011) 6:e21216. doi: 10.1371/journal.pone.0021216
129. Jin X, Sun J, Yu B, Wang Y, Sun WJ, Yang J, et al. Daidzein Stimulates Osteogenesis Facilitating Proliferation, Differentiation, and Antiapoptosis in Human Osteoblast-Like MG-63 Cells *via* Estrogen Receptor–Dependent MEK/ERK and PI3K/Akt Activation. *Nutr Res* (2017) 42:20–30. doi: 10.1016/j.nutres.2017.04.009
130. Hu B, Yu B, Tang DZ, Li SY, Wu Y, Chen M. Daidzein Promotes Proliferation and Differentiation in Osteoblastic OCT1 Cells *via* Activation of the BMP-2/Smads Pathway. *Pharmazie* (2017) 72:35–40. doi: 10.1691/ph.2017.6502
131. Karieb S, Fox SW. Phytoestrogens Directly Inhibit TNF-α-Induced Bone Resorption in RAW264.7 Cells by Suppressing C-Fos-Induced NFATc1 Expression. *J Cell Biochem* (2011) 112:476–87. doi: 10.1002/jcb.22935
132. Kim TH, Jung JW, Ha BG, Hong JM, Park EK, Kim HJ, et al. The Effects of Luteolin on Osteoclast Differentiation, Function *In Vitro* and Ovariectomy-Induced Bone Loss. *J Nutr Biochem* (2011) 22:8–15. doi: 10.1016/j.jnutbio.2009.11.002
133. Lee JW, Ahn JY, Hasegawa SI, Cha BY, Yonezawa T, Nagai K, et al. Inhibitory Effect of Luteolin on Osteoclast Differentiation and Function. *Cytotechnology* (2009) 61:125–34. doi: 10.1007/s10616-010-9253-5
134. Balci Yuce H, Toker H, Yildirim A, Tekin MB, Gevrek F, Altunbas N. The Effect of Luteolin in Prevention of Periodontal Disease in Wistar Rats. *J Periodontol* (2019) 90:1481–9. doi: 10.1002/JPER.18-0584
135. Nash LA, Sullivan PJ, Peters SJ, Ward WE. Rooibos Flavonoids, Orientin and Luteolin, Stimulate Mineralization in Human Osteoblasts Through the Wnt Pathway. *Mol Nutr Food Res* (2015) 59:443–53. doi: 10.1002/mnfr.201400592
136. Choi EM. Modulatory Effects of Luteolin on Osteoblastic Function and Inflammatory Mediators in Osteoblastic MC3T3-E1 Cells. *Cell Biol Int* (2007) 31:870–7. doi: 10.1016/j.cellbi.2007.01.038
137. Quan H, Dai X, Liu M, Wu C, Wang D. Luteolin Supports Osteogenic Differentiation of Human Periodontal Ligament Cells. *BMC Oral Health* (2019) 19:1–10. doi: 10.1186/s12903-019-0926-y
138. Yang H, Liu Q, Ahn JH, Kim SB, Kim YC, Sung SH, et al. Luteolin Downregulates IL-1β-Induced MMP-9 and-13 Expressions in Osteoblasts *via* Inhibition of ERK Signalling Pathway. *J Enzyme Inhib Med Chem* (2012) 27:261–6. doi: 10.3109/14756366.2011.587415
139. Chen CY, Peng WH, Tsai KD, Hsu SL. Luteolin Suppresses Inflammation-Associated Gene Expression by Blocking NF-κB and AP-1 Activation Pathway in Mouse Alveolar Macrophages. *Life Sci* (2007) 81:1602–14. doi: 10.1016/j.lfs.2007.09.028
140. Abbasi N, Khosravi A, Aidy A, Shafiei M. Biphasic Response to Luteolin in MG-63 Osteoblast-Like Cells Under High Glucose-Induced Oxidative Stress. *Iran J Med Sci* (2016) 41:118–25.
141. Dixon RA, Ferreira D. Genistein. *Phytochemistry* (2002) 60:205–11. doi: 10.1016/S0031-9422(02)00116-4
142. Albertazzi P, Steel SA, Bottazzi M. Effect of Pure Genistein on Bone Markers and Hot Flushes. *Climacteric* (2005) 8:371–9. doi: 10.1080/13697130500345257
143. Anderson JJB, Ambrose WW, Garner SC. Biphasic Effects of Genistein on Bone Tissue in the Ovariectomized, Lactating Rat Model. *Proc Soc Exp Biol Med* (1998) 217:345–50. doi: 10.3181/00379727-217-44243
144. Li YQ, Xing XH, Wang H, Weng XL, Bin YS, Dong GY. Dose-Dependent Effects of Genistein on Bone Homeostasis in Rats' Mandibular Subchondral Bone. *Acta Pharmacol Sin* (2012) 33:66–74. doi: 10.1038/aps.2011.136
145. Fanti P, Monier-Faugere MC, Geng Z, Schmidt J, Morris PE, Cohen D, et al. The Phytoestrogen Genistein Reduces Bone Loss in Short-Term Ovariectomized Rats. *Osteoporos Int* (1998) 8:274–81. doi: 10.1007/s001980050065
146. Kim M, Lim J, Lee J-H, Lee K-M, Kim S, Park KW, et al. Understanding the Functional Role of Genistein in the Bone Differentiation in Mouse Osteoblastic Cell Line MC3T3-E1 by RNA-Seq Analysis. *Sci Rep* (2018) 8:1:1–12. doi: 10.1038/s41598-018-21601-9
147. Heim M, Frank O, Kampmann G, Sochocky N, Pennimpede T, Fuchs P, et al. The Phytoestrogen Genistein Enhances Osteogenesis and Represses Adipogenic Differentiation of Human Primary Bone Marrow Stromal Cells. *Endocrinology* (2004) 145:848–59. doi: 10.1210/en.2003-1014
148. Lu R, Zheng Z, Yin Y, Jiang Z. Genistein Prevents Bone Loss in Type 2 Diabetic Rats Induced by Streptozotocin. *Food Nutr Res* (2020) 64:1–12. doi: 10.29219/fnr.v64.3666
149. Liao QC, Xiao ZS, Qin YF, Zhou HH. Genistein Stimulates Osteoblastic Differentiation *via* P38 MAPK-Cbfa1 Pathway in Bone Marrow Culture. *Acta Pharmacol Sin* (2007) 28:1597–602. doi: 10.1111/j.1745-7254.2007.00632.x
150. Pan W, Quarles LD, Song LH, Yu YH, Jiao C, Tang HB, et al. Genistein Stimulates the Osteoblastic Differentiation *via* NO/cGMP in Bone Marrow Culture. *J Cell Biochem* (2005) 94:307–16. doi: 10.1002/jcb.20308
151. Trzeciakiewicz A, Habauzit V, Mercier S, Barron D, Urpi-Sarda M, Manach C, et al. Molecular Mechanism of Hesperetin-7-O-Glucuronide, the Main Circulating Metabolite of Hesperidin, Involved in Osteoblast Differentiation. *J Agric Food Chem* (2010) 58:668–75. doi: 10.1021/jf902680n

152. Trzeciakiewicz A, Habauzit V, Mercier S, Lebecque P, Davicco M, Coxam V, et al. Hesperetin Stimulates Differentiation of Primary Rat Osteoblasts Involving the BMP Signalling Pathway. *J Nutr Biochem* (2010) 21:424–31. doi: 10.1016/j.jnutbio.2009.01.017
153. Kim SY, Lee JJCJY, Park YD, Kang KL, Lee JJCJY, Heo JS. Hesperetin Alleviates the Inhibitory Effects of High Glucose on the Osteoblastic Differentiation of Periodontal Ligament Stem Cells. *PloS One* (2013) 8:1–11. doi: 10.1371/journal.pone.0067504
154. Horcajada MN, Habauzit V, Trzeciakiewicz A, Morand C, Gil-Izquierdo A, Mardon J, et al. Hesperidin Inhibits Ovariectomized-Induced Osteopenia and Shows Differential Effects on Bone Mass and Strength in Young and Adult Intact Rats. *J Appl Physiol* (2021) 104:648–54. doi: 10.1152/japplphysiol.00441.2007
155. Habauzit V, Maria S, Gil-izquierdo A, Trzeciakiewicz A, Morand C, Barron D, et al. Differential Effects of Two Citrus Fl Avanones on Bone Quality in Senescent Male Rats in Relation to Their Bioavailability and Metabolism. *Bone* (2011) 49:1108–16. doi: 10.1016/j.bone.2011.07.030
156. Goto T, Hagiwara K, Shirai N, Yoshida K, Hagiwara H. Apigenin Inhibits Osteoblastogenesis and Osteoclastogenesis and Prevents Bone Loss in Ovariectomized Mice. *Cytotechnology* (2015) 67:357–65. doi: 10.1007/s10616-014-9694-3
157. Jung WW. Protective Effect of Apigenin Against Oxidative Stress-Induced Damage in Osteoblastic Cells. *Int J Mol Med* (2014) 33:1327–34. doi: 10.3892/ijmm.2014.1666
158. Choi EM. Apigenin Increases Osteoblastic Differentiation and Inhibits Tumor Necrosis Factor-α-Induced Production of Interleukin-6 and Nitric Oxide in Osteoblastic MC3T3-E1 Cells. *Pharmazie* (2007) 62:216–20. doi: 10.1691/ph.2007.3.6629
159. Lee JH, Zhou HY, Cho SY, Kim YS, Lee YS, Jeong CS. Anti-Inflammatory Mechanisms of Apigenin: Inhibition of Cyclooxygenase-2 Expression, Adhesion of Monocytes to Human Umbilical Vein Endothelial Cells, and Expression of Cellular Adhesion Molecules. *Arch Pharm Res* (2007) 30:1318–27. doi: 10.1007/BF02980273
160. Zhang X, Wang G, Gurley EC, Zhou H. Flavonoid Apigenin Inhibits Lipopolysaccharide-Induced Inflammatory Response Through Multiple Mechanisms in Macrophages. *PloS One* (2014) 9:1–18. doi: 10.1371/journal.pone.0107072
161. Zhang X, Zhou C, Zha X, Xu Z, Li L, Liu Y, et al. Apigenin Promotes Osteogenic Differentiation of Human Mesenchymal Stem Cells Through JNK and P38 MAPK Pathways. *Mol Cell Biochem* (2015) 407:41–50. doi: 10.1007/s11010-015-2452-9
162. Zhang Y, Zeng X, Zhang L, Zheng X. Stimulatory Effect of Puerarin on Bone Formation Through Activation of PI3K/Akt Pathway in Rat Calvaria Osteoblasts. *Planta Med* (2007) 73:341–7. doi: 10.1055/s-2007-967168
163. Zhou Y, Lian H, Liu K, Wang D, Xiu X, Sun Z. Puerarin Improves Graft Bone Defect Through microRNA-155-3p-Mediated P53/TNF-α/STAT1 Signaling Pathway. *Int J Mol Med* (2020) 46:239–51. doi: 10.3892/ijmm.2020.4595
164. Liu L, Liu L, Bo T, Li S, Zhu Z, Cui R, et al. Puerarin Suppress Apoptosis of Human Osteoblasts *via* ERK Signaling Pathway. *Int J Endocrinol* (2013) 2013:1–6. doi: 10.1155/2013/786574
165. Zhang G, Wang Y, Tang G, Ma Y. Puerarin Inhibits the Osteoclastogenesis by Inhibiting RANKL-Dependent and –Independent Autophagic Responses. *BMC Complement Altern Med* (2019) 19:269. doi: 10.1186/s12906-019-2691-5
166. Xiao L, Zhong M, Huang Y, Zhu J, Tang W, Li D, et al. Puerarin Alleviates Osteoporosis in the Ovariectomy-Induced Mice by Suppressing Osteoclastogenesis *via* Inhibition of TRAF6/ROS-Dependent MAPK/NF-κB Signaling Pathways. *Aging* (2020) 12:21706–29. doi: 10.18632/aging.103976
167. Nagaoka M, Maeda T, Moriwaki S, Nomura A, Kato Y, Niida S, et al. Petunidin, a B-Ring 50-O-Methylated Derivative of Delphinidin, Stimulates Osteoblastogenesis and Reduces Srankl-Induced Bone Loss. *Int J Mol Sci* (2019) 20:2795. doi: 10.3390/ijms20112795
168. Kaczmarczyk-Sedlak I, Wojnar W, Zych M, Ozimina-Kamińska E, Taranowicz J, Siwek A. Effect of Formononetin on Mechanical Properties and Chemical Composition of Bones in Rats With Ovariectomy-Induced Osteoporosis. *Evid Based Complement Alternat Med* (2013) 2013:1–10. doi: 10.1155/2013/457052
169. Singh KB, Dixit M, Dev K, Maurya R, Singh D. Formononetin, a Methoxy Isoflavone, Enhances Bone Regeneration in a Mouse Model of Cortical Bone Defect. *Br J Nutr* (2017) 117:1511–22. doi: 10.1017/S0007114517001556
170. Soundharrajan I, Kim DH, Kuppusamy P, Choi KC. Modulation of Osteogenic and Myogenic Differentiation by a Phytoestrogen Formononetin *via* P38mapk-Dependent JAK-STAT and Smad-1/5/8 Signaling Pathways in Mouse Myogenic Progenitor Cells. *Sci Rep* (2019) 9:1–12. doi: 10.1038/s41598-019-45793-w
171. Huh JE, Lee WI, Kang JW, Nam D, Choi DY, Park DS, et al. Formononetin Attenuates Osteoclastogenesis *via* Suppressing the RANKL-Induced Activation of NF-κB, C-Fos, and Nuclear Factor of Activated T-Cells Cytoplasmic 1 Signaling Pathway. *J Natural Prod* (2014) 77:2423–31. doi: 10.1021/np500417d
172. Huh JE, Kwon NH, Baek YH, Lee JD, Choi DY, Jingushi S, et al. Formononetin Promotes Early Fracture Healing Through Stimulating Angiogenesis by Up-Regulating VEGFR-2/Flk-1 in a Rat Fracture Model. *Int Immunopharmacol* (2009) 9:1357–65. doi: 10.1016/j.intimp.2009.08.003
173. Wang Y, Bai S, Cheng Q, Zeng Y, Xu X, Guan G. Naringenin Promotes Sdf-1/Cxcr4 Signaling Pathway in Bmscs Osteogenic Differentiation. *Folia Histochem Cytobiol* (2021) 59:66–73. doi: 10.5603/FHC.a2021.0008
174. Quan GH, Wang H, Cao J, Zhang Y, Wu D, Peng Q, et al. Calycosin Suppresses RANKL-Mediated Osteoclastogenesis Through Inhibition of MAPKs and NF-κB. *Int J Mol Sci* (2015) 16:29496–507. doi: 10.3390/ijms161226179
175. Folwarczna J, Zych M, Trzeciak HI. Effects of Curcumin on the Skeletal System in Rats. *Pharmacol Rep* (2010) 62:900–9. doi: 10.1016/S1734-1140(10)70350-9
176. Rohanizadeh R, Deng Y, Verron E. Therapeutic Actions of Curcumin in Bone Disorders. *Bonekey Rep* (2016) 5:793. doi: 10.1038/bonekey.2016.20
177. Li H, Yue L, Xu H, Li N, Li J, Zhang Z, et al. Curcumin Suppresses Osteogenesis by Inducing miR-126a-3p and Subsequently Suppressing the WNT/LRP6 Pathway. *Aging (Albany NY)* (2019) 11:6983–98. doi: 10.18632/aging.102232
178. Dong J, Tao L, Abourehab M, Z H-I. Modified Nanoparticles for Combo-Delivery of Curcumin and Alendronate: Fabrication, Characterization, and Cellular and Molecular Evidences of Enhanced Bone. (2018). Elsevier. doi: 10.1016/j.ijbiomac.2018.05.116
179. Tiwari S, Agarwal S, Seth B, Yadav A. Curcumin-Loaded Nanoparticles Potently Induce Adult Neurogenesis and Reverse Cognitive Deficits in Alzheimer's Disease Model *via* Canonical Wnt/β-Catenin. *ACS Publ* (2013) 8:76–103. doi: 10.1021/nn405077y
180. Tiwari SK, Agarwal S, Tripathi A, Chaturvedi RK. Bisphenol-A Mediated Inhibition of Hippocampal Neurogenesis Attenuated by Curcumin *via* Canonical Wnt Pathway. *Mol Neurobiol* (2016) 53:3010–29. doi: 10.1007/s12035-015-9197-z
181. Vallée A, Lecarpentier Y, Vallée JN. Curcumin: A Therapeutic Strategy in Cancers by Inhibiting the Canonical WNT/β-Catenin Pathway. *J Exp Clin Cancer Res* (2019) 38:323. doi: 10.1186/s13046-019-1320-y
182. Dou H, Shen R, Tao J, Huang L, Shi H, Chen H, et al. Curcumin Suppresses the Colon Cancer Proliferation by Inhibiting Wnt/β-Catenin Pathways *via* miR-130a. *Front Pharmacol* (2017) 8. doi: 10.3389/fphar.2017.00877
183. Ashrafizadeh M, Ahmadi Z, Mohamamdinejad R, Yaribeygi H, Serban M-C, Orafai HM, et al. Curcumin Therapeutic Modulation of the Wnt Signaling Pathway. *Curr Pharm Biotechnol* (2020) 21:1006–15. doi: 10.2174/1389201021666200305115101
184. Zhang F, Lu C, Xu W, Shao J, Wu L, Lu Y, et al. Curcumin Raises Lipid Content by Wnt Pathway in Hepatic Stellate Cell. *J Surg Res* (2016) 200:460–6. doi: 10.1016/j.jss.2015.08.040
185. Prasad C, Rath G, Mathur S, Bhatnagar D, Ralhan R. Potent Growth Suppressive Activity of Curcumin in Human Breast Cancer Cells: Modulation of Wnt/β-Catenin Signaling. *Chem Biol Interact* (2009) 181:263–71.doi: 10.1016/j.cbi.2009.06.012
186. Zhang Z, Chen H, Xu C, Song L, Huang L, Lai Y, et al. Curcumin Inhibits Tumor Epithelial-Mesenchymal Transition by Downregulating the Wnt Signaling Pathway and Upregulating NKD2 Expression in Colon Cancer. *Oncol Rep* (2016) 35:2615–23. span doi: 10.3892/or.2016.4669
187. Ryu M-J, Cho M, Song J-Y, Yun Y-S, Choi I-W, Kim D-E, et al. Natural Derivatives of Curcumin Attenuate the Wnt/β-Catenin Pathway Through Down-Regulation of the Transcriptional Coactivator P300. *Biochem Biophys Res Commun* (2008) 377:1304–8. doi: 10.1016/j.bbrc.2008.10.171
188. Notoya M, Nishimura H, Woo JT, Nagai K, Ishihara Y, Hagiwara H. Curcumin Inhibits the Proliferation and Mineralization of Cultured Osteoblasts. *Eur J Pharmacol* (2006) 534:55–62. doi: 10.1016/j.ejphar.2006.01.028

189. Yamaguchi M, Moore TW, Sun A, Snyder JP, Shoji M. Novel Curcumin Analogue UBS109 Potently Stimulates Osteoblastogenesis and Suppresses Osteoclastogenesis: Involvement in Smad Activation and NF-κB Inhibition. *Integr Biol* (2012) 4:905–13. doi: 10.1039/c2ib20045g
190. Oh S, Kyung TW, Choi HS. Curcumin Inhibits Osteoclastogenesis by Decreasing Receptor Activator of Nuclear factor-kappaB Ligand (RANKL) in Bone Marrow Stromal Cells. *Mol Cells* (2008) 26(5):486–9.
191. Lin S-Y, Kang L, Chen J, Wang C. Phytomedicine H. Molecules (–)-Epigallocatechin-3-Gallate (EGCG) Enhances Osteogenic Differentiation of Human Bone Marrow Mesenchymal Stem Cells. *Molecules* (2018) 23:3221. doi: 10.3390/molecules23123221
192. CH C, ML H, JK C, SH H, GJ W. Green Tea Catechin Enhances Osteogenesis in a Bone Marrow Mesenchymal Stem Cell Line. *Osteoporos Int* (2005) 16:2039–45. doi: 10.1007/s00198-005-1995-0
193. Sun W, Frost B, Liu J. Oleuropein, Unexpected Benefits! *Oncotarget* (2017) 8:17409. doi: 10.18632/oncotarget.15538
194. MM T, H BY, O K, F G, H T. Evaluation of the Effect of Oleuropein on Alveolar Bone Loss, Inflammation, and Apoptosis in Experimental Periodontitis. *J Periodontal Res* (2019) 54:624–32. doi: 10.1111/jre.12662
195. Woźniak M, Michalak B, Wyszomierska J, Dudek MK, Kiss AK. Effects of Phytochemically Characterized Extracts From Syringa Vulgaris and Isolated Secoiridoids on Mediators of Inflammation in a Human Neutrophil Model. *Front Pharmacol* (2018) 9:349. doi: 10.3389/fphar.2018.00349
196. Feng Z, Li X, Lin J, Zheng W, Hu Z, Xuan J, et al. Oleuropein Inhibits the IL-1β-Induced Expression of Inflammatory Mediators by Suppressing the Activation of NF-κB and MAPKs in Human Osteoarthritis Chondrocytes. *Food Funct* (2017) 8:3737–44. doi: 10.1039/C7FO00823F
197. Castejón ML, Rosillo MÁ, Montoya T, González-Benjumea A, Fernández-Bolaños JG, Alarcón-de-la-Lastra C. Oleuropein Down-Regulated IL-1β-Induced Inflammation and Oxidative Stress in Human Synovial Fibroblast Cell Line SW982. *Food Funct* (2017) 8:1890–8. doi: 10.1039/C7FO00210F
198. Filip R, Possemiers S, Heyerick A, Pinheiro I, Raszewski G, Davicco MJ, et al. Twelve-Month Consumption of a Polyphenol Extract From Olive (Olea Europaea) in a Double Blind, Randomized Trial Increases Serum Total Osteocalcin Levels and Improves Serum Lipid Profiles in Postmenopausal Women With Osteopenia. *J Nutr Health Aging* (2015) 19:77–86. doi: 10.1007/s12603-014-0480-x
199. Fujita K, Otsuka T, Yamamoto N, Kainuma S, Ohguchi R, Kawabata T, et al. (-)-Epigallocatechin Gallate But Not Chlorogenic Acid Upregulates Osteoprotegerin Synthesis Through Regulation of Bone Morphogenetic Protein-4 in Osteoblasts. *Exp Ther Med* (2017) 14:417–23. doi: 10.3892/etm.2017.4491
200. Torre E, Iviglia G, Cassinelli C, Morra M. "Potentials of Polyphenols in Bone-Implant Devices", in *Polyphenols*. InTech (2018). pp.s 1–28. doi: 10.5772/intechopen.76319

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Bone Response to Weight Loss Following Bariatric Surgery

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Obesity is a global health challenge that warrants effective treatments to avoid its multiple comorbidities. Bariatric surgery, a cornerstone treatment to control bodyweight excess and relieve the health-related burdens of obesity, can promote accelerated bone loss and affect skeletal strength, particularly after malabsorptive and mixed surgical procedures, and probably after restrictive surgeries. The increase in bone resorption markers occurs early and persist for up to 12 months or longer after bariatric surgery, while bone formation markers increase but to a lesser extent, suggesting a potential uncoupling process between resorption and formation. The skeletal response to bariatric surgery, as investigated by dual-energy X-ray absorptiometry (DXA), has shown significant loss in bone mineral density (BMD) at the hip with less consistent results for the lumbar spine. Supporting DXA studies, analyses by high-resolution peripheral quantitative computed tomography (HR-pQCT) showed lower cortical density and thickness, higher cortical porosity, and lower trabecular density and number for up to 5 years after bariatric surgery. These alterations translate into an increased risk of fall injury, which contributes to increase the fracture risk in patients who have been subjected to bariatric surgery procedures. As bone deterioration continues for years following bariatric surgery, the fracture risk does not seem to be dependent on acute weight loss but, rather, is a chronic condition with an increasing impact over time. Among the post-bariatric surgery mechanisms that have been claimed to act globally on bone health, there is evidence that micro- and macro-nutrient malabsorptive factors, mechanical unloading and changes in molecules partaking in the crosstalk between adipose tissue, bone and muscle may play a determining role. Given these circumstances, it is conceivable that bone health should be adequately investigated in candidates to bariatric surgery through bone-specific work-up and dedicated postsurgical follow-up. Specific protocols of nutrients supplementation, motor activity, structured rehabilitative programs and, when needed, targeted therapeutic strategies should be deemed as an integral part of post-bariatric surgery clinical support.

Keywords: bone loss, bone turnover, bone mineral density, bariatric surgery, fracture risk, rehabilitation

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INTRODUCTION

Obesity is a disease (1) and a global health challenge, now included among the global noncommunicable disease targets identified by the World Health Organization (2). Bariatric surgery constitutes a remarkable tool against obesity and its comorbidities (3). The current epidemic proportions reached by obesity make it a cornerstone treatment to contrast obesity if resistant to standard weight-loss approaches, especially when significant weight loss results are mandatory to control obesity-related impaired health conditions. A number of recent clinical practice guidelines exist on bariatric surgery in adults with obesity (4–6) and the evolution of surgical trends in the past 10 years shows similarities and disparities in the number and types of surgical and endoluminal interventions (7). As for trends of standard bariatric surgery, sleeve gastrectomy (SG) shows a continuous upward trend, while Roux-en-Y gastric bypass (RYGB) and laparoscopic adjustable gastric band (AGB) have trended downward (5). In parallel, an increasing number of gastroenterologists are performing bariatric endoscopic procedures that include placement of intragastric balloons, plications and suturing of the stomach, and insertion of a duodenal-jejunal bypass liner, among other emerging procedures (8).

To ensure long-term postoperative success, patients must be prepared to adopt comprehensive lifestyle changes, yet a number of endogenous and exogenous factors are known to influence bariatric surgery outcomes, as summarized in **Table 1** (5, 9–25). It is known that postsurgical weight loss is associated with extended health benefits in terms of arterial hypertension, diabetes mellitus, cardiopulmonary problems, dyslipidemia, susceptibility to neoplasms, osteoarticular disabilities, gastroesophageal reflux disease, psychosocial wellbeing, quality of life, as well as decreased odds of cardiovascular fatalities, strokes and all-cause-mortality (26–29). Nevertheless, there is growing interest on the potential impact of bariatric surgery on bone health, as changes have accumulated to suggest the postsurgical development of accelerated bone loss and skeletal fragility (30). After gastric bypass, an increase in bone resorption markers occurs as early as 10 days postoperatively (31), then marker levels peak by 6 to 12 months (31, 32) and remain elevated thereafter (33). Biochemical markers of bone formation increase but to a lesser extent, suggesting a potential "uncoupling" of resorption from formation (34–37). Several post-bariatric surgery mechanisms have been claimed to occur and act globally on bone health, including mechanical unloading due to bodyweight loss, adipocyte-derived and gastro-enteric hormone changes, and malabsorptive factors (30, 34, 37, 38). Concerns exist on the key role of bariatric surgery on micronutrient intake, diminished calcium and vitamin D absorption leading to secondary hyperparathyroidism, and restricted energy delivery (30, 37). Secondary hyperparathyroidism, which often occurs before bariatric surgery due to the highly prevalent vitamin D deficiency in obesity, progressively increases its prevalence following bariatric surgery, going from 21% at baseline to 35.4% at 1 year and 63.3% at 5 years after surgery (39). The mechanism resides in calcium malabsorption which, in association with vitamin D deficiency, promotes secondary hyperparathyroidism leading to bone resorption. There are even claims that, in the very long term, post-bariatric surgery hypocalcemia may overstimulate parathyroid gland and lead to the anectodical development of a parathyroid adenoma (40). Further, intestinal adaptation mechanisms, local effect of the Wnt/β-catenin signaling pathway, changes in carrier proteins, and the effect of adipokines are other calcium-related mechanisms potentially involved in bone loss after bariatric surgery (40, 41).

While the skeletal response to the effects of bariatric surgery has been collectively confirmed in systematic reviews and meta-analyses, discrepancies still exist in terms of affected bone regions and timing of dynamic bone changes (30, 42, 43). As such, dual-energy X-ray absorptiometry (DXA) studies have shown significant BMD loss at the hip with less consistent results for the lumbar spine (44, 45). Despite weight stabilization and maintenance of metabolic parameters, bone loss and deterioration in bone strength continued years following bariatric surgery, supporting the hypothesis that fracture risk

TABLE 1 | Baseline and peri-operative factors associated with higher and more durable total weight loss after bariatric surgery.

Factors	Outcomes
Age	Younger patients tend to experience greater results than elderlies
Gender	Higher absolute weight loss occurs in men, greater BMI loss in women
Presurgical bodyweight	Higher preoperative BMI (particularly super-obesity) is associated with less weight loss
Surgical approach	Effectiveness on total weight loss varies as follows: BPD > RYGB > SG > AGB
Motivations and expectations	Physiological, emotional, cognitive, and interpersonal/environmental factors can strengthen bariatric surgery outcomes
Eating behaviors	Disordered eating is associated with poorer weight loss and greater weight regain in the long term
Adherence to dietary guidelines	Presurgery nutritional evaluation, dietary adherence and postsurgery nutritional follow-up are associated with successful postsurgical weight loss.
Gastroenteric environment	Gut hormones, bile acids and gut microbiota predict responses to successful weight loss
Sarcopenia	No interaction is suggested between obesity sarcopenia and postsurgical skeletal muscle loss as compared to nonsarcopenic persons
Muscle mass maintenance and propensity to physical exercise	Physical activity after bariatric surgery is associated with enhanced weight loss outcomes

AGB, laparoscopic adjustable gastric banding; BMI, body mass index; BPD, bilio-pancreatic diversion; RYGB, Roux-en-Y gastric bypass; SG, sleeve gastrectomy.

does not appear to be dependent on weight loss, and rather it increases with observation time (46). Moreover, an increased risk of fall injury has been reported after bariatric surgery, which contributes to increase the fracture risk in patients undergoing bariatric surgery procedures (47, 48). Supporting DXA studies, analyses by high-resolution peripheral quantitative computed tomography (HR-pQCT) also showed lower cortical density and thickness, higher cortical porosity, and lower trabecular density and number for up to 5 years after gastric bypass (49). Given these circumstances, it is conceivable that bone health should be accounted for before deciding on the type of bariatric surgery. Little is known about the role of lifestyle intervention, rehabilitation and pharmacological treatments to prevent or treat post-bariatric surgery bone loss and fracture outcome.

The aim of the present review is to update the current data regarding the mechanisms and determinants of bone damage after bariatric surgery and the strategies of prevention and treatment.

CROSSTALK BETWEEN OBESITY AND BONE HEALTH

The crosstalk between adipose tissue and bone is regulated by a large number of interacting factors (**Figure 1**). A common stromal cell origin of osteoblasts and adipocytes has been proposed as a possible link between adipose tissue and bone (50). Despite being acknowledged as beneficial to bone mineral density (BMD) due to the mechanical loading effect of weight excess (51), obesity is consistently emerging as a potential detrimental factor for bone health, particularly appendicular bones. Studies highlighted the impact of fat overload on bone strength (52–54) and cortical rearrangement through insulin resistance (55). Osteoporosis is associated with obesity in one out of three women (56), and nearly one out of four postmenopausal women with fractures can present with obesity, particularly in the case of ankle and femur fractures (57). Further, femoral neck BMD is reduced and risk of non-vertebral fragility fractures increased in obese postmenopausal women compared to lean counterparts (58). Collectively, potential mechanisms claimed to explain the interaction between obesity, menopause and bone metabolism (59) include: 1) visceral fat accumulation (60) and sarcopenia (61) compromise the mechanical loading effect (62); 2) lower vitamin D levels and secondary hyperparathyroidism can favor osteoporosis (63); 3) obesity affects endocrine somatotroph, adrenal and thyroid signals active on the bone (64–66); 4) increased risk of type 2 diabetes mellitus (T2DM) can impair femoral neck strength (67, 68).

It is noteworthy that several molecules can serve as signaling triggers between adipose and bone tissue. These include several products and determinants, i.e. adipocytokines released by the adipose tissue (AT) and its macrophage-rich stromal fraction, osteoblast- and osteoclast-derived proteins, as well as several vitamins (69–75). Leptin and other adipokines secreted by the adipose tissue can modulate bone cells through major inhibition of bone remodeling, whereas molecules activating the peroxisome proliferator-activated receptor-γ can drive mesenchymal stem cell differentiation from osteoblastic towards adipocyte lineage (76). Obesity-associated leptin resistance has been linked to decreased bone mass, as seen in case of hypoleptinemia due to extreme leanness (77). Leptin actions involve inhibition of osteoblastic bone formation through its binding to a specific receptor located in the hypothalamus (69, 70). A study conducted by Ducy et al. showed that leptin receptor expression is associated with noradrenalin release and activation of β2 adrenergic receptor in osteoblasts, thus reducing their activity (70). In mice, leptin also inhibits endocrine function of osteoblasts by sympathetic enhancement of the Esp gene expression, thereby decreasing osteocalcin bioactivity and leading to hyperinsulinemia and glucose intolerance (78).

Importantly, insulin partakes in the feedback loop between pancreas and osteoblasts (79), enhances bone remodeling and promotes the decarboxylation of osteocalcin. Osteoblast-derived osteocalcin circulates both as carboxylated (cOC) and undercarboxylated (ucOC) isoforms (69). ucOC possesses extra-skeletal effects, as it stimulates insulin expression and secretion, β-cells proliferation and adiponectin expression in adipocytes, thus resulting in improved glucose tolerance (69,

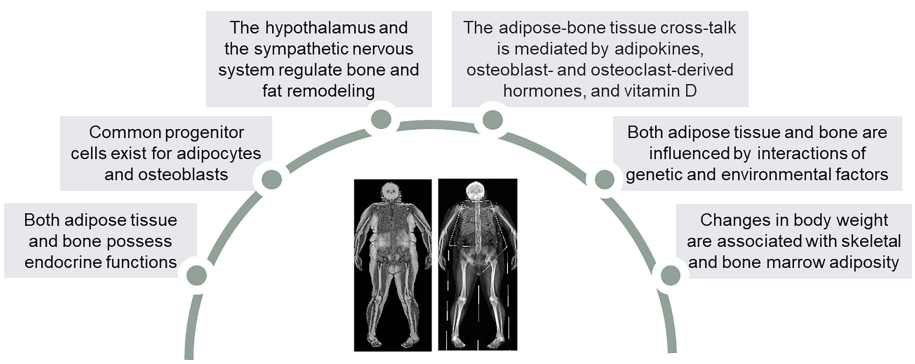

FIGURE 1 | Similarities and homologies between adipose tissue and bone.

80). In 1998, Rosato et al. (81) reported that osteocalcin levels were lower in patients with T2DM than healthy subjects, while others observed lower serum osteocalcin levels in patients with poor metabolic control as compared to those with adequate metabolic control and to healthy subjects (82). In rodents and humans, ucOC has also been found to reduce fat mass accumulation through an increase in adiponectin production (83–88). In osteocalcin-deficient homozygous mice (Ocn -/-), Lee et al. (71) documented higher blood glucose and lower insulin levels than in wild-type or heterozygous mice. More, Ocn -/- mice showed an increase in fat mass, adipocyte number and serum triglyceride levels than wild-type mates. In children and adolescents with obesity, serum osteocalcin is reported to be inversely associated with markers of metabolic health and meta-inflammation, i.e. HOMA-IR, HbA1c, triglycerides, C-reactive protein, and fibrinogen, as well as with measures of adiposity, i.e. body mass index (BMI), body fat and waist circumference (87).

Recent studies emphasized a potential role for the osteocyte-secreted product of the SOST gene, sclerostin, in the relationship linking adiposity, T2DM and bone (89, 90). Biologically, sclerostin antagonizes the Wnt/β catenin signaling pathway, which regulates positively osteoprogenitor cell and osteoblast activity (91–93), and plays a role in adipocyte differentiation and metabolic homeostasis (94). Sclerostin predicts bone loss in relation to age, gender and menopause (95), prolonged immobilization (96), and postmenopausal hip fracture risk (97). Even if no difference appears to exist between obese and controls (98), serum sclerostin was found to be negatively associated with insulin sensitivity in obese but not lean subjects, suggesting a potential role for the Wnt/β-catenin pathway in regulating insulin sensitivity in obesity (99). Moreover, sclerostin is increased in states of unloading and possibly mediates changes in bone metabolism associated with weight loss and exercise (100). A negative association relates sclerostin to skeletal muscle mass after adjusting for multiple confounders (101). Finally, in pre- and postmenopausal women with obesity sclerostin positively predicted lumbar spine BMD. Although this relationship seems to conflict with the intrinsic osteopenic effects of sclerostin, this finding suggests a potential role of this hormone in the protective effects elicited by obesity on lumbar spine at menopause (102). Further studies are required to clarify this issue.

Another relevant link between bone, adipose tissue and glucose metabolism is vitamin D. It has been demonstrated that 25-hydroxyvitamin D (25(OH)D) concentrations are positively associated with adiponectin (103) and negatively associated with indices of insulin resistance (103, 104), BMI, and leptin (105). Debatedly, the adipose tissue (AT) is capable of storing vitamin D and, in case of AT excess, it is deemed as responsible of leading to a reduction in its circulating levels (106, 107). Moreover, there is suggestion that vitamin D deficiency promotes greater adiposity by elevating PTH release, which has been shown to increase intracellular calcium accumulation in adipocytes, thereby enhancing lipogenesis (108). After cholecalciferol administration, a change in multimeric adiponectin is also seen (109). Adiponectin is regulated by osteocalcin and has insulin-sensitizing effects (110), with a well-known negative correlation with parameters of the metabolic syndrome (111–114).

In summary, the crosstalk between bone metabolism and adipose tissue involves multiple factors, which could exert different regulatory mechanisms that affect the skeletal health. However, these mechanisms still need to be clarified.

BONE TURNOVER MARKERS AFTER BARIATRIC SURGERY

Bariatric surgery is characterized by rapid and dramatic changes in body composition and nutritional factors that are paralleled by changes in bone turnover markers (37, 115). Bone turnover can be inspected and monitored through circulating markers that include serum C-terminal cross-linked telopeptide of type I collagen (CTX), procollagen type I N-terminal propeptide (PINP), bone-specific alkaline phosphatase (BALP), and osteocalcin.

These factors dramatically increase from the first 3-10 days, peak after 6-24 months, and remain elevated until 7 years following bariatric surgery (116–118). Both SG and RYGB promote increases in bone turnover markers, with the latter eliciting the strongest effects and leading to an increase in CTX by 50%-300% (37, 49, 119). Comparative analyses between RYBP and SG showed a significantly higher increase in CTX, P1NP, TRAcP5b with the former, and a greater increase in total OC and uOC with the latter, suggesting a predominating bone resorption over bone formation markers during RYGB (36). A randomized triple-blind trial showed an approximately 100% higher increase in P1NP and CTX-1 levels after RYGB than SG at 1-year post surgery (45). Likewise, studies after biliopancreatic diversion (BPD) showed that CTX increased significantly at 3 days (+ 66%), 3 months (+ 219%), and 12 months (+ 295%), while OC decreased at 3 days (- 19%) then increased at 3 months (+ 69%) and 12 months (+ 164%), suggesting an earlier and greater increase in bone resorption over bone formation markers with BPD (120). Although both CTX and P1NP start to decline after 12-24 months since surgery, they do not tend to return to presurgical levels (116, 121). Oppositely, BALP is a less varying bone turnover marker (10-25%) which predominantly changes during the first year after surgery and more after RYGB than SG (49, 118, 119). **Figure 2** summarizes visually the changes occurring over time in bone turnover markers after bariatric surgery. Interestingly, the magnitude of variation in bone turnover markers is reportedly similar between diabetic and non-diabetic cohorts undergoing bariatric surgery, yet patients T2DM patients carry per se a higher risk of osteoporosis (122, 123). Because alterations in CTX and P1NP levels have also been reported in adolescents after RYGB (124) and SG (125), uncertainties remain on the final effect of bariatric surgery on bone mass peak and subsequent adult risk of osteoporosis.

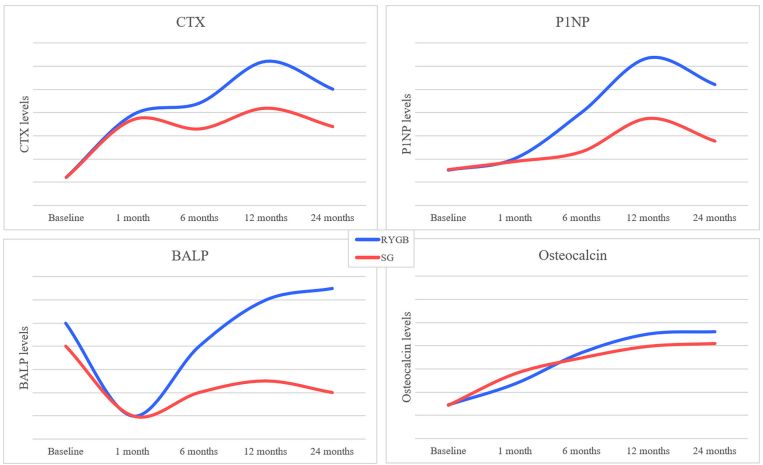

FIGURE 2 | Visual graph of changes over time of bone turnover markers levels after bariatric surgery procedures (RYGB and SG). Data extracted from the references (116, 118,120).

BONE MINERAL DENSITY AFTER BARIATRIC SURGERY

A negative impact of bariatric surgery on bone mineral density (BMD) has been described since the 1980s. Hey et al. described the case of a 38-year old woman who underwent a jejunoileal bypass for severe obesity. Despite vitamin D supplementation, five years after the surgical procedure, the patient develop a fragility fracture of the distal forearm, which healed only after intestinal reanastomosis. The authors hypothesized that the surgery-related malabsorption could lead to alterations in bone metabolism with an increased fracture risk (126). Several studies and meta-analyses on the relationship between bariatric surgery and bone loss have clearly demonstrated that significant declines in BMD occur already within the first year after bariatric surgery (127). Following any type of bariatric surgery, the BMD at the femoral neck has been found significantly lower as compared to controls, with a mean difference (MD) of -0.05 g/cm2 (95% CI -0.07 to -0.02) (43). Oppositely, no difference in BMD was found at column between the two groups.

Growing evidence suggested that the negative effect of bariatric surgery on BMD is strictly dependent on the type of the surgical procedure. A systematic review and meta-analysis by Rodriguez-Carmona et al. demonstrated a significant decrease of -0.03 g/cm2 (95% CI -0.06 to 0.00) in total BMD in patients undergoing mixed restrictive-malabsorptive surgical procedures, but not in those undergoing restrictive surgery (128). In particular, BMD was reduced by -0.07 g/cm2 (95% CI -0.11 to -0.03) at the lumbar spine, -0.12 g/cm2 (95% CI -0.15 to -0.10) at the hip and -0.03 g/cm2 (95% CI -0.04 to -0.02) at the forearm.

BMD changes following bariatric procedures differ depending on skeletal sites and time passed since the operation. At the hip, bone loss following RYGB reaches -3 to -5% after 6 months (129–132) and -6 to -11% after 9-12 months (34, 35, 46, 133–135). Similar results were reported for bone loss at the femoral neck, with a decrease in BMD of -1 to -5% after 6 months and -2 to -9% after 9-12 months, mostly in patients undergoing malabsorptive procedures (34, 35, 38, 49, 130–138). A decline in BMD at the hip and femoral neck has also been observed after restrictive procedures, equivalent to -2 to -8% after 6-24 months (130, 131). At the lumbar spine, some authors did not observe significant variations in BMD at 6-12 month after RYGB (34, 129, 135), whereas others showed a significant reduction in BMD equivalent to -2 to -6% at 6 months and -3 to -7% at 9-12 months (33, 133, 134, 138). Nogues et al. observed a mild difference in BMD loss at the lumbar spine between SG and RYGB (-4.6% vs -6.3%, respectively) (139), while others reported a significant reduction of BMD at this level only after restrictive procedures (130, 140). Further, Maghrabi and co-workers conducted a randomized controlled trial on patients with T2DM to evaluate BMD after 2 years since RYGB and SG, as compared to intensive medical treatment (141). At the hip, BMD loss was significantly higher in patients undergoing SG (-9.2%) and RYGB (-9.5%) than intensive medical therapy group (-0.3%), whereas at lumbar spine a significant decrease in BMD was only observed in the SG group (-2.3%), without changes in RYGB (0.4%) and intensive medical therapy groups (0.8%). A subsequent randomized controlled trial in patients with obesity and T2DM demonstrated that subjects undergoing RYGB had a higher decrease in BMD at the femoral neck (mean between-group difference -2.8%, 95% CI -4.7 to -0.8), total hip (mean between-group difference -3.0%, 95% CI -5.0 to -0.9) and lumbar spine (mean between-group difference -4.2%, 95% CI -6.4 to -2.1) than patients undergoing SG (45).

Overall, the decrease in BMD mainly occurs in the first years after bariatric surgery during the period of rapid weight loss, but it continues even after reaching a stable weight, suggesting that the impact of bariatric surgery on BMD is not completely explained by weight loss (142). Losses appear to be heavier after malabsorptive surgery. Several mechanisms have been

hypothesized to explain the negative effects of bariatric surgery on bone metabolism, including mechanical unloading, malabsorption of macro and micronutrients, alterations in gut-derived hormones and microbiota, and changes in body composition (30, 37), which have been extensively described in the following paragraphs.

BONE MICRO-ARCHITECTURE AFTER BARIATRIC SURGERY

As seen in many observational studies and clinical trials investigating bone loss associated with bariatric surgery, attention is primarily focused on areal BMD (aBMD) evaluated by using dual-energy X-rays absorptiometry (DXA). However, in the setting of a marked weight loss, DXA results could be influenced by changes in the composition of the bone surrounding tissues (37, 143). In addition, DXA is unable to assess bone microarchitecture, as it cannot discriminate trabecular from cortical bone compartments (37). With the aim of overcoming such limitations, more recent studies have focused on HR-pQCT to evaluate post-bariatric volumetric BMD (vBMD) and bone microarchitecture (38, 46, 119, 135, 144–146). A pioneer study by Stein and co-workers (38) assessed HR-pQCT at the distal radius and tibia in 22 women who underwent RYGB (n=14) and restrictive procedures (n=8). Compared to baseline, after 12 months from surgery trabecular parameters remained stable in both sites, while cortical bone deterioration was observed as exemplified by reductions at the tibia in cortical density (-1.7%), cortical thickness (-2.1%) and cortical area (-2.7%). These changes in cortical compartment were noticed to be more pronounced after RYGB and were independently predicted by the increase in PTH levels, thus suggesting a preferential endocortical bone resorption (38).

In a prospective cohort study on 30 obese adults and 20 non-surgical controls, Yu et al. evaluated the rate of bone loss and microarchitectural alterations occurring in 24 months after RYGB (144). Their results showed that total vBMD progressively decreased after surgery both at the radius and tibia, with a 9% decrease of bone strength as compared to the control group. At the radius, the decrease in bone strength was associated with a greater reduction in trabecular vBMD together with an increase in trabecular heterogeneity, while similar reductions in cortical and trabecular vBMD were seen at the tibia. In addition, the authors observed that the impairment in bone microarchitecture, density and strength observed after the first 12 months was maintained or even worsened after 24 months following surgery, despite a bodyweight plateau reached after 6 months. This temporal connection highlights the potential complex origin of bone loss associated with bariatric surgery.

Subsequent studies (46, 119, 135, 145), underscored an increased porosity in the bone cortical compartment associated, in some instances, with a significant decrease in cortical and trabecular vBMD both at the radius and tibia (46, 49, 119, 135, 145) (**Figure 3**). The effects of bariatric surgery on bone microarchitecture and vBMD are early and occur within the first months, with a progressive deterioration over the following years (46, 119, 135, 145). It has been also hypothesized that the changes in the cortical compartment of weight bearing and non-weight bearing sites could be modulated by estrogen concentrations in the bone microenvironment (46, 135).

A recent comparative study on vBMD and bone microarchitecture 10 years after RYGB and adjustable gastric banding (AGB) in comparison to age, sex and BMI-matched non-surgical controls (146) documented that total vBMD at the tibia and radius were 17% and 19% lower than in controls, respectively. Alterations were prominent in trabecular microarchitecture and consisted of lower trabecular number and thinner trabeculae. Moreover, in RYGB group, trabecular bone was less axially aligned at both radius and tibia with a decrease in plate bone volume fraction and density as compared to matched controls. No significant differences were found in terms of bone morphology and microarchitecture between the AGB group and controls (146).

In summary, all the evidences confirmed that the decrease in vBMD after bariatric surgery is strongly related to a significant deterioration in bone microarchitecture and strength, thus predisposing to greater bone fragility.

FRACTURE RISK AFTER BARIATRIC SURGERY

Despite the large body of evidence suggesting that obesity is associated with an increase in BMD, both relating to higher estrogens levels in the adipose tissue and due to the mechanical effect of weight increment (115), this increase in BMD does not reflect a functional improvement of bone microarchitecture, and several studies have demonstrated a higher risk of fracture in obese patients (147). This "obesity paradox" (148) has been attributed to mechanisms involving an increase in bone fragility caused by adiposity and higher risk of falls (115, 149–153) (**Figure 4**).

Opposed to what would be expected, weight loss is not associated with a decrease in bone fractures risk. In fact, some authors have demonstrated that weight loss, both unintentional and intentional, leads to a decrease in BMD at the proximal humerus and the hip (154–156), with a consequent increase of fracture risk in these sites (157–159). Ensrud and co-workers demonstrated that women achieving intentional and non-intentional weight loss had 1.8 times the risk of subsequent hip fracture (95% CI 1.43–2.24) than those with stable or increasing weight (154). Moreover, a decline of 35% in hip BMD for every 5 kg lost has been observed. The consequences of bariatric surgery are strictly connected with the effect of weight loss, and many studies suggested its potential negative effects on bone metabolism (160). A summary of the results of observational studies and interventional trials is reported in **Table 2** (48, 141, 161–169).

One of the first studies designed to evaluate the increased fracture risk in patients undergoing bariatric surgery was

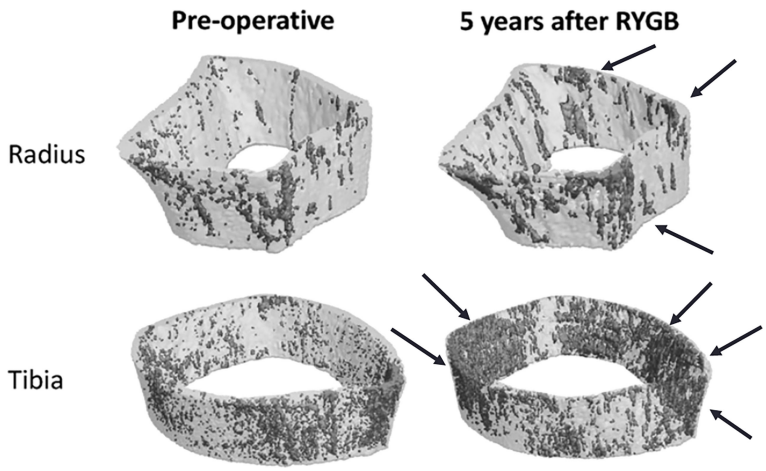

FIGURE 3 | Prospective 5-year observational study of cortical porosity at the distal radius and tibia after RYGB in 21 adults with severe obesity. Declines in cortical and trabecular microarchitecture led to decreases in estimated failure load of -20% and -13% at the radius and tibia (46).

published in 2014 by Nakamura and co-workers (162). The authors observed that the relative risk (RR) for any fracture was increased by 2.3-fold as compared to non-surgical controls and that the standardized incidence ratios (SIRs) for a first fracture in osteoporotic sites, including the spine, hip, wrist, or humerus, was nearly doubled (SIR, 1.9; 95% CI, 1.1-2.9). Many subsequent retrospective and interventional studies confirmed the strong association between bariatric surgery and bone

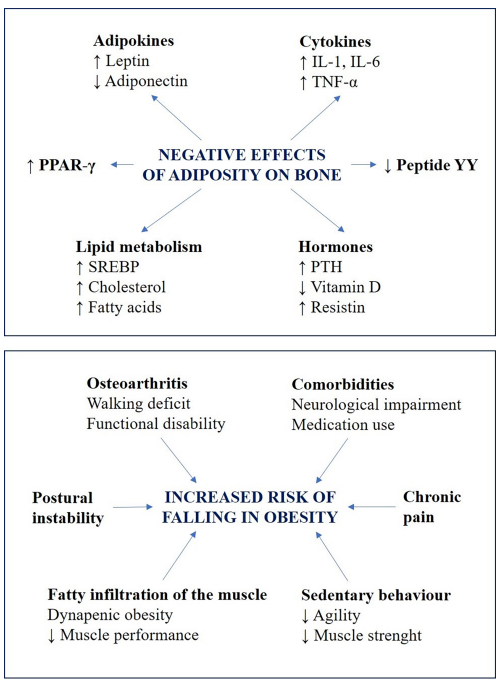

FIGURE 4 | Summary of the two hypothesized mechanisms to explain the susceptibility of obese patients to bone fractures. The negative effects of adiposity on bone fragility are reported in the upper box: obesity is associated with alterations in adipokines and cytokines levels, deregulation of peptides and hormones related to bone metabolism, and dyslipidaemia (112, 146). All these factors contribute to alter bone resorption and formation, by acting directly on osteoclast and osteoblast or indirectly through different molecular pathways. The factors that influence the risk of falling in obesity are reported in the lower box: the mechanistic links between falls and obesity include chronic health conditions, medication use and sedentary behaviour, which lead to a reduction in muscle strength and agility (147). Moreover, biomechanical alterations including poor muscle quality, impaired postural control and osteoarthritis, may reduce postural stability and muscle performances, thus inducing walking deficit and functional disability (148–150).

TABLE 2 | Summary of the observational and interventional studies on the fracture risk after bariatric surgery.

References	Study design	Participants	Type of surgery	Follow-up (years) Mean (SD) or Median (IQR)	Fracture risk for bariatric surgeryrisk ratio (95% CI)
Lalmohamed A 2012 (158)	Retrospective cohort study	Bariatric surgery: 2079 Control group: 10442	AGB: 1249 RYGB: 613 Other: 217	Bariatric surgery: 2.2 (2.1) Control group: 2.3 (2.2)	Adjusted RR (bariatric vs control group): - any fracture: 0.89 (0.60-1.33) - fragility fracture: 0.67 (0.34-1.32) - non-fragility fracture: 0.90 (0.56-1.45)
Nakamura KM 2014 (159)	Retrospective cohort study	Bariatric surgery: 258 No control group	RYGB: 243 VBG: 13 BPD: 1 PBD: 1	Bariatric surgery: 8.9 (4.8) Control group:/	SIR (bariatric vs control group): - any fracture: 2.3 (1.8-2.8) - osteoportic sites: 2.0 (1.3-3.0) - non-osteoporotic sites: 2.4 (1.8-3.0)
Douglas IJ 2015 (160)	Retrospective cohort study	Bariatric surgery: 3882 Control group: 3882	GB: 1829 RYGB: 1421 SG: 613 GS: 6 SP: 5 DS: <5 VBG: <5	Bariatric surgery: 3.4 (2.3) Control group: 3.4 (2.4)	HR for any fracture (bariatric vs control group): 1.28 (0.81-2.02)
Lu CW 2015 (161)	Observational cohort study	Bariatric surgery: 2064 Control group: 5027	Malabsorptive: 289 Restrictive: 1775	Bariatric surgery: 4.8 (2.3) Control group: 4.9 (2.1)	Adjusted HR for any fracture (bariatric vs control group): - all procedures: 1.21 (1.01-1.44) - malabsorptive procedures: 1.47 (1.01-2.15) - restrictive procedures: 1.17 (0.97-1.41)
Maghrabi AH 2015 (138)	Randomized control trial	Bariatric surgery: 37 Control group: 17	SG: 19 RYGB: 18	12 and 24 months	RR for peripheral fractures (bariatric vs control group): 2.12 (0.44-10.16)
Rousseau C 2016 (45)	Case-control study	Bariatric surgery: 12676 Control group: - Obese: 38028 - Non-obese: 126760	AGB: 3887 SG: 2554 BPD: 1986 RYGB: 873	4.4 (range <1-13)	Adjusted RR for any fracture: - Bariatric vs non-obese group: 1.44 (1.29-1.59) - Bariatric vs obese group: 1.38 (1.23-1.55)
Fashandi AZ 2018 (162)	Retrospective cohort study	Bariatric surgery: 3439 Control group: 3380	RYGB: 2729 AGB: 385 SG: 268 Other: 57	From 3 to 22 years	OR for any fracture (bariatric vs control group): - all procedures: 2.36 (1.72-2.23) - RYGB (vs SG): 2.17 (1.04-4.52)
Javanainen M 2018 (163)	Retrospective cohort study	Bariatric surgery: 395 Control group: 199	RYGB: 253 SG: 142	12 and 24 months	HR for any fracture (bariatric vs control group): 5.49 (1.76-17.15)
Yu EW 2019 (164)	Retrospective cohort study	Bariatric surgery: 42345	RYGB: 29624 AGB: 12721	RYGB: 3.3 (2.2) AGB: 3.9 (2.1)	Adjusted HR for non-vertebral fractures (RYGB vs AGB): 1.73 (1.45-2.08)
Ahlin S 2020 (165)	Nonrandomized controlled intervention study	Bariatric surgery: 2007 Control group: 2040	VBG: 1365 GB: 376 RYGB: 266	From 6 months to 20 years	Adjusted HR for any fracture: - VBG vs controls: 1.20 (1.00-1.43) - GB vs controls: 1.30 (0.97-1.74) - RYGB vs controls: 2.58 (2.02-3.31) Adjusted HR for osteoporotic fractures: - VBG vs controls: 1.15 (0.87-1.51) - GB vs controls: 1.85 (1.27-2.70) - RYGB vs controls: 3.60 (2.56-5.05)
Khalid SI 2020 (166)	Retrospective cohort study	Bariatric surgery: 32742 Control group: 16371	RYGB: 16371 SG: 16371	3 years	OR for any fractures: - RYGB vs controls: 0.95 (0.84-1.07) - SG vs controls: 0.53 (0.46-0.62) - RYGB vs SG: 1.79 (1.55-2.06)
Paccou J 2020 (167)	Retrospective cohort study	Bariatric surgery: 40992 Control group: 40992	SG: 18635 RYGB: 14532 AGB: 5178 VBG: 2647	Bariatric surgery: 6.19 years Control group: 5.26 years	Adjusted HR for major osteoporotic fractures: - Bariatric surgery vs controls: 1.22 (1.08-1.39) - SG vs controls: 0.95 (0.79-1.14) - RYGB vs controls: 1.70 (1.46-1.98) - AGB vs controls: 0.95 (0.72-1.25) - VBG vs controls: 0.95 (0.68-1.31)
Alsaed OS 2021 (168)	Case-controlled study	Bariatric surgery: 403	SG: 334 RYGB: 69	8.6 years (mean)	OR for any fracture: 2.71 (1.69-4.36)

(Continued)

TABLE 2 | Continued

References	Study design	Participants	Type of surgery	Follow-up (years) Mean (SD) or Median (IQR)	Fracture risk for bariatric surgeryrisk ratio (95% CI)
Chin WL 2021 (169)	Retrospective cohort study	Control group: 806 Bariatric surgery: 1322 Non-surgical group: 1322 General population: 4359	Not specified	87.55 months (median)	Adjusted HR for any fracture: - Bariatric surgery vs non-surgical group: 0.77 (0.54-1.11) - Bariatric surgery vs general population: 2.21 (1.57-3.11) Adjusted HR for non-traffic accident-related fractures: - Bariatric surgery vs non-surgical group: 0.54 (0.34-0.87) - Bariatric surgery vs general population: 1.69 (1.08-2.66)

AGB, adjustable gastric banding; RYGB, roux-en-Y gastric bypass; VBG, vertical-banded gastroplasty; BPD, biliopancreatic diversion; PBD, pancreatobiliary diversion; SIR, standardized incidence ratios; GB, gastric band; SG, sleeve gastrectomy; GS, gastric stapling; SP, stomach partition; HR, hazard ratio; OR, odds ratio; SG, sleeve gastrectomy.

fractures risk, also in long-term follow-up (47, 48, 164–166, 168, 170, 171). A recent meta-analysis of 10 observational studies was conducted to compare the fracture risk between 116,205 subjects who underwent bariatric surgery and 134,637 non-surgical patients (121). The results showed that the risk of any fractures was significantly increase by 20% in the group of bariatric surgery than the control counterpart. Despite the low rating on the risk of bias assessment scales, the analysis on three interventional trials shows a trend toward an increase in the fracture risk in patients who underwent bariatric surgery (RR 1.16, CI 95% 1.00-1.33).

Alternatively, Lalmohamed at al. failed to observe a significant increase in the fracture risk in patients undergoing bariatric surgery as compared to control group (adjusted relative risk 0.89, 95% CI 0.60-1.33). However, the authors found a trend towards an increased fracture risk after 3 to 5 years following surgery and in patients with a greater weight loss (161). A lack of association between bariatric surgery and an increased fracture risk was also observed in three subsequent retrospective cohort studies (163, 169, 172). The results of a randomized controlled trial aiming at investigating the 2-year outcomes of bariatric surgery vs intensive medical therapy, have reported a lower total and hip BMD in bariatric surgery but the number of bone fractures did not differ between groups (141). It is important to note that these studies predominantly included patients who underwent restrictive surgery with few cases of malabsorptive procedures (161, 163). Some suggested that the degree of damage to bone microarchitecture varies according to the type of surgery (48, 162). Paccou and co-workers, in a population-based cohort study including 81,948 patients (40,992 in the bariatric surgery group, and 40,992 matched controls), observed a 70% increased risk of fragility fractures only for RYGB within 10-year after surgery (170), while no association between the risk of fragility fractures and SG, AGB and vertical banded gastroplasty (VBG) was seen. An average 1.4-fold higher fracture risk was also observed in a Bayesian network metanalysis, with differences emerging across the various surgical procedures (173), as subjects receiving mixed restrictive/malabsorptive procedures tended to suffer from an increased risk of fracture as compared with those undergoing restrictive procedures (RR 1.54, 95% CI 0.96-2.46). A meta-analysis by Chaves et al. confirmed that malabsorptive procedures elicited a high fracture risk as compared to controls (RR 1.53, CI 95% 1.13-2.07), and RYGB group had a higher risk as compared to SG group (RR 1.77, CI 95% 1.48-2.12) (118). It has been suggested that the higher fracture risk related to malabsorptive or combined procedures could be attributable to neurohormonal changes and malabsorption (37).

POTENTIAL MECHANISMS ASSOCIATED WITH BONE LOSS AFTER BARIATRIC SURGERY

A number of mechanisms have been hypothetically linked to postsurgical bone loss, which may involve nutrient absorption deficits, mechanical unloading, alterations in bone marrow adipose tissue (BMAT), as well as changes in adipokines and gut-derived hormones (**Table 3**) (30, 92, 174–195).

In 1985, Parfitt et al. described bone histomorphometric changes in patients undergoing intestinal bypass and collected evidence from literature of 2.5-25% rate of osteomalacia after shunt operations, commenting that osteomalacia after intestinal bypass surgery has similar clinical, biochemical and histologic features as in other causes of net intestinal malabsorption (196). Bariatric surgery impairs the ability of the digestive tract to secrete hydrochloric acid required for digestion and absorption of nutrients which are required for bone formation and healthy bone remodeling, such as trace elements, essential minerals, water-soluble and fat-soluble vitamins. Many of these disorders are inadequately replaced after surgery, especially if extended gastric bypass surgery, duodenal switch or biliopancreatic diversion are involved (197). Among these, calcium is absorbed passively as well as actively in the small intestine, and a study using a dual stable calcium isotope method (198) demonstrated a significant reduction in calcium absorption following RYGB

TABLE 3 | Summary of the mechanisms hypothesized to explain the negative effects of bariatric surgery on bone metabolism.

Factors	Mechanisms	Changes after bariatric surgery	Expected effect on bone
Mechanical loading	The skeletal adaptation to mechanical strain and loading is fundamental to preserve bone mass and microarchitecture. Weight loss reduces mechanical loading on bone structure, thus favoring bone loss (171).	↑ Mechanical unloading	Upregulation of bone turnover/ BMD loss
Nutritional factors			
Vitamin D/ calcium	A high prevalence of hypovitaminosis D have been documented in obese patients (172). Vitamin D deficiency is worsened by bariatric surgery and calcium absorption is impaired, particularly after malabsorptive procedures (30).	↓ Vitamin D levels ↓ Calcium levels ↑ PTH	Upregulation of bone turnover/ BMD loss
Amino-acids	The early post-surgery phase after malabsorptive procedures is characterized by protein depletion (173).	↓ Muscle mass ↑ amino-acids levels	BMD loss
Neuroendocrine and gut-derived hormones			
Peptide YY (PYY)	PYY is produced and secreted by the enteroendocrine L-cells of the colon and ileum to counteract caloric intake (174). Serum concentrations of this hormone increase are directly associated with a higher bone turnover (175).	↑ PYY levels (in contrast with conventional weight loss)	BMD loss
Glucose-dependent insulinotropic polypeptide (GIP)	The incretin hormone GIP is produces and secreted from duodenum and jejunum (176). Animal studies have shown that GIP increase in bone formation. To date, studies with the aim of investigating the post-surgical changes of this hormone in correlation with BMD variations are lacking (30).	↓ GIP levels	BMD loss
Glucagon-like peptide type 1 (GLP-1)	The incretin hormone GLP-1 is produced and secreted by the enteroendocrine L-cells located in the distal ileum and colon. Only few studies correlate the post-bariatric increase in GLP-1 levels with bone metabolism. A recent interventional study has suggested that GLP-1 variations after bariatric surgery do not significantly affect bone metabolism (177).	↑ GLP-1 levels (in contrast with conventional weight loss)	No significant role in bone turnover and BMD loss
Ghrelin	Ghrelin is a 28-amino acid peptide mainly released from the oxyntic cells of the stomach mucosa in response to fasting. While *in vitro* ghrelin promotes osteoblast differentiation and inhibits osteoclastogenesis, in (178), in humans there is no association between BMD and ghrelin levels (179).	↓ Ghrelin levels (in contrast with conventional weight loss)	No significant role in bone turnover and BMD loss
Amylin	Amylin is a pancreatic hormone with pleiotropic effects in different organs. It stimulates osteoblasts activity and inhibits bone reabsorption (180).	↓ Amylin secretion	Upregulation of bone turnover/ BMD loss
Insulin	Insulin is secreted by pancreatic beta cells and represents a potential regulator of bone metabolism, considering that insulin receptors are expressed on osteoblasts (181). While *in vitro* studies have shown that insulin promotes osteoblast proliferation and differentiation (182), insulin signaling in human osteoblasts stimulates bone reabsorption by reducing osteoprotegerin levels (183). However, associative studies have shown that insulin levels are directly associated with bone density (181).	↓ Insulin levels	Upregulation of bone turnover/ BMD loss
Adipokines and other hormones			
Adiponectin	Adiponectin is secreted by adipose tissue and is negatively associated with fat mass. Observational studies have reported that adiponectin levels are negatively correlated to BMD (184, 185).	↑ Adiponectin	BMD loss
Leptin	Leptin is secreted by adipose tissue and its circulating levels are positively associated with fat mass. This peptide regulates energy expenditure and plays a pivotal role in bone metabolism, by increasing bone formation and reducing bone resorption (181).	↓ Leptin levels	Upregulation of bone turnover
Visfatin	Visfatin is a multifaced adipokine whose serum levels are increased in obese subjects and associated with insulin resistance (186). Observational studies did not find any association between visfatin concentrations and BMD (187, 188).	↓, ↑ or ↔ Visfatin levels	Unclear role
Sclerostin	Sclerostin is the osteocyte-product of the SOST gene and represents a major inhibitor of the osteogenic Wnt signaling pathway (89).	↑ Sclerostin levels	BMD loss
Estrogen	Obesity is characterized by hyperestrogenism and weight loss induces a significant reduction in total and free estradiol. Estrogens exert a fundamental role in promoting osteoblastic activity and in regulating bone turnover (189).	↓ Estrogen levels	Upregulation of bone turnover/ BMD loss
Body composition	Bariatric surgery is characterized by a decrease of both fat mass and muscle mass (190, 191). The relationship between the loss of muscle mass and the impairment of bone health is widley known.	↓ Muscle mass	Alterations in bone

(Continued)

TABLE 3 | Continued

Factors	Mechanisms	Changes after bariatric surgery	Expected effect on bone
Bone marrow adiposity (BMA)	Contrary to what expected, BMA is increased in weight loss and is related to a lower BMD and vertebral fractures (192).	↑ BMA	microarchitecture/ BMD loss BMD loss

↑, *increased;* ↓, *reduced;* ↔, *unchanged.*

despite adequate vitamin D and calcium intake. With concern to vitamin D, even solely restrictive procedures like SG can lead to postoperative vitamin D deficiency in as much as 39% of patients despite daily multivitamin supplementation (199), while malabsorptive surgeries pose a higher risk for nutrient deficiencies (200). In a retrospective study, a 73% incidence of vitamin D deficiency was observed following BPD (201).

Weight loss following bariatric surgery instigates a condition of mechanical unloading, that causes a net loss of bone mass by reducing osteoblast function and bone formation. The mechanism likely involves the aforementioned sclerostin and its negative regulation of Wnt/β-catenin signaling pathway, which is important for osteoblast differentiation and function (202). Maintenance of bone strength and density is indeed dependent on adequate muscle mass and function, which reflects intake of high-quality protein (203). Bariatric patients ingest less than 60–120 g of protein recommended daily (197, 204). The combination of postsurgical malnutrition, negative skeletal muscle protein balance, and rapid weight loss initiates a net loss of fat-free mass especially during the first postoperative year, which can last up to 36 months or even up to 9 years post-surgery, and is correlated with loss of handgrip strength (205). Post-operative decrease in fat-free mass and fat mass does not differ between RYGBP and LSG (206). While the biomechanical interaction between nutrition, muscle and bone after bariatric surgery is of key relevance, also important is the biochemical communication existing between muscle and bone which involves secreted factors that act bidirectionally with autocrine/paracrine effects locally as well as through the endocrine system (207). Together, these mechanisms and cross-talks potentially represent the theoretical basis to explain the bone-directed benefit of protein supplementation and mechanical loading through exercise after bariatric surgery. In addition to the previous, a role for bone marrow adipose tissue (BMAT) has also suggested in this scenario. Identified over 100 years ago, BMAT represents up to 70% of bone marrow volume in humans and is a metabolically active, insulin-sensitive and molecularly distinct fat depot that may play a role in whole body energy metabolism (208). Several factors are known to impact bone marrow fat such as obesity, T2DM, estrogen deficiency, caloric restriction, aging, chronic kidney disease, radiotherapy and glucocorticoids. In obese individuals, BMAT fraction is negatively associated with aBMD and the change described for BMAT fraction following RYGB is positively associated with changes in BMI and total body fat (209). RYGB decreases both BMAT and vBMD, yet their changes are unrelated (210).

Finally, a role for adipokines and gut hormones through their central and peripheral receptors has been described (211–216), with evidence indeed coming mainly from animal and *in vitro* studies (**Figure 5**).

In humans, bariatric surgery modulates a change in gut hormones, the magnitude of which is dependent of the surgical technique and has a predictive value on postsurgical weight loss and metabolic improvements by mechanisms other than restriction and malabsorption (24). In rodents, GLP-1 administration elicited an anabolic effect on bone, while GLP-2 was associated with reduction in bone resorption markers (217, 218). *In vitro*, it was further observed that ghrelin administration suppressed osteoclastogenesis and stimulated proliferation and differentiation of osteoblasts (219), while a study in bariatric surgery patients reported an association between ghrelin reduction and BMD loss after RYGB and SG (220). Also, PYY has been positively associated with bone resorption (221), whereas a positive correlation linking PYY to CTX and P1NP levels was observed in patients following RYGB and AGB. In a 12-month study on patients subjected to RYGB, SG or greater curvature plication, postsurgical bone mineral content at the lumbar spine was inversely correlated both with fasting ghrelin and GLP-1, while BMD was positively correlated with post-surgical fasting glucagon and insulin at the femoral neck and inversely with GLP-1 at the lumbar spine.

SUPPLEMENTATION AND ANTIRESORPTIVE TREATMENTS

Supplementation Treatment

In the last two decades, increasing evidence has showed that post-bariatric surgery patients need a specific protocol of supplementation in order to prevent bone loss and the risk of osteoporosis in the following years.

Calcium balance and vitamin D levels are considered as the main components for maintaining bone mass after bariatric surgery. Nevertheless, a high proportion of subjects do not reach an adequate intake, especially after the surgery. It is estimated that, 4 years following a malabsorptive bariatric procedure, calcium deficiency develops in 25–48% of patients, and vitamin D deficiency in 50–63% of cases. For this reason, before

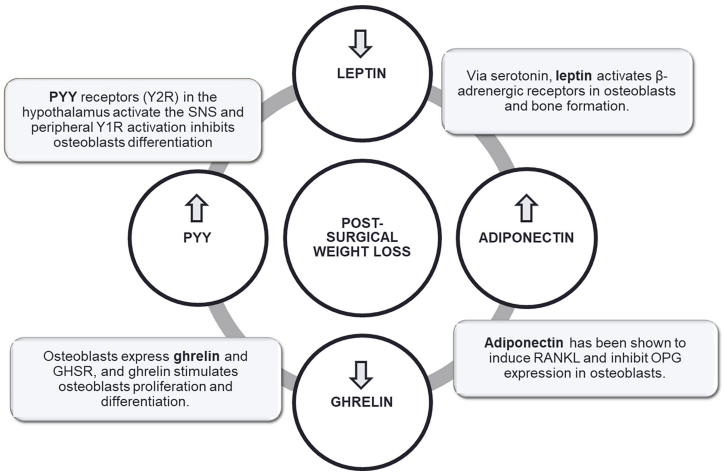

FIGURE 5 | Putative mechanisms linking post-bariatric surgery weight loss to changes in bone cells.

administration of antiresorptive agents, normal vitamin D and calcium levels should be guaranteed even by means of aggressive supplementation, especially when hypocalcaemia and hyperparathyroidism are present (127, 222). In general, GB is associated with higher prevalence of hyperparathyroidism and hypocalcaemia compared to SG and therefore the recommended doses are typically higher. On the other hand, patients subjected to BPD/duodenal switch (DS) show the highest needs in terms of supplementation (222, 223).

As far as the recommended doses are concerned, guidelines established a minimal daily intake of 1,200-1,500 mg/day for SG and RYGB, and 1,800-2,400 mg/day for BPD with or without DS of elemental calcium in the diet or as supplement (5, 222). Calcium citrate is generally recommended over calcium carbonate and should be given in divided doses to enhance absorption.

For vitamin D, a recent meta-analysis reported that at least 800 UI/die can be sufficient to maintain a level of vitamin D sufficiency (224). Due to the high heterogeneity of patients, therapies should start from 1000 UI/die and go up to 2,000-6,000 IU daily, depending on the malabsorption level. A target vitamin D level of 30 ng/ml is desirable. In the case of severe vitamin D malabsorption, an initial oral dose of vitamin D equivalent to 50,000 IU should be administered 1 to 3 times weekly (5, 204, 222). In case of secondary hyperparathyroidism, the Endocrine Society Clinical Practice Guidelines suggested that a weekly 100,000 IU of parenteral ergocalciferol could be useful, until the target Vitamin D level ≥ 30 ng/ml is achieved, resorting to calcitriol if bone loss or elevated PTH persisted (5).

Magnesium, has also shown to be slightly increased in blood after gastric bypass, even if it requires HCl from secreting parietal cells to be solubilized and its absorption is compromised due to the reduction of fatty acids which are bound with. However, the supplementation of at least 100 mg/day of magnesium is highly recommended since its increase in blood, like calcium, could be associated to a higher bone resorption (225–227).

Antiresorptive *Treatments*

In spite of the negative bone effects of bariatric surgery, the optimal medical management for these patients has not been elucidated yet (5). In fact, use of antiresorptive therapy (i.e., bisphosphonates and denosumab) is potentially burdened by a high risk of adverse events in this particular population (228).

The major risks for oral bisphosphonates are reflux and anastomotic ulceration (229). On the other hand, the administration of intravenous bisphosphonate and denosumab may be complicated by severe hypocalcemia and tetany in patients without adequate calcium or vitamin D levels.

According to clinical practice guidelines of American Association of Clinical Endocrinologists, the Obesity Society, and the American Society for Metabolic and Bariatric Surgery for the peri-operative nutritional, metabolic and nonsurgical support of bariatric surgery (224), bisphosphonates may be a considered in bariatric surgery patients affected by osteoporosis after appropriate assessment and treatment for calcium and vitamin D insufficiency. Moreover, if oral malabsorption is suspected or potential anastomotic ulceration risk is evaluated, intravenously bisphosphonates should be preferred. Recommended dosages of orally and intravenous administered bisphosphonates in bariatric surgery patients with osteoporosis are summarized in **Table 4**. In spite of this, no clinical trial data regarding the use of bisphosphonates in post-bariatric patients are available to date.

The risk related to malabsorption is the failure in reaching the optimal blood level and the to obtain the therapeutic effect. If malabsorption is suspected, a safe choice should be risedronate, as a pharmacokinetic study in non-bariatric surgery patients demonstrated that it was absorbed along the small bowel independently of the site of administration (stomach, duodenum or terminal ileum), and the range of absorption is not affected by the rate of administration (aqueous solution or iv infusion) (230). A trial on risedronate in sleeve gastrectomy

TABLE 4 | Recommended dosages of orally and intravenously bisphosphonates in bariatric surgery patients affected by osteoporosis.

Type of bisphosphonates	Route of administration	Dose	Frequency
Alendronate	os	70 mg	week
Risedronate	os	35 mg	week
	os	150 mg	month
Ibandronate	os	150 mg	month
	iv	3 mg	3 months
Zoledronate	iv	5 mg	year

patients is ongoing (NCT03411902), and study design only has been published (231).

Considering the risk of gastric ulceration, studies in non-bariatric patients demonstrated that bisphosphonates differ in their potential effect in damaging the gastroesophageal mucosa. In fact, in postmenopausal women received 5 mg risedronate or 10 mg alendronate daily for 2 weeks, risedronate was associated with fewer endoscopically detected gastric ulcers than alendronate (232), probably related to the structural differences in the nitrogen-containing group.

Thus, intravenously bisphosphonates have been proposed for bone management in postoperative bariatric patients. The safety considerations for zoledronate 5 mg once a year and ibandronate 3 mg every three months includes the onset of flu-like syndrome (low-grade fever, muscle and joint pain) and renal adverse events, particularly in high-risk patients (i.e., dehydration, concomitant nephrotoxic medications, myeloma kidney) (233). Recently, a pilot study explored the safety and efficacy of zoledronic acid in preoperative post-menopausal women who were planning RYGB: a single dose of zoledronate appeared to transiently (2 weeks) reduce bone turnover markers, but at 24 weeks after surgery an increase in CTX versus baseline was observed, although the rise was less than that observed in the controls, even without differences in total hip BMD (234). Moreover, a trial to determine the efficacy of zoledronic acid in preventing bone loss associated with sleeve gastrectomy is ongoing (NCT04279392).

A major concern in the administration of intravenously bisphosphonates is the occurrence of hypocalcemia, in particular after gastric bypass; thus, adequate vitamin D level should be ensured because a bypassed small bowel may not be able to absorb calcium enough to compensate the effects of bisphosphonate binding to bone matrix (235).

In this framework, data on denosumab are even more scanty. A randomized placebo-controlled trial to establish the role of denosumab to prevent bone loss after RYGB or sleeve gastrectomy is ongoing (NCT04087096). The same observations of iv bisphosphonates regarding the risk of hypocalcemia should be considered for denosumab treatment (236).

PHYSICAL EXERCISE AND REHABILITATION

As previously mentioned, bariatric surgery induces a decline in muscle mass, which is responsible for the 10-28% of total body weight loss (237, 238). A prospective cohort study on 184 patients who underwent SG showed that the prevalence of sarcopenia increases from 8% up to 32% within one year after surgical procedure (239). Muscle waist and sarcopenia were found to be independently associated with several adverse outcomes, including functional decline, a higher rate of falls as well as a higher risk of hospitalization and mortality (OR 3.596, 95% CI 2.96-4.37) (240). Therefore, during follow-up of patients after bariatric surgery, the assessment of BMD, muscle mass and strength, physical activity and fitness should be considered (241). With the aim at improving these parameters, individual interventions of physical activity and rehabilitation have been proven to be effective in preserving muscle mass and endurance capacity, in reducing the risk of bone fractures and in improving quality of life (242–245).

In this context, several studies have demonstrated that physical activity during weight loss is able to prevent the reduction of BMD (100, 246, 247). In 2016, Muschitz et al. conducted an interventional study in 220 patients after RYGB and SG procedures with the aim of assessing the differences in serum markers of bone turnover and BMD between an intervention group (supplementation of vitamin D, calcium, protein, and physical exercise) and a non-intervention group after 2 years from surgical procedure (248). The physical activity intervention consisted of an aerobic and strength exercise program including Nordic walking for 45 minutes for at least 3 times a week and strength training for 30 minutes for at least 2 times a week, for two years. The results showed that the supplementation combined with physical exercise exert a positive effect on long-term outcome in bone protection after bariatric surgery, by modulating serum levels of sclerostin, CTX, DKK-1 and PTH, and counteracting the loss of the spine, hip and total body BMD. One year later, Campanha-Versiani and co-workers evaluated the role of physical exercise after RYGB in influencing BMD and bone turnover markers in a group of patients undergoing a regular and supervised exercise program compared to a control group (249). In this study, physical exercise combined weight-bearing and aerobic exercises two times a week for 36 weeks. One year following RYGB, the intervention group showed a lower decrease in total BMD and at the lumbar spine and hip than the control group, without significant differences in terms of serum concentrations of bone remodeling markers.

Recently, Diniz-Sousa et al. conducted a systematic review and meta-analysis to extensively evaluate the effect of physical exercise and training on BMD at clinically relevant skeletal sites

during the first year after bariatric surgery (250). Meta-analysis showed that the decrease in BMD after bariatric surgery can be attenuated from 0.7 to 3.7 percentage points with an exercise training intervention. In particular, exercise training induced a positive effect on BMD at femoral neck [standardized mean difference (SMD)=0.63 (95% CI 0.19-1.06)], total hip [SMD=0.37 (95% CI 0.02-0.71)], lumbar spine [SMD=0.41 (95% CI 0.19-0.62)], and 1/3 radius [SMD=0.58 (95% CI 0.19, 0.97)] as compared to standard medical interventions.

Overall, the evidences suggest that physical exercise after bariatric surgery is fundamental, as it prevents bone loss and muscle depletion during the drastic weight reduction period (142, 205, 251). Exercise programs that include high-impact loading, resistance and strength training as well as aerobic exercises seem to be effective in counteracting the negative effects of bariatric surgery on BMD and bone microarchitecture (142, 252, 253).

Being bariatric surgery patients exposed to multiple systemic risks and particularly for cardiovascular diseases and musculoskeletal impairment, a tailored rehabilitation program through a multidisciplinary approach is key to optimize post-bariatric surgery management (254). The multidisciplinary approach integrates different clinical specialties including endocrinology, clinical nutrition, psychiatry, rehabilitation medicine, as well as health professionals such as nursing, physiotherapy and occupational therapy (255). A post-bariatric surgery rehabilitation project should comprise a number of goals: 1) preventing surgical-related complications, 2) enhancing physical function through adapted physical activity, 3) addressing bariatric-related disabilities as well as socio-environmental and psychological barriers, 4) promoting education on nutritional management, and 5) providing primary or secondary prevention for cardiovascular diseases (254). The rehabilitation program should thus combine musculoskeletal reconditioning, functional mobility, balance training, muscle strengthening, aerobic exercises or physical endurance, activity of daily living (ADL) training, nutritional and psychological support, weight management, and monitoring of clinical aspects (254).

CONCLUSIONS

Bone changes after bariatric surgery may have effects that go well beyond the acute phase of weight loss. Bariatric surgery, especially malabsorptive procedures, increases bone turnover, decreases bone mass, and enhances the fracture risk. These consequences advocate the need to adequately study, monitor and support skeletal health and micronutrient supply in bariatric surgery patients to avoid short- and long-term damage in bone density. There is collective evidence that bone density estimation by DXA can be improved by HR-pQCT to better classify patients at risk of osteoporosis. To compensate for nutritional and mechanical deficits after surgery, replenishment with calcium citrate and high-dose vitamin D plus scheduled exercise programs are mandatory. Noticeably, absorption problems and potential ulceration of anastomosis should be considered before prescribing oral bisphosphonates. In patients who fail to achieve BMD improvements post-surgically, intravenous bisphosphonates and/or denosumab should be considered, with calcium and vitamin D being critical to avoid hypocalcemia. Yet, RCTs are needed to determine whether anti-osteoporotic therapy is effective and safe for preventing high-turnover bone loss and treating osteoporosis in this population. Finally, it is of utmost importance to consider bone health before deciding the type of bariatric surgery a patient with obesity should be subjected to.

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Conceptualization and methodology, CM, MC, and PM. Original draft preparation, CM, MC, AF, TD, BC, DS, and FP. Review and editing, CM, GA, and PM. Supervision, GA, AN, and PM. All authors contributed to the article and approved the submitted version.

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REFERENCES

1. Council of the Obesity Society. Obesity as a Disease: The Obesity Society Council Resolution. *Obes (Silver Spring)* (2008) 16:1151. doi: 10.1038/oby.2008.246
2. World Health Organization. *Global Action Plan for the Prevention and Control of Noncommunicable Diseases 2013-2020* (2013). World Health Organization. Available at: https://www.who.int/publications/i/item/9789241506236 (Accessed April 11, 2022).
3. Swinburn BA, Sacks G, Hall KD, McPherson K, Finegood DT, Moodie ML, et al. The Global Obesity Pandemic: Shaped by Global Drivers and Local Environments. *Lancet* (2011) 378:804–14. doi: 10.1016/S0140-6736(11)60813-1
4. Di Lorenzo N, Antoniou SA, Batterham RL, Busetto L, Godoroja D, Iossa A, et al. Clinical Practice Guidelines of the European Association for Endoscopic Surgery (EAES) on Bariatric Surgery: Update 2020 Endorsed by IFSO-EC, EASO and ESPCOP. *Surg Endosc* (2020) 34:2332–58. doi: 10.1007/s00464-020-07555-y
5. Mechanick JI, Apovian C, Brethauer S, Timothy Garvey W, Joffe AM, Kim J, et al. Clinical Practice Guidelines for the Perioperative Nutrition, Metabolic, and Nonsurgical Support of Patients Undergoing Bariatric Procedures - 2019 Update: Cosponsored by American Association of Clinical Endocrinologists/American College of Endocrinology, The Obesity Society, American Society for Metabolic and Bariatric Surgery, Obesity Medicine Association, and American Society of Anesthesiologists. *Obes (Silver Spring)* (2020) 28:O1–O58. doi: 10.1002/oby.22719
6. Wharton S, Lau DCW, Vallis M, Sharma AM, Biertho L, Campbell-Scherer D, et al. Obesity in Adults: A Clinical Practice Guideline. *CMAJ* (2020) 192:E875–91. doi: 10.1503/cmaj.191707
7. Angrisani L, Santonicola A, Iovino P, Ramos A, Shikora S, Kow L. Bariatric Surgery Survey 2018: Similarities and Disparities Among the 5 IFSO Chapters. *Obes Surg* (2021) 31:1937–48. doi: 10.1007/s11695-020-05207-7
8. Sullivan S, Edmundowicz SA, Thompson CC. Endoscopic Bariatric and Metabolic Therapies: New and Emerging Technologies. *Gastroenterology* (2017) 152:1791–801. doi: 10.1053/j.gastro.2017.01.044

9. Livhits M, Mercado C, Yermilov I, Parikh JA, Dutson E, Mehran A, et al. Preoperative Predictors of Weight Loss Following Bariatric Surgery: Systematic Review. *Obes Surg* (2012) 22:70–89. doi: 10.1007/s11695-011-0472-4
10. Scozzari G, Passera R, Benvenga R, Toppino M, Morino M. Age as a Long-Term Prognostic Factor in Bariatric Surgery. *Ann Surg* (2012) 256:724–8. doi: 10.1097/SLA.0b013e3182734113
11. Tymitz K, Kerlakian G, Engel A, Bollmer C. Gender Differences in Early Outcomes Following Hand-Assisted Laparoscopic Roux-En-Y Gastric Bypass Surgery: Gender Differences in Bariatric Surgery. *Obes Surg* (2007) 17:1588–91. doi: 10.1007/s11695-007-9296-7
12. Ranasinghe WK, Wright T, Attia J, McElduff P, Doyle T, Bartholomew M, et al. Effects of Bariatric Surgery on Urinary and Sexual Function. *BJU Int* (2011) 107:88–94. doi: 10.1111/j.1464-410X.2010.09509.x
13. Still CD, Benotti P, Wood GC, Gerhard GS, Petrick A, Reed M, et al. Outcomes of Preoperative Weight Loss in High-Risk Patients Undergoing Gastric Bypass Surgery. *Arch Surg* (2007) 142:994–8. doi: 10.1001/archsurg.142.10.994
14. Sjöström L. Bariatric Surgery and Reduction in Morbidity and Mortality: Experiences From the SOS Study. *Int J Obes (Lond)* (2008) 32 Suppl 7:S93–7. doi: 10.1038/ijo.2008.244
15. King WC, Hinerman AS, Belle SH, Wahed AS, Courcoulas AP. Comparison of the Performance of Common Measures of Weight Regain After Bariatric Surgery for Association With Clinical Outcomes. *JAMA* (2018) 320:1560–9. doi: 10.1001/jama.2018.14433
16. O'Brien PE, Hindle A, Brennan L, Skinner S, Burton P, Smith A, et al. Long-Term Outcomes After Bariatric Surgery: A Systematic Review and Meta-Analysis of Weight Loss at 10 or More Years for All Bariatric Procedures and a Single-Centre Review of 20-Year Outcomes After Adjustable Gastric Banding. *Obes Surg* (2019) 29:3–14. doi: 10.1007/s11695-018-3525-0
17. Kalarchian MA, King WC, Devlin MJ, Marcus MD, Garcia L, Chen JY, et al. Psychiatric Disorders and Weight Change in a Prospective Study of Bariatric Surgery Patients: A 3-Year Follow-Up. *Psychosom Med* (2016) 78:373–81. doi: 10.1097/PSY.0000000000000277
18. Herring LY, Stevinson C, Davies MJ, Biddle SJ, Sutton C, Bowrey D, et al. Changes in Physical Activity Behaviour and Physical Function After Bariatric Surgery: A Systematic Review and Meta-Analysis. *Obes Rev* (2016) 17:250–61. doi: 10.1111/obr.12361
19. Egberts K, Brown WA, Brennan L, O'Brien PE. Does Exercise Improve Weight Loss After Bariatric Surgery? A Systematic Review. *Obes Surg* (2012) 22:335–41. doi: 10.1007/s11695-011-0544-5
20. Sarwer DB, Dilks RJ, West-Smith L. Dietary Intake and Eating Behavior After Bariatric Surgery: Threats to Weight Loss Maintenance and Strategies for Success. *Surg Obes Relat Dis* (2011) 7:644–51. doi: 10.1016/j.soard.2011.06.016
21. Mastino D, Robert M, Betry C, Laville M, Gouillat C, Disse E. Bariatric Surgery Outcomes in Sarcopenic Obesity. *Obes Surg* (2016) 26:2355–62. doi: 10.1007/s11695-016-2102-7
22. Lent MR, Bailey-Davis L, Irving BA, Wood GC, Cook AM, Hirsch AG, et al. Bariatric Surgery Patients and Their Families: Health, Physical Activity, and Social Support. *Obes Surg* (2016) 26:2981–8. doi: 10.1007/s11695-016-2228-7
23. Conceição EM, Utzinger LM, Pisetsky EM. Eating Disorders and Problematic Eating Behaviours Before and After Bariatric Surgery: Characterization, Assessment and Association With Treatment Outcomes. *Eur Eat Disord Rev* (2015) 23:417–25. doi: 10.1002/erv.2397
24. Pucci A, Batterham RL. Mechanisms Underlying the Weight Loss Effects of RYGB and SG: Similar, Yet Different. *J Endocrinol Invest* (2019) 42:117–28. doi: 10.1007/s40618-018-0892-2
25. Cohn I, Raman J, Sui Z. Patient Motivations and Expectations Prior to Bariatric Surgery: A Qualitative Systematic Review. *Obes Rev* (2019) 20:1608–18. doi: 10.1111/obr.12919
26. Buchwald H, Avidor Y, Braunwald E, Jensen MD, Pories W, Fahrbach K, et al. Bariatric Surgery: A Systematic Review and Meta-Analysis. *JAMA* (2004) 292:1724–37. doi: 10.1001/jama.292.14.1724
27. Carlsson LMS, Sjöholm K, Jacobson P, Andersson-Assarsson JC, Svensson PA, Taube M, et al. Life Expectancy After Bariatric Surgery in the Swedish Obese Subjects Study. *N Engl J Med* (2020) 383:1535–43. doi: 10.1056/NEJMoa2002449
28. Sjöström L. Review of the Key Results From the Swedish Obese Subjects (SOS) Trial - a Prospective Controlled Intervention Study of Bariatric Surgery. *J Intern Med* (2013) 273:219–34. doi: 10.1111/joim.12012
29. Wiggins T, Guidozzi N, Welbourn R, Ahmed AR, Markar SR. Association of Bariatric Surgery With All-Cause Mortality and Incidence of Obesity-Related Disease at a Population Level: A Systematic Review and Meta-Analysis. *PLos Med* (2020) 17:e1003206. doi: 10.1371/journal.pmed.1003206
30. Paccou J, Tsourdi E, Meier C, Palermo A, Pepe J, Body JJ, et al. Bariatric Surgery and Skeletal Health: A Narrative Review and Position Statement for Management by the European Calcified Tissue Society (ECTS). *Bone* (2022) 154:116236. doi: 10.1016/j.bone.2021.116236
31. Yu EW, Wewalka M, Ding SA, Simonson DC, Foster K, Holst JJ, et al. Effects of Gastric Bypass and Gastric Banding on Bone Remodeling in Obese Patients With Type 2 Diabetes. *J Clin Endocrinol Metab* (2016) 101:714–22. doi: 10.1210/jc.2015-3437
32. Muschitz C, Kocijan R, Marterer C, Nia AR, Muschitz GK, Resch H, et al. Sclerostin Levels and Changes in Bone Metabolism After Bariatric Surgery. *J Clin Endocrinol Metab* (2015) 100:891–901. doi: 10.1210/jc.2014-3367
33. Vilarrasa N, Gómez JM, Elio I, Gómez-Vaquero C, Masdevall C, Pujol J, et al. Evaluation of Bone Disease in Morbidly Obese Women After Gastric Bypass and Risk Factors Implicated in Bone Loss. *Obes Surg* (2009) 19:860–6. doi: 10.1007/s11695-009-9843-5
34. Fleischer J, Stein EM, Bessler M, Della Badia M, Restuccia N, Olivero-Rivera L, et al. The Decline in Hip Bone Density After Gastric Bypass Surgery is Associated With Extent of Weight Loss. *J Clin Endocrinol Metab* (2008) 93:3735–40. doi: 10.1210/jc.2008-0481
35. Yu EW, Bouxsein ML, Roy AE, Baldwin C, Cange A, Neer RM, et al. Bone Loss After Bariatric Surgery: Discordant Results Between DXA and QCT Bone Density. *J Bone Miner Res* (2014) 29:542–50. doi: 10.1002/jbmr.2063
36. Ivaska KK, Huovinen V, Soinio M, Hannukainen JC, Saunavaara V, Salminen P, et al. Changes in Bone Metabolism After Bariatric Surgery by Gastric Bypass or Sleeve Gastrectomy. *Bone* (2017) 95:47–54. doi: 10.1016/j.bone.2016.11.001
37. Gagnon C, Schafer AL. Bone Health After Bariatric Surgery. *JBMR Plus* (2018) 2:121–33. doi: 10.1002/jbm4.10048
38. Stein EM, Carrelli A, Young P, Bucovsky M, Zhang C, Schrope B, et al. Bariatric Surgery Results in Cortical Bone Loss. *J Clin Endocrinol Metab* (2013) 98:541–9. doi: 10.1210/jc.2012-2394
39. Wei JH, Lee WJ, Chong K, Lee YC, Chen SC, Huang PH, et al. High Incidence of Secondary Hyperparathyroidism in Bariatric Patients: Comparing Different Procedures. *Obes Surg* (2018) 28:798–804. doi: 10.1007/s11695-017-2932-y
40. Keskin M, Öztürk D, Koca AO, Ertuğrul DT, Bulus H. Does Bariatric Surgery Increase the Formation of Parathyroid Adenoma. *Bariatr Surg Pract Patient Care* (2020) 15:155–9. doi: 10.1089/bari.2019.0043
41. Corbeels K, Verlinden L, Lannoo M, Simoens C, Matthys C, Verstuyf A, et al. Thin Bones: Vitamin D and Calcium Handling After Bariatric Surgery. *Bone Rep* (2018) 8:57–63. doi: 10.1016/j.bonr.2018.02.002
42. Scibora LM. Skeletal Effects of Bariatric Surgery: Examining Bone Loss, Potential Mechanisms and Clinical Relevance. *Diabetes Obes Metab* (2014) 16:1204–13. doi: 10.1111/dom.12363
43. Ko BJ, Myung SK, Cho KH, Park YG, Kim SG, Kim do H, et al. Relationship Between Bariatric Surgery and Bone Mineral Density: A Meta-Analysis. *Obes Surg* (2016) 26:1414–21. doi: 10.1007/s11695-015-1928-8
44. Bredella MA, Greenblatt LB, Eajazi A, Torriani M, Yu EW. Effects of Roux-En-Y Gastric Bypass and Sleeve Gastrectomy on Bone Mineral Density and Marrow Adipose Tissue. *Bone* (2017) 95:85–90. doi: 10.1016/j.bone.2016.11.014
45. Hofsø D, Hillestad TOW, Halvorsen E, Fatima F, Johnson LK, Lindberg M, et al. Bone Mineral Density and Turnover After Sleeve Gastrectomy and Gastric Bypass: A Randomized Controlled Trial (Oseberg). *J Clin Endocrinol Metab* (2021) 106:501–11. doi: 10.1210/clinem/dgaa808
46. Shanbhogue VV, Støving RK, Frederiksen KH, Hanson S, Brixen K, Gram J, et al. Bone Structural Changes After Gastric Bypass Surgery Evaluated by HR-pQCT: A Two-Year Longitudinal Study. *Eur J Endocrinol* (2017) 176:685–93. doi: 10.1530/EJE-17-0014
47. Axelsson KF, Werling M, Eliasson B, Szabo E, Näslund I, Wedel H, et al. Fracture Risk After Gastric Bypass Surgery: A Retrospective Cohort Study. *J Bone Miner Res* (2018) 33:2122–31. doi: 10.1002/jbmr.3553

48. Rousseau C, Jean S, Gamache P, Lebel S, Mac-Way F, Biertho L, et al. Change in Fracture Risk and Fracture Pattern After Bariatric Surgery: Nested Case-Control Study. *BMJ* (2016) 354:i3794. doi: 10.1136/bmj.i3794
49. Lindeman KG, Greenblatt LB, Rourke C, Bouxsein ML, Finkelstein JS, Yu EW. Longitudinal 5-Year Evaluation of Bone Density and Microarchitecture After Roux-En-Y Gastric Bypass Surgery. *J Clin Endocrinol Metab* (2018) 103:4104–12. doi: 10.1210/jc.2018-01496
50. Reid R. Relationships Among Body Mass, its Components, and Bone. *Bone* (2002) 31:547–55. doi: 10.1016/S8756-3282(02)00864-5
51. Villareal DT, Apovian CM, Kushner RF, Klein SAmerican Society for Nutrition. NAASO, The Obesity Society Obesity in Older Adults: Technical Review and Position Statement of the American Society for Nutrition and NAASO, The Obesity Society. *Am J Clin Nutr* (2005) 82:923–34. doi: 10.1093/ajcn/82.5.923
52. Janicka A, Wren TA, Sanchez MM, Dorey F, Kim PS, Mittelman SD, et al. Fat Mass is Not Beneficial to Bone in Adolescents and Young Adults. *J Clin Endocrinol Metab* (2007) 92:143–7. doi: 10.1210/jc.2006-0794
53. Kim JH, Choi HJ, Kim MJ, Shin CS, Cho NH. Fat Mass is Negatively Associated With Bone Mineral Content in Koreans. *Osteoporos Int* (2012) 23:2009–16. doi: 10.1007/s00198-011-1808-6
54. Gilsanz V, Chalfant J, Mo AO, Lee DC, Dorey FJ, Mittelman SD. Reciprocal Relations of Subcutaneous and Visceral Fat to Bone Structure and Strength. *J Clin Endocrinol Metab* (2009) 94:3387–93. doi: 10.1210/jc.2008-2422
55. Leidig-Bruckner G, Ziegler R. Diabetes Mellitus a Risk for Osteoporosis? *Exp Clin Endocrinol Diabetes* (2001) 109:S493–514. doi: 10.1055/s-2001-18605
56. Greco EA, Fornari R, Rossi F, Santiemma V, Prossomariti G, Annoscia C, et al. Is Obesity Protective for Osteoporosis? Evaluation of Bone Mineral Density in Individuals With High Body Mass Index. *Int J Clin Pract* (2010) 64:817–20. doi: 10.1111/j.1742-1241.2009.02301.x
57. Compston JE, Watts NB, Chapurlat R, Cooper C, Boonen S, Greenspan S, et al. Obesity is Not Protective Against Fracture in Postmenopausal Women: GLOW. *Am J Med* (2011) 124:1043–50. doi: 10.1016/j.amjmed.2011.06.013
58. Premaor MO, Ensrud K, Lui L, Parker RA, Cauley J, Hillier TA, et al. Study of Osteoporotic Fractures. Risk Factors for Nonvertebral Fracture in Obese Older Women. *J Clin Endocrinol Metab* (2011) 96:2414–21. doi: 10.1210/jc.2011-0076
59. Sadie-Van Gijsen H, Crowther NJ, Hough FS, Ferris WF. The Interrelationship Between Bone and Fat: From Cellular See-Saw to Endocrine Reciprocity. *Cell Mol Life Sci* (2013) 70:2331–49. doi: 10.1007/s00018-012-1211-2
60. Lee CG, Carr MC, Murdoch SJ, Mitchell E, Woods NF, Wener MH, et al. Adipokines, Inflammation, and Visceral Adiposity Across the Menopausal Transition: A Prospective Study. *J Clin Endocrinol Metab* (2009) 94:1104–10. doi: 10.1210/jc.2008-0701
61. Messier V, Rabasa-Lhoret R, Barbat-Artigas S, Elisha B, Karelis AD, Aubertin-Leheudre M. Menopause and Sarcopenia: A Potential Role for Sex Hormones. *Maturitas* (2011) 68:331–6. doi: 10.1016/j.maturitas.2011.01.014
62. Frost HM. On Our Age-Related Bone Loss: Insights From a New Paradigm. *J Bone Miner Res* (1997) 12:1539–46. doi: 10.1359/jbmr.1997.12.10.1539
63. Holick MF. Vitamin D Deficiency. *N Engl J Med* (2007) 357:266–81. doi: 10.1056/NEJMra070553
64. Scacchi M, Pincelli AI, Cavagnini F. Growth Hormone in Obesity. *Int J Obes Relat Metab Disord* (1999) 23:260–71. doi: 10.1038/sj.ijo.0800807
65. Morton NM. Obesity and Corticosteroids: 11beta-Hydroxysteroid Type 1 as a Cause and Therapeutic Target in Metabolic Disease. *Mol Cell Endocrinol* (2010) 316:154–64. doi: 10.1016/j.mce.2009.09.024
66. Marzullo P, Minocci A, Tagliaferri MA, Guzzaloni G, Di Blasio A, De Medici C, et al. Investigations of Thyroid Hormones and Antibodies in Obesity: Leptin Levels are Associated With Thyroid Autoimmunity Independent of Bioanthropometric, Hormonal, and Weight-Related Determinants. *J Clin Endocrinol Metab* (2010) 95:3965–72. doi: 10.1210/jc.2009-2798
67. Ishii S, Cauley JA, Crandall CJ, Srikanthan P, Greendale GA, Huang MH, et al. Diabetes and Femoral Neck Strength: Findings From the Hip Strength Across the Menopausal Transition Study. *J Clin Endocrinol Metab* (2012) 97:190–7. doi: 10.1210/jc.2011-1883
68. Crespo CJ, Smit E, Snelling A, Sempos CT, Andersen RE. NHANES III Hormone Replacement Therapy and its Relationship to Lipid and Glucose Metabolism in Diabetic and Nondiabetic Postmenopausal Women: Results From the Third National Health and Nutrition Examination Survey (NHANES III). *Diabetes Care* (2002) 25:1675–80. doi: 10.2337/diacare.25.10.1675
69. Giudici KV, Fisberg RM, Marchioni DML, Peters BSE, Martini LA. Crosstalk Between Bone and Fat Tissue: Associations Between Vitamin D, Osteocalcin, Adipokines, and Markers of Glucose Metabolism Among Adolescents. *J Am Coll Nutr* (2017) 36:273–80. doi: 10.1080/07315724.2016.1274923
70. Ducy P, Amling M, Takeda S, Priemel M, Schilling AF, Beil FT, et al. Leptin Inhibits Bone Formation Through a Hypothalamic Relay: A Central Control of Bone Mass. *Cell* (2000) 100(2):197–207. doi: 10.1016/S0092-8674(00)81558-5
71. Lee NK, Sowa H, Hinoi E, Ferron M, Ahn JD, Confavreux C, et al. Endocrine Regulation of Energy Metabolism by the Skeleton. *Cell* (2007) 130:456–69. doi: 10.1016/j.cell.2007.05.047
72. Carvallo L, Henríquez B, Paredes R, Olate J, Onate S, Van Wijnen AJ, et al. 1α,25-Dihydroxy Vitam in D3-Enhanced Expression of the Osteocalcin Gene Involves Increased Promoter Occupancy of Basal Transcription Regulators and Gradual Recruitment of the 1α,25-Dihydroxy Vitamin D 3 Receptor-SRC-1 Coactivator Complex. *J Cell Physiol* (2008) 214:740–9. doi: 10.1002/jcp.21267
73. Ferron M, Hinoi E, Karsenty G, Ducy P, Proc Natl Acad Sci USA. Osteocalcin Differentially Regulates Beta Cell and Adipocyte Gene Expression and Affects the Development of Metabolic Diseases in Wild-Type Mice. *Proc Natl Acad Sci USA* (2008) 105:5266–70. doi: 10.1073/pnas.0711119105
74. Confavreux CB, Levine RL, Karsenty G. A Paradigm of Integrative Physiology, the Crosstalk Between Bone and Energy Metabolisms. *Mol Cell Endocrinol* (2009) 310:21–9. doi: 10.1016/j.mce.2009.04.004
75. Abseyi N, Şıklar Z, Berberoğlu M, Hacıhamdioğlu B, Savaş Erdeve Ş, Öçal G. Relationships Between Osteocalcin, Glucose Metabolism, and Adiponectin in Obese Children: Is There Crosstalk Between Bone Tissue and Glucose Metabolism? *J Clin Res Pediatr Endocrinol* (2012) 4:182–8. doi: 10.4274/Jcrpe.831
76. Zaidi M, Buettner C, Sun L, Iqbal J. The Link Between Fat and Bone: Does Mass Beget Mass? *Endocrinology* (2012) 153:2070–5. doi: 10.1210/en.2012-1022
77. Upadhyay J, Farr OM, Mantzoros CS. The Role of Leptin in Regulating Bone Metabolism. *Metabolism* (2015) 64:105–13. doi: 10.1016/j.metabol.2014.10.021
78. Hinoi E, Gao N, Jung DY, Yadav V, Yoshizawa T, Myers MGJr, et al. The Sympathetic Tone Mediates Leptin's Inhibition of Insulin Secretion by Modulating Osteocalcin Bioactivity. *J Cell Biol* (2008) 183:1235–42. doi: 10.1083/jcb.200809113
79. Schwetz V, Pieber T, Obermayer-Pietsch B. The Endocrine Role of the Skeleton: Background and Clinical Evidence. *Eur J Endocrinol* (2012) 166:959–67. doi: 10.1530/EJE-12-0030
80. Kanazawa I. Osteocalcin as a Hormone Regulating Glucose Metabolism. *World J Diabetes* (2015) 6:1345–54. doi: 10.4239/wjd.v6.i18.1345
81. Rosato MT, Schneider SH, Shapses SA. Bone Turnover and Insulin-Like Growth Factor I Levels Increase After Improved Glycemic Control in Noninsulin-Dependent Diabetes Mellitus. *Calcif Tissue Int* (1998) 63:107–11. doi: 10.1007/s002239900498
82. Kanazawa I, Yamaguchi T, Tada Y, Yamauchi M, Yano S, Sugimoto T. Serum Osteocalcin Level is Positively Associated With Insulin Sensitivity and Secretion in Patients With Type 2 Diabetes. *Bone* (2011) 48:720–5. doi: 10.1016/j.bone.2010.12.020
83. Nakashima R, Kamei N, Yamane K, Nakanishi S, Nakashima A, Kohno N. Decreased Total and High Molecular Weight Adiponectin are Independent Risk Factors for the Development of Type 2 Diabetes in Japanese-Americans. *J Clin Endocrinol Metab* (2006) 91:3873–7. doi: 10.1210/jc.2006-1158
84. Mather KJ, Funahashi T, Matsuzawa Y, Edelstein S, Bray GA, Kahn SE, et al. Adiponectin, Change in Adiponectin, and Progression to Diabetes in the Diabetes Prevention Program. *Diabetes* (2008) 57:980–6. doi: 10.2337/db07-1419
85. Pittas AG, Harris SS, Eliades M, Stark P, Dawson-Hughes B. Association Between Serum Osteocalcin and Markers of Metabolic Phenotype. *J Clin Endocrinol Metab* (2009) 94:827–32. doi: 10.1210/jc.2008-1422

86. Zhou M, Ma X, Li H, Pan X, Tang J, Gao Y, et al. Serum Osteocalcin Concentrations in Relation to Glucose and Lipid Metabolism in Chinese Individuals. *Eur J Endocrinol* (2009) 161:723–9. doi: 10.1530/EJE-09-0585
87. Garanty-Bogacka B, Syrenicz M, Rać M, Krupa B, Czaja-Bulsa G, Walczak M, et al. Association Between Serum Osteocalcin, Adiposity and Metabolic Risk in Obese Children and Adolescents. *Endokrynol Pol* (2013) 64:346–52. doi: 10.5603/EP.2013.0016
88. Rinehr T, Roth CL. A New Link Between Skeleton, Obesity and Insulin Resistance: Relationships Between Osteocalcin, Leptin and Insulin Resistance in Obese Children Before and After Weight Loss. *Int J Obes* (2010) 34:852–8. doi: 10.1038/ijo.2009.282
89. Urano T, Shiraki M, Ouchi Y, Inoue S. Association of Circulating Sclerostin Levels With Fat Mass and Metabolic Disease–Related Markers in Japanese Postmenopausal Women. *J Clin Endocrinol Metab* (2012) 97:1473–7. doi: 10.1210/jc.2012-1218
90. García-Martín A, Rozas-Moreno P, Reyes-García R, Morales-Santana S, García-Fontana B, García-Salcedo JA, et al. Circulating Levels of Sclerostin are Increased in Patients With Type 2 Diabetes Mellitus. *J Clin Endocrinol Metab* (2012) 97:234–41. doi: 10.1210/jc.2011-2186
91. Clevers H. Wnt/beta-Catenin Signaling in Development and Disease. *Cell* (2006) 127:469–80. doi: 10.1016/j.cell.2006.10.018
92. Barons R, Rawadi G. Targeting the Wnt/beta-Catenin Pathway to Regulate Bone Formation in the Adult Skeleton. *Endocrinology* (2007) 148:2635–43. doi: 10.1210/en.2007-0270
93. Balemans W, Ebeling M, Patel N, Van Hul E, Olson P, Dioszegi M, et al. Increased Bone Density in Sclerosteosis is Due to the Deficiency of a Novel Secreted Protein (SOST). *Hum Mol Genet* (2001) 10:537–43. doi: 10.1093/hmg/10.5.537
94. MacDonald BT, Tamai K, He X. Wnt/β-Catenin Signaling: Components, Mechanisms, and Diseases. *Dev Cell* (2009) 17:9–26. doi: 10.1016/j.devcel.2009.06.016
95. Mödder UI, Hoey KA, Amin S, McCready LK, Achenbach SJ, Riggs BL, et al. Relation of Age, Gender, and Bone Mass to Circulating Sclerostin Levels in Women and Men. *J Bone Miner Res* (2011) 26:373–9. doi: 10.1002/jbmr.217
96. Gaudio A, Pennisi P, Bratengeier C, Torrisi V, Lindner B, Mangiafico RA, et al. Increased Sclerostin Serum Levels Associated With Bone Formation and Resorption Markers in Patients With Immobilization-Induced Bone Loss. *J Clin Endocrinol Metab* (2010) 95:2248–53. doi: 10.1210/jc.2010-0067
97. Arasu A, Cawthon PM, Lui LY, Do TP, Arora PS, Cauley JA, et al. Serum Sclerostin and Risk of Hip Fracture in Older Caucasian Women. *J Clin Endocrinol Metab* (2012) 97:2027–32. doi: 10.1210/jc.2011-3419
98. Grethen E, Hill KM, Jones R, Cacucci BM, Gupta CE, Acton A, et al. Serum Leptin, Parathyroid Hormone, 1,25-Dihydroxyvitamin D, Fibroblast Growth Factor 23, Bone Alkaline Phosphatase, and Sclerostin Relationships in Obesity. *J Clin Endocrinol Metab* (2012) 97:1655–62. doi: 10.1210/jc.2011-2280
99. Aznou A, Meijer R, van Raalte D, den Heijer M, Heijboer A, de Jongh R. Serum Sclerostin is Negatively Associated With Insulin Sensitivity in Obese But Not Lean Women. *Endocr Connect* (2021) 10:131–8. doi: 10.1530/EC-20-0535
100. Armamento-Villareal R, Sadler C, Napoli N, Shah K, Chode S, Sinacore DR, et al. Weight Loss in Obese Older Adults Increases Serum Sclerostin and Impairs Hip Geometry But Both are Prevented by Exercise Training. *J Bone Miner Res* (2012) 27:1215–21. doi: 10.1002/jbmr.1560
101. Kim JA, Roh E, Hong SH, Lee YB, Kim NH, Yoo HJ, et al. Association of Serum Sclerostin Levels With Low Skeletal Muscle Mass: The Korean Sarcopenic Obesity Study (KSOS). *Bone* (2019) 128:115053. doi: 10.1016/j.bone.2019.115053
102. Marzullo P, Mele C, Mai S, Nardone A, Scacchi M, Aimaretti G. Obesity and Bone Loss at Menopause: The Role of Sclerostin. *Diagn (Basel)* (2021) 11:1914. doi: 10.3390/diagnostics11101914
103. Liu E, Meigs JB, Pittas AG, McKeown NM, Economos CD, Booth SL, et al. Plasma 25-Hydroxyvitamin D is Associated With Markers of the Insulin Resistant Phenotype in Nondiabetic Adults. *J Nutr* (2009) 139:329–34. doi: 10.3945/jn.108.093831
104. Pinelli NR, Jaber LA, Brown MB, Herman WH. Serum 25-Hydroxy Vitamin D and Insulin Resistance, Metabolic Syndrome, and Glucose Intolerance Among Arab Americans. *Diabetes Care* (2010) 33:1373–5. doi: 10.2337/dc09-2199
105. Gomez JM, Maravall FJ, Gomez N, Navarro MA, Casamitjana R, Soler J. Relationship Between 25-(OH)D3, the IGF-1 System, Leptin, Anthropometric and Body Composition Variables in a Healthy, Randomly Selected Population. *Horm Metab Res* (2004) 36:48–53. doi: 10.1055/s-2004-814103
106. Hypponen E, Power C. Vitamin D Status and Glucose Status Homeostasis in the 1958 British Birth Cohort: The Role of Obesity. *Diabetes Care* (2006) 29:2244–6. doi: 10.2337/dc06-0946
107. Wortsman J, Matsuoka LY, Chen TC, Lu Z, Holick MF. Decreased Bioavailability of Vitamin D in Obesity. *Am J Clin Nutr* (2000) 72:690–3. doi: 10.1093/ajcn/72.3.690
108. McCarthy MF, Thomas CA. PTH Excess may Promote Weight Gain by Impeding Catecholamine-Induced Lipolysis—Implications for the Impact of Calcium, Vitamin D, and Alcohol on Body Weight. *Med Hypotheses* (2003) 61:535–42. doi: 10.1016/S0306-9877(03)00227-5
109. Mai S, Walker GE, Vietti R, Cattaldo S, Mele C, Priano L, et al. Acute Vitamin D_3 Supplementation in Severe Obesity: Evaluation of Multimeric Adiponectin. *Nutrients* (2017) 9:459. doi: 10.3390/nu9050459
110. Diez JJ, Iglesias P. The Role of the Novel Adipocyte-Derived Hormone Adiponectin in Human Disease. *Eur J Endocrinol* (2003) 148:293–300. doi: 10.1530/eje.0.1480293
111. Mi J, Munkonda MN, Li M, Zhang MX, Zhao XY, Fouejeu PC, et al. Adiponectin and Leptin Metabolic Biomarkers in Chinese Children and Adolescents. *J Obes* (2010) 2010:892081. doi: 10.1155/2010/892081
112. Magge SN, Stettler N, Koren D, Levitt Katz LE, Gallagher PR, Mohler ER3rd, et al. Adiponectin is Associated With Favorable Lipoprotein Profile, Independent of BMI and Insulin Resistance, in Adolescents. *J Clin Endocrin Metab* (2011) 96:1549–54. doi: 10.1210/jc.2010-2364
113. Riestra P, Garcia-Anguita A, Lasuncion MA, Cano B, de Oya M, Garces C. Relationship of Adiponectin With Metabolic Syndrome Components in Pubertal Children. *Atherosclerosis* (2011) 216:467–70. doi: 10.1016/j.atherosclerosis.2011.02.031
114. Morrison JA, Glueck CJ, Daniels S, Wang P, Stroop D. Paradoxically High Adiponectin in Obese 16-Year-Old Girls Protects Against Appearance of the Metabolic Syndrome and its Components Seven Years Later. *J Pediatr* (2011) 158:208–14. doi: 10.1016/j.jpeds.2010.08.012
115. Rinonapoli G, Pace V, Ruggiero C, Ceccarini P, Bisaccia M, Meccariello L, et al. Obesity and Bone: A Complex Relationship. *Int J Mol Sci* (2021) 22:13662. doi: 10.3390/ijms222413662
116. Paccou J, Thuillier D, Courtalin M, Pigny P, Labreuche J, Cortet B, et al. A Comparison of Changes in Bone Turnover Markers After Gastric Bypass and Sleeve Gastrectomy, and Their Association With Markers of Interest. *Surg Obes Relat Dis* (2022) 18:373–83. doi: 10.1016/j.soard.2021.12.004
117. Paccou J, Caiazzo R, Lespessailles E, Cortet B. Bariatric Surgery and Osteoporosis. *Calcif Tissue Int* (2022) 110(5):576–91. doi: 10.1007/s00223-020-00798-w
118. Salman MA, Aradaib M, Salman A, Elewa A, Tourky M, Shaaban HE. Effects of Gastric Bypass and Sleeve Gastrectomy on Bone Mineral Density and Bone Turnover Markers: A Systematic Review and Meta-Analysis. *World J Surg* (2022) 46:865–75. doi: 10.1007/s00268-021-06429-1
119. Hansen S, Jørgensen NR, Hermann AP, Støving RK. Continuous Decline in Bone Mineral Density and Deterioration of Bone Microarchitecture 7 Years After Roux-En-Y Gastric Bypass Surgery. *Eur J Endocrinol* (2020) 182:303–11. doi: 10.1530/EJE-19-0741
120. Turcotte AF, Grenier-Larouche T, Ung RV, Simonyan D, Carreau AM, Carpentier AC, et al. Effects of Biliopancreatic Diversion on Bone Turnover Markers and Association With Hormonal Factors in Patients With Severe Obesity. *Obes Surg* (2019) 29:990–8. doi: 10.1007/s11695-018-3617-x
121. Chaves Pereira de Holanda N, de Lima Carlos I, Chaves de Holanda Limeira C, Cesarino de Sousa D, Serra de Lima Junior FA, Telis de Vilela Araújo A, et al. Fracture Risk After Bariatric Surgery: A Systematic Literature Review and Meta-Analysis. *Endocr Pract* (2022) 28:58–69. doi: 10.1016/j.eprac.2021.09.007
122. Crawford MR, Pham N, Khan L, Bena JF, Schauer PR, Kashyap SR. INCREASED BONE TURNOVER IN TYPE 2 DIABETES PATIENTS RANDOMIZED TO BARIATRIC SURGERY VERSUS MEDICAL

THERAPY AT 5 YEARS. *Endocr Pract* (2018) 24:256–64. doi: 10.4158/EP-2017-0072

123. Huang TW, Chen JY, Wu YL, Kao CC, Yeh SC, Lin YC. Alterations of Bone Markers in Obese Patients With Type 2 Diabetes After Bariatric Surgery: A Meta-Analysis and Systemic Review of Randomized Controlled Trials and Cohorts. *Med (Baltimore)* (2021) 100:e26061. doi: 10.1097/MD.0000000000026061
124. Beamish AJ, Gronowitz E, Olbers T, Flodmark CE, Marcus C, Dahlgren J. Body Composition and Bone Health in Adolescents After Roux-En-Y Gastric Bypass for Severe Obesity. *Pediatr Obes* (2017) 12:239–46. doi: 10.1111/ijpo.12134
125. Weiner A, Cowell A, McMahon DJ, Tao R, Zitsman J, Oberfield SE, et al. The Effects of Adolescent Laparoscopic Adjustable Gastric Band and Sleeve Gastrectomy on Markers of Bone Health and Bone Turnover. *Clin Obes* (2020) 10:e12411. doi: 10.1111/cob.12411
126. Hey H, Lund B, Sorensen OH. Delayed Fracture Healing Following Jejunoileal Bypass Surgery for Obesity. *Calcif Tissue Int* (1982) 34:13–5. doi: 10.1007/BF02411201
127. Liu C, Wu D, Zhang JF, Xu D, Xu WF, Chen Y, et al. Changes in Bone Metabolism in Morbidly Obese Patients After Bariatric Surgery: A Meta-Analysis. *Obes Surg* (2016) 26:91–7. doi: 10.1007/s11695-015-1724-5
128. Rodríguez-Carmona Y, López-Alavez FJ, González-Garay AG, Solís-Galicia C, Meléndez G, Serralde-Zúñiga AE. Bone Mineral Density After Bariatric Surgery. *A Syst Rev Int J Surg* (2014) 12:976–82. doi: 10.1016/j.ijsu.2014.08.002
129. Kim TY, Schwartz AV, Li X, Xu K, Black DM, Petrenko DM, et al. Bone Marrow Fat Changes After Gastric Bypass Surgery Are Associated With Loss of Bone Mass. *J Bone Miner Res* (2017) 32:2239–47. doi: 10.1002/jbmr.3212
130. Giusti V, Gasteyger C, Suter M, Heraief E, Gaillard RC, Burckhardt P. Gastric Banding Induces Negative Bone Remodelling in the Absence of Secondary Hyperparathyroidism: Potential Role of Serum C Telopeptides for Follow-Up. *Int J Obes (Lond)* (2005) 29:1429–35. doi: 10.1038/sj.ijo.0803040
131. Adamczyk P, Bužga M, Holéczy P, Švagera Z, Šmajstrla V, Zonča P, et al. Bone Mineral Density and Body Composition After Laparoscopic Sleeve Gastrectomy in Men: A Short-Term Longitudinal Study. *Int J Surg* (2015) 23:101–7. doi: 10.1016/j.ijsu.2015.09.048
132. Geoffroy M, Charlot-Lambrecht I, Chrusciel J, Gaubil-Kaladjian I, Diaz-Cives A, Eschard JP, et al. Impact of Bariatric Surgery on Bone Mineral Density: Observational Study of 110 Patients Followed Up in a Specialized Center for the Treatment of Obesity in France. *Obes Surg* (2019) 29:1765–72. doi: 10.1007/s11695-019-03719-5
133. Coates PS, Fernstrom JD, Fernstrom MH, Schauer PR, Greenspan SL. Gastric Bypass Surgery for Morbid Obesity Leads to an Increase in Bone Turnover and a Decrease in Bone Mass. *J Clin Endocrinol Metab* (2004) 89:1061–5. doi: 10.1210/jc.2003-031756
134. Johnson JM, Maher JW, Samuel I, Heitshusen D, Doherty C, Downs RW. Effects of Gastric Bypass Procedures on Bone Mineral Density, Calcium, Parathyroid Hormone, and Vitamin D. *J Gastrointest Surg* (2005) 9:1106–10. doi: 10.1016/j.gassur.2005.07.012
135. Schafer AL, Kazakia GJ, Vittinghoff E, Stewart L, Rogers SJ, Kim TY, et al. Effects of Gastric Bypass Surgery on Bone Mass and Microarchitecture Occur Early and Particularly Impact Postmenopausal Women. *J Bone Miner Res* (2018) 33:975–86. doi: 10.1002/jbmr.3371
136. Carlin AM, Rao DS, Yager KM, Parikh NJ, Kapke A. Treatment of Vitamin D Depletion After Roux-En-Y Gastric Bypass: A Randomized Prospective Clinicaltrial. *Surg Obes Relat Dis* (2009) 5:444–9. doi: 10.1016/j.soard.2008.08.004
137. Pluskiewicz W, Bužga M, Holéczy P, Bortlík L, Šmajstrla V, Adamczyk P. Bone Mineral Changes in Spine and Proximal Femur in Individual Obese Women After Laparoscopic Sleeve Gastrectomy: A Short-Term Study. *Obes Surg* (2012) 22:1068–76. doi: 10.1007/s11695-012-0654-8
138. Casagrande DS, Repetto G, Mottin CC, Shah J, Pietrobon R, Worni M, et al. Changes in Bone Mineral Density in Women Following 1-Year Gastric Bypass Surgery. *Obes Surg* (2012) 22:1287–92. doi: 10.1007/s11695-012-0687-z
139. Nogués X, Goday A, Peña MJ, Benaiges D, de Ramón M, Crous X, et al. Bone Mass Loss After Sleeve Gastrectomy: A Prospective Comparative Study With Gastric Bypass. *Cir Esp* (2010) 88:103–9. doi: 10.1016/S2173-5077(10)70015-6
140. Ruiz-Tovar J, Oller I, Priego P, Arroyo A, Calero A, Diez M, et al. Short- and Mid-Term Changes in Bone Mineral Density After Laparoscopic Sleeve Gastrectomy. *Obes Surg* (2013) 23:861–6. doi: 10.1007/s11695-013-0866-6
141. Maghrabi AH, Wolski K, Abood B, Licata A, Pothier C, Bhatt DL, et al. Two-Year Outcomes on Bone Density and Fracture Incidence in Patients With T2DM Randomized to Bariatric Surgery Versus Intensive Medical Therapy. *Obes (Silver Spring)* (2015) 23:2344–8. doi: 10.1002/oby.21150
142. Ben-Porat T, Elazary R, Sherf-Dagan S, Goldenshluger A, Brodie R, Mintz Y, et al. Bone Health Following Bariatric Surgery: Implications for Management Strategies to Attenuate Bone Loss. *Adv Nutr* (2018) 9:114–27. doi: 10.1093/advances/nmx024
143. Tothill P, Hannan WJ, Cowen S, Freeman CP. Anomalies in the Measurement of Changes in Total-Body Bone Mineral by Dual-Energy X-Ray Absorptiometry During Weight Change. *J Bone Miner Res* (1997) 12:1908–21. doi: 10.1359/jbmr.1997.12.11.1908
144. Yu EW, Bouxsein ML, Putman MS, Monis EL, Roy AE, Pratt JS, et al. Two-Year Changes in Bone Density After Roux-En-Y Gastric Bypass Surgery. *J Clin Endocrinol Metab* (2015) 100:1452–9. doi: 10.1210/jc.2014-4341
145. Frederiksen KD, Hanson S, Hansen S, Brixen K, Gram J, Jørgensen NR, et al. Bone Structural Changes and Estimated Strength After Gastric Bypass Surgery Evaluated by HR-pQCT. *Calcif Tissue Int* (2016) 98:253–62. doi: 10.1007/s00223-015-0091-5
146. Lindeman KG, Rushin CC, Cheney MC, Bouxsein ML, Hutter MM, Yu EW. Bone Density and Trabecular Morphology at Least 10 Years After Gastric Bypass and Gastric Banding. *J Bone Miner Res* (2020) 35:2132–42. doi: 10.1002/jbmr.4112
147. Sadeghi O, Saneei P, Nasiri M, Larijani B, Esmaillzadeh A. Abdominal Obesity and Risk of Hip Fracture: A Systematic Review and Meta-Analysis of Prospective Studies. *Adv Nutr* (2017) 8:728–38. doi: 10.3945/an.117.015545
148. Fassio A, Idolazzi L, Rossini M, Gatti D, Adami G, Giollo A, et al. The Obesity Paradox and Osteoporosis. *Eat Weight Disord* (2018) 23:293–302. doi: 10.1007/s40519-018-0505-2
149. Hou J, He C, He W, Yang M, Luo X, Li C. Obesity and Bone Health: A Complex Link. *Front Cell Dev Biol* (2020) 8:600181. doi: 10.3389/fcell.2020.600181
150. Mitchell RJ, Lord SR, Harvey LA, Close JC. Obesity and Falls in Older People: Mediating Effects of Disease, Sedentary Behavior, Mood, Pain and Medication Use. *Arch Gerontol Geriatr* (2015) 60:52–8. doi: 10.1016/j.archger.2014.09.006
151. Vilaça KH, Carneiro JA, Ferriolli E, Lima NK, de Paula FJ, Moriguti JC. Body Composition, Physical Performance and Muscle Quality of Active Elderly Women. *Arch Gerontol Geriatr* (2014) 59:44–8. doi: 10.1016/j.archger.2014.02.004
152. Neri SGR, Gadelha AB, de David AC, Ferreira AP, Safons MP, Tiedemann A, et al. The Association Between Body Adiposity Measures, Postural Balance, Fear of Falling, and Fall Risk in Older Community-Dwelling Women. *J Geriatr Phys Ther* (2019) 42:E94–E100. doi: 10.1519/JPT.0000000000000165
153. Hooker ER, Shrestha S, Lee CG, Cawthon PM, Abrahamson M, Ensrud K, et al. Obesity and Falls in a Prospective Study of Older Men: The Osteoporotic Fractures in Men Study. *J Aging Health* (2017) 29:1235–50. doi: 10.1177/0898264316660412
154. Ensrud KE, Ewing SK, Stone KL, Cauley JA, Bowman PJ, Cummings SR, et al. Intentional and Unintentional Weight Loss Increase Bone Loss and Hip Fracture Risk in Older Women. *J Am Geriatr Soc* (2003) 51:1740–7. doi: 10.1046/j.1532-5415.2003.51558.x
155. Ensrud KE, Cauley J, Lipschutz R, Cummings SR. Weight Change and Fractures in Older Women. Study of Osteoporotic Fractures Research Group. *Arch Intern Med* (1997) 157:857–63. doi: 10.1001/archinte.157.8.857
156. Ensrud KE, Fullman RL, Barrett-Connor E, Cauley JA, Stefanick ML, Fink HA, et al. Voluntary Weight Reduction in Older Men Increases Hip Bone Loss: The Osteoporotic Fractures in Men Study. *J Clin Endocrinol Metab* (2005) 90:1998–2004. doi: 10.1210/jc.2004-1805
157. Villareal DT, Shah K, Banks MR, Sinacore DR, Klein S. Effect of Weight Loss and Exercise Therapy on Bone Metabolism and Mass in Obese Older Adults: A One-Year Randomized Controlled Trial. *J Clin Endocrinol Metab* (2008) 93:2181–7. doi: 10.1210/jc.2007-1473

158. Santanasto AJ, Glynn NW, Newman MA, Taylor CA, Brooks MM, Goodpaster BH, et al. Impact of Weight Loss on Physical Function With Changes in Strength, Muscle Mass, and Muscle Fat Infiltration in Overweight to Moderately Obese Older Adults: A Randomized Clinical Trial. *J Obes* (2011) 2011:516576. doi: 10.1155/2011/516576
159. Kelleher JL, Beavers DP, Henderson RM, Yow D, Crotts C, Kiel J, et al. Weighted Vest Use During Dietary Weight Loss on Bone Health in Older Adults With Obesity. *J Osteoporos Phys Act* (2017) 5:210. doi: 10.4172/2329-9509.1000210
160. Krez AN, Stein EM. The Skeletal Consequences of Bariatric Surgery. *Curr Osteoporos Rep* (2020) 18:262–72. doi: 10.1007/s11914-020-00579-2
161. Lalmohamed A, de Vries F, Bazelier MT, Cooper A, van Staa TP, Cooper C, et al. Risk of Fracture After Bariatric Surgery in the United Kingdom: Population Based, Retrospective Cohort Study. *BMJ* (2012) 345:e5085. doi: 10.1136/bmj.e5085
162. Nakamura KM, Haglind EG, Clowes JA, Achenbach SJ, Atkinson EJ, Melton LJ3rd, et al. Fracture Risk Following Bariatric Surgery: A Population-Based Study. *Osteoporos Int* (2014) 25:151–8. doi: 10.1007/s00198-013-2463-x
163. Douglas IJ, Bhaskaran K, Batterham RL, Smeeth L. Bariatric Surgery in the United Kingdom: A Cohort Study of Weight Loss and Clinical Outcomes in Routine Clinical Care. *PLos Med* (2015) 12:e1001925. doi: 10.1371/journal.pmed.1001925
164. Lu CW, Chang YK, Chang HH, Kuo CS, Huang CT, Hsu CC, et al. Fracture Risk After Bariatric Surgery: A 12-Year Nationwide Cohort Study. *Med (Baltimore)* (2015) 94:e2087. doi: 10.1097/MD.0000000000002087
165. Fashandi AZ, Mehaffey JH, Hawkins RB, Schirmer B, Hallowell PT. Bariatric Surgery Increases Risk of Bone Fracture. *Surg Endosc* (2018) 32:2650–5. doi: 10.1007/s00464-017-5628-4
166. Javanainen M, Pekkarinen T, Mustonen H, Scheinin T, Leivonen M. Two-Year Nutrition Data in Terms of Vitamin D, Vitamin B12, and Albumin After Bariatric Surgery and Long-Term Fracture Data Compared With Conservatively Treated Obese Patients: A Retrospective Cohort Study. *Obes Surg* (2018) 28:2968–75. doi: 10.1007/s11695-018-3336-3
167. Yu EW, Kim SC, Sturgeon DJ, Lindeman KG, Weissman JS. Fracture Risk After Roux-En-Y Gastric Bypass vs Adjustable Gastric Banding Among Medicare Beneficiaries. *JAMA Surg* (2019) 154:746–53. doi: 10.1001/jamasurg.2019.1157
168. Ahlin S, Peltonen M, Sjöholm K, Anveden Å, Jacobson P, Andersson-Assarsson JC, et al. Fracture Risk After Three Bariatric Surgery Procedures in Swedish Obese Subjects: Up to 26 Years Follow-Up of a Controlled Intervention Study. *J Intern Med* (2020) 287:546–57. doi: 10.1111/joim.13020
169. Khalid SI, Omotosho PA, Spagnoli A, Torquati A. Association of Bariatric Surgery With Risk of Fracture in Patients With Severe Obesity. *JAMA Netw Open* (2020) 3:e207419. doi: 10.1001/jamanetworkopen.2020.7419
170. Paccou J, Martignène N, Lespessailles E, Babykina E, Pattou F, Cortet B, et al. Gastric Bypass But Not Sleeve Gastrectomy Increases Risk of Major Osteoporotic Fracture: French Population-Based Cohort Study. *J Bone Miner Res* (2020) 35:1415–23. doi: 10.1002/jbmr.4012
171. Alsaed OS, Al-Allaf AW, Elgenaied I, Jebril RA, Sasi S, Ahmed AO, et al. Increased Fracture Risk After Bariatric Surgery: A Case-Controlled Study With a Long-Term Follow-Up. *Obes Surg* (2021) 31:4853–60. doi: 10.1007/s11695-021-05655-9
172. Chin WL, Chi PJ, Hung WC, Lin CW, Chen CY, Chen JH. Bariatric Surgery Decreases the Risk of Non-Traffic Accident-Related Fractures in Patients With Obesity: Real-World Data From Taiwan. *Obes Surg* (2021) 31:2231–40. doi: 10.1007/s11695-021-05262-8
173. Zhang Q, Chen Y, Li J, Chen D, Cheng Z, Xu S, et al. A Meta-Analysis of the Effects of Bariatric Surgery on Fracture Risk. *Obes Rev* (2018) 19:728–36. doi: 10.1111/obr.12665
174. Shapses SA, Sukumar D. Bone Metabolism in Obesity and Weight Loss. *Annu Rev Nutr* (2012) 32:287–309. doi: 10.1146/annurev.nutr.012809.104655
175. Censani M, Stein EM, Shane E, Oberfield SE, McMahon DJ, Lerner S, et al. Vitamin D Deficiency Is Prevalent in Morbidly Obese Adolescents Prior to Bariatric Surgery. *ISRN Obes* (2013) 2013:284516. doi: 10.1155/2013/284516
176. Nicoletti CF, Morandi Junqueira-Franco MV, dos Santos JE, Marchini JS, Salgado WJr, Nonino CB. Protein and Amino Acid Status Before and After Bariatric Surgery: A 12-Month Follow-Up Study. *Surg Obes Relat Dis* (2013) 9:1008–12. doi: 10.1016/j.soard.2013.07.004
177. Adrian TE, Savage AP, Sagor GR, Allen JM, Bacarese-Hamilton AJ, Tatemoto K, et al. Effect of Peptide YY on Gastric, Pancreatic, and Biliary Function in Humans. *Gastroenterology* (1985) 89:494–9. doi: 10.1016/0016-5085(85)90442-1
178. Misra M, Klibanski A. Anorexia Nervosa and Osteoporosis. *Rev Endocr Metab Disord* (2006) 7:91–9. doi: 10.1007/s11154-006-9005-1
179. Yip RG, Wolfe MM. GIP Biology and Fat Metabolism. *Life Sci* (2000) 66:91–103. doi: 10.1016/S0024-3205(99)00314-8
180. Guerrero-Pérez F, Casajoana A, Gómez-Vaquero C, Virgili N, López-Urdiales R, Hernández-Montoliu L, et al. Changes in Bone Mineral Density in Patients With Type 2 Diabetes After Different Bariatric Surgery Procedures and the Role of Gastrointestinal Hormones. *Obes Surg* (2020) 30:180–8. doi: 10.1007/s11695-019-04127-5
181. Maccarinelli G, Sibilia V, Torsello A, Raimondo F, Pitto M, Giustina A, et al. Ghrelin Regulates Proliferation and Differentiation of Osteoblastic Cells. *J Endocrinol* (2005) 184:249–56. doi: 10.1677/joe.1.05837
182. Biver E, Salliot C, Combescure C, Gossec L, Hardouin P, Legroux-Gerot I, et al. Influence of Adipokines and Ghrelin on Bone Mineral Density and Fracture Risk: A Systematic Review and Meta-Analysis. *J Clin Endocrinol Metab* (2011) 96:2703–13. doi: 10.1210/jc.2011-0047
183. Naot D, Cornish J. Cytokines and Hormones That Contribute to the Positive Association Between Fat and Bone. *Front Endocrinol (Lausanne)* (2014) 5:70. doi: 10.3389/fendo.2014.00070
184. Reid IR. Relationships Between Fat and Bone. *Osteoporos Int* (2008) 19:595–606. doi: 10.1007/s00198-007-0492-z
185. Yang J, Zhang X, Wang W, Liu J. Insulin Stimulates Osteoblast Proliferation and Differentiation Through ERK and PI3K in MG-63 Cells. *Cell Biochem Funct* (2010) 28:334–41. doi: 10.1002/cbf.1668
186. Ferron M, Wei J, Yoshizawa T, Del Fattore A, DePinho RA, Teti A, et al. Insulin Signaling in Osteoblasts Integrates Bone Remodeling and Energy Metabolism. *Cell* (2010) 142:296–308. doi: 10.1016/j.cell.2010.06.003
187. Tanna N, Patel K, Moore AE, Dulnoan D, Edwards S, Hampson G. The Relationship Between Circulating Adiponectin, Leptin and Vaspin With Bone Mineral Density (BMD), Arterial Calcification and Stiffness: A Cross-Sectional Study in Post-Menopausal Women. *J Endocrinol Invest* (2017) 40:1345–53. doi: 10.1007/s40618-017-0711-1
188. Mpalaris V, Anagnostis P, Anastasilakis AD, Goulis DG, Doumas A, Iakovou I. Serum Leptin, Adiponectin and Ghrelin Concentrations in Post-Menopausal Women: Is There an Association With Bone Mineral Density? *Maturitas* (2016) 88:32–6. doi: 10.1016/j.maturitas.2016.03.004
189. Ha CH, Swearingin B, Jeon YK. Relationship of Visfatin Level to Pancreatic Endocrine Hormone Level, HOMA-IR Index, and HOMA β-Cell Index in Overweight Women Who Performed Hydraulic Resistance Exercise. *J Phys Ther Sci* (2015) 27:2965–9. doi: 10.1589/jpts.27.2965
190. Peng XD, Xie H, Zhao Q, Wu XP, Sun ZQ, Liao EY. Relationships Between Serum Adiponectin, Leptin, Resistin, Visfatin Levels and Bone Mineral Density, and Bone Biochemical Markers in Chinese Men. *Clin Chim Acta* (2008) 387:31–5. doi: 10.1016/j.cca.2007.08.012
191. Zhang H, Xie H, Zhao Q, Xie GQ, Wu XP, Liao EY, et al. Relationships Between Serum Adiponectin, Apelin, Leptin, Resistin, Visfatin Levels and Bone Mineral Density, and Bone Biochemical Markers in Post-Menopausal Chinese Women. *J Endocrinol Invest* (2010) 33:707–11. doi: 10.1007/BF03346674
192. Väänänen HK, Härkönen PL. Estrogen and Bone Metabolism. *Maturitas* (1996) 23:S65–9. doi: 10.1016/0378-5122(96)01015-8
193. Carey DG, Pliego GJ, Raymond RL, Skau KB. Body Composition and Metabolic Changes Following Bariatric Surgery: Effects on Fat Mass, Lean Mass and Basal Metabolic Rate. *Obes Surg* (2006) 16:469–77. doi: 10.1381/096089206776327378
194. Zalesin KC, Franklin BA, Lillystone MA, Shamoun T, Krause KR, Chengelis DL, et al. Differential Loss of Fat and Lean Mass in the Morbidly Obese After Bariatric Surgery. *Metab Syndr Relat Disord* (2010) 8:15–20. doi: 10.1089/met.2009.0012
195. Paccou J, Penel G, Chauveau C, Cortet B, Hardouin P. Marrow Adiposity and Bone: Review of Clinical Implications. *Bone* (2019) 118:8–15. doi: 10.1016/j.bone.2018.02.008

196. Parfitt AM, Pødenphant J, Villanueva AR, Frame B. Metabolic Bone Disease With and Without Osteomalacia After Intestinal Bypass Surgery: A Bone Histomorphometric Study. *Bone* (1985) 6:211–20. doi: 10.1016/8756-3282(85)90003-1
197. Bal BS, Finelli FC, Shope TR, Koch TR. Nutritional Deficiencies After Bariatric Surgery. *Nat Rev Endocrinol* (2012) 8:544–56. doi: 10.1038/nrendo.2012.48
198. Schafer AL, Weaver CM, Black DM, Wheeler AL, Chang H, Szefc GV, et al. Intestinal Calcium Absorption Decreases Dramatically After Gastric Bypass Surgery Despite Optimization of Vitamin D Status. *J Bone Miner Res* (2015) 30:1377–85. doi: 10.1002/jbmr.2467
199. Aarts EO, Janssen IM, Berends FJ. The Gastric Sleeve: Losing Weight as Fast as Micronutrients? *Obes Surg* (2011) 21:207–11. doi: 10.1007/s11695-010-0316-7
200. Hewitt S, Kristinsson J, Aasheim ET, Blom-Høgestøl IK, Aaseth E, Jahnsen J, et al. Relationships Between Vitamin D Status and PTH Over 5 Years After Roux-En-Y Gastric Bypass: A Longitudinal Cohort Study. *Obes Surg* (2020) 30:3426–34. doi: 10.1007/s11695-020-04582-5
201. Khandalavala BN, Hibma PP, Fang X. Prevalence and Persistence of Vitamin D Deficiency in Biliopancreatic Diversion Patients: A Retrospective Study. *Obes Surg* (2010) 20:881–4. doi: 10.1007/s11695-010-0185-0
202. Folli F, Sabowitz BN, Schwesinger W, Fanti P, Guardado-Mendoza R, Muscogiuri G. Bariatric Surgery and Bone Disease: From Clinical Perspective to Molecular Insights. *Int J Obes (Lond)* (2012) 36:1373–9. doi: 10.1038/ijo.2012.115
203. Wolfe RR. The Underappreciated Role of Muscle in Health and Disease. *Am J Clin Nutr* (2006) 84:475–82. doi: 10.1093/ajcn/84.3.475
204. Heber D, Greenway FL, Kaplan LM, Livingston E, Salvador J, Still C, et al. Endocrine and nutritional management of the post-bariatric surgery patient: an Endocrine Society Clinical Practice Guideline. *J Clin Endocrinol Metab* (2010) 95:4823–43. doi: 10.1210/jc.2009-2128
205. Thibault R, Huber O, Azagury DE, Pichard C. Twelve Key Nutritional Issues in Bariatric Surgery. *Clin Nutr* (2016) 35:12–7. doi: 10.1016/j.clnu.2015.02.012
206. Otto M, Elrefai M, Krammer J, Weiß C, Kienle P, Hasenberg T. Sleeve Gastrectomy and Roux-En-Y Gastric Bypass Lead to Comparable Changes in Body Composition After Adjustment for Initial Body Mass Index. *Obes Surg* (2016) 26:479–85. doi: 10.1007/s11695-015-1792-6
207. Kirk B, Feehan J, Lombardi G, Duque G. Muscle, Bone, and Fat Crosstalk: The Biological Role of Myokines, Osteokines, and Adipokines. *Curr Osteoporos Rep* (2020) 18:388–400. doi: 10.1007/s11914-020-00599-y
208. Pham TT, Ivaska KK, Hannukainen JC, Virtanen KA, Lidell ME, Enerbäck S, et al. Human Bone Marrow Adipose Tissue Is a Metabolically Active and Insulin-Sensitive Distinct Fat Depot. *J Clin Endocrinol Metab* (2020) 105:2300–10. doi: 10.1210/clinem/dgaa216
209. Blom-Høgestøl IK, Mala T, Kristinsson JA, Hauge EM, Brunborg C, Gulseth HL, et al. Changes in Bone Marrow Adipose Tissue One Year After Roux-En-Y Gastric Bypass: A Prospective Cohort Study. *J Bone Miner Res* (2019) 34:1815–23. doi: 10.1002/jbmr.3814
210. Beekman KM, Akkerman EM, Streekstra GJ, Veldhuis-Vlug AG, Acherman Y, Gerdes VE, et al. The Effect of Roux-En-Y Gastric Bypass on Bone Marrow Adipose Tissue and Bone Mineral Density in Postmenopausal, Nondiabetic Women. *Obes (Silver Spring)* (2021) 29:1120–7. doi: 10.1002/oby.23171
211. Leitch VD, Brassill MJ, Rahman S, Butterfield NC, Ma P, Logan JG, et al. PYY Is a Negative Regulator of Bone Mass and Strength. *Bone* (2019) 127:427–35. doi: 10.1016/j.bone.2019.07.011
212. Casimiro I, Sam S, Brady MJ. Endocrine Implications of Bariatric Surgery: A Review on the Intersection Between Incretins, Bone, and Sex Hormones. *Physiol Rep* (2019) 7:e14111. doi: 10.14814/phy2.14111
213. Reid IR, Baldock PA, Cornish J. Effects of Leptin on the Skeleton. *Endocr Rev* (2018) 39:938–59. doi: 10.1210/er.2017-00226
214. Luo XH, Guo LJ, Xie H, Yuan LQ, Wu XP, Zhou HD, et al. Adiponectin Stimulates RANKL and Inhibits OPG Expression in Human Osteoblasts Through the MAPK Signaling Pathway. *J Bone Miner Res* (2006) 21:1648–56. doi: 10.1359/jbmr.060707
215. Delhanty PJ, van der Eerden BC, van Leeuwen JP. Ghrelin and Bone. *Biofactors* (2014) 40:41–8. doi: 10.1002/biof.1120
216. Takeda S, Elefteriou F, Levasseur R, Liu X, Zhao L, Parker KL, et al. Leptin Regulates Bone Formation *via* the Sympathetic Nervous System. *Cell* (2002) 111:305–17. doi: 10.1016/S0092-8674(02)01049-8
217. Nuche-Berenguer B, Moreno P, Esbrit P, Dapía S, Caeiro JR, Cancelas J, et al. Effect of GLP-1 Treatment on Bone Turnover in Normal, Type 2 Diabetic, and Insulin-Resistant States. *Calcif Tissue Int* (2009) 84:453–61. doi: 10.1007/s00223-009-9220-3
218. Henriksen DB, Alexandersen P, Byrjalsen I, Hartmann B, Bone HG, Christiansen C, et al. Reduction of Nocturnal Rise in Bone Resorption by Subcutaneous GLP-2. *Bone* (2004) 34:140–7. doi: 10.1016/j.bone.2003.09.009
219. Fukushima N, Hanada R, Teranishi H, Fukue Y, Tachibana T, Ishikawa H, et al. Ghrelin Directly Regulates Bone Formation. *J Bone Miner Res* (2005) 20:790–8. doi: 10.1359/JBMR.041237
220. Carrasco F, Basfi-Fer K, Rojas P, Valencia A, Csendes A, Codoceo J, et al. Changes in Bone Mineral Density After Sleeve Gastrectomy or Gastric Bypass: Relationships With Variations in Ghrelin, and Adiponectin Levels. *Obes Surg* (2014) 24:877–84. doi: 10.1007/s11695-014-1179-0
221. Wong IP, Driessler F, Khor EC, Shi YC, Hörmer B, Nguyen AD, et al. Peptide YY Regulates Bone Remodeling in Mice: A Link Between Gut and Skeletal Biology. *PLos One* (2012) 7:e40038. doi: 10.1371/journal.pone.0040038
222. Mechanick JI, Kushner RF, Sugerman HJ, Gonzalez-Campoy JM, Collazo-Clavell ML, Spitz AF, et al. American Association of Clinical Endocrinologists, The Obesity Society, and American Society for Metabolic & Bariatric Surgery Medical Guidelines for Clinical Practice for the Perioperative Nutritional, Metabolic, and Nonsurgical Support of the Bariatric Surgery Patient. *Obes (Silver Spring)* (2009) 17:S1–70. doi: 10.1038/oby.2009.28
223. Tian Z, Fan XT, Li SZ, Zhai T, Dong J. Changes in Bone Metabolism After Sleeve Gastrectomy Versus Gastric Bypass: A Meta-Analysis. *Obes Surg* (2020) 30:77–86. doi: 10.1007/s11695-019-04119-5
224. Mechanick JI, Youdim A, Jones DB, Garvey WT, Hurley DL, McMahon MM, et al. Clinical Practice Guidelines for the Perioperative Nutritional, Metabolic, and Nonsurgical Support of the Bariatric Surgery Patient–2013 Update: Cosponsored by American Association of Clinical Endocrinologists, the Obesity Society, and American Society for Metabolic & Bariatric Surgery. *Endocr Pract* (2013) 19:337–72. doi: 10.4158/EP12437.GL
225. Alejo Ramos M, Cano Rodríguez IM, Urioste Fondo AM, Pintor de la Maza B, Barajas Galindo DE, Fernández Martínez P, et al. Secondary Hyperparathyroidism in Patients With Biliopancreatic Diversion After 10 Years of Follow-Up, and Relationship With Vitamin D and Serum Calcium. *Obes Surg* (2019) 29:999–1006. doi: 10.1007/s11695-018-03624-3
226. Johansson HE, Zethelius B, Ohrvall M, Sundbom M, Haenni A. Serum Magnesium Status After Gastric Bypass Surgery in Obesity. *Obes Surg* (2009) 19:1250–5. doi: 10.1007/s11695-008-9536-5
227. Wawrzyniak A, Krotki M. The Need and Safety of Mineral Supplementation in Adults With Obesity Post Bariatric Surgery-Sleeve Gastrectomy (Sg). *Obes Surg* (2021) 31:4502–10. doi: 10.1007/s11695-021-05639-9
228. Stein EM, Silverberg SJ. Bone Loss After Bariatric Surgery: Causes, Consequences, and Management. *Lancet Diabetes Endocrinol* (2014) 2:165–74. doi: 10.1016/S2213-8587(13)70183-9
229. Lanza FL. Gastrointestinal Adverse Effects of Bisphosphonates: Etiology, Incidence and Prevention. *Treat Endocrinol* (2002) 1:37–43. doi: 10.2165/00024677-200201010-00004
230. Mitchell DY, Eusebio RA, Dunlap LE, Pallone KA, Nesbitt JD, Russell DA, et al. Risedronate Gastrointestinal Absorption Is Independent of Site and Rate of Administration. *Pharm Res* (1998) 15:228–32. doi: 10.1023/A:1011910517200
231. Swafford AA, Ard JD, Beavers DP, Gearren PC, Fernandez AZ, Ford SA, et al. Risedronate to Prevent Bone Loss After Sleeve Gastrectomy: Study Design and Feasibility Report of a Pilot Randomized Controlled Trial. *JBMR Plus* (2020) 4:e10407. doi: 10.1002/jbm4.10407
232. Lanza FL, Hunt RH, Thomson AB, Provenza JM, Blank MA. Endoscopic Comparison of Esophageal and Gastroduodenal Effects of Risedronate and Alendronate in Postmenopausal Women. *Gastroenterology* (2000) 119:631–8. doi: 10.1053/gast.2000.16517
233. Maricic M. New and Emerging Treatments for Osteoporosis. *Curr Opin Rheumatol* (2007) 19:364–9. doi: 10.1097/BOR.0b013e328172dc1a

234. Liu Y, Côté MM, Cheney MC, Lindeman KG, Rushin CC, Hutter MM, et al. Zoledronic Acid for Prevention of Bone Loss in Patients Receiving Bariatric Surgery. *Bone Rep* (2021) 14:100760. doi: 10.1016/j.bonr.2021.100760
235. Collazo-Clavell ML, Jimenez A, Hodgson SF, Sarr MG. Osteomalacia After Roux-En-Y Gastric Bypass. *Endocr Pract* (2004) 10:195–8. doi: 10.4158/EP.10.3.195
236. Schmucker AM, Green DE, Montemuro PM. Denosumab-Induced Hypocalcemia After Billroth II Gastric Bypass Surgery. *Case Rep Endocrinol* (2020) 2020:8833723. doi: 10.1155/2020/8833723
237. Hue O, Berrigan F, Simoneau M, Marcotte J, Marceau P, Marceau S, et al. Muscle Force and Force Control After Weight Loss in Obese and Morbidly Obese Men. *Obes Surg* (2008) 18:1112–8. doi: 10.1007/s11695-008-9597-5
238. Ito MK, Gonçalves VSS, Faria SLCM, Moizé V, Porporatti AL, Guerra ENS, et al. Effect of Protein Intake on the Protein Status and Lean Mass of Post-Bariatric Surgery Patients: A Systematic Review. *Obes Surg* (2017) 27:502–12. doi: 10.1007/s11695-016-2453-0
239. Voican CS, Lebrun A, Maitre S, Lainas P, Lamouri K, Njike-Nakseu M, et al. Predictive Score of Sarcopenia Occurrence One Year After Bariatric Surgery in Severely Obese Patients. *PLos One* (2018) 13:e0197248. doi: 10.1371/journal.pone.0197248
240. Beaudart C, Zaaria M, Pasleau F, Reginster JY, Bruyère O. Health Outcomes of Sarcopenia: A Systematic Review and Meta-Analysis. *PLos One* (2017) 12:e0169548. doi: 10.1371/journal.pone.0169548
241. Hansen D, Decroix L, Devos Y, Nocca D, Cornelissen V, Dillemans B, et al. Towards Optimized Care After Bariatric Surgery by Physical Activity and Exercise Intervention: A Review. *Obes Surg* (2020) 30:1118–25. doi: 10.1007/s11695-020-04390-x
242. Pouwels S, Wit M, Teijink JA, Nienhuijs SW. Aspects of Exercise Before or After Bariatric Surgery: A Systematic Review. *Obes Facts* (2015) 8:132–46. doi: 10.1159/000381201
243. Sellberg F, Possmark S, Willmer M, Tynelius P, Persson M, Berglind D. Meeting Physical Activity Recommendations Is Associated With Health-Related Quality of Life in Women Before and After Roux-En-Y Gastric Bypass Surgery. *Qual Life Res* (2019) 28:1497–507. doi: 10.1007/s11136-019-02120-0
244. Fontana AD, Lopes AD, Lunardi AC. Bariatric Surgery Associated With Practice of Moderate to Intense Physical Activity Related to Weight Loss, Activity Level in Daily Life, Dyspnea, and Quality of Life of Sedentary Individuals With Morbid Obesity: A Prospective Longitudinal Study. *Obes Surg* (2019) 29:2442–8. doi: 10.1007/s11695-019-03859-8
245. Bellicha A, Ciangura C, Roda C, Torcivia A, Portero P, Oppert JM. Changes in Cardiorespiratory Fitness After Gastric Bypass: Relations With Accelerometry-Assessed Physical Activity. *Obes Surg* (2019) 29:2936–41. doi: 10.1007/s11695-019-03932-2
246. Shah K, Armamento-Villareal R, Parimi N, Chode S, Sinacore DR, Hilton TN, et al. Exercise Training in Obese Older Adults Prevents Increase in Bone Turnover and Attenuates Decrease in Hip Bone Mineral Density Induced by Weight Loss Despite Decline in Bone-Active Hormones. *J Bone Miner Res* (2011) 26:2851–9. doi: 10.1002/jbmr.475
247. Villareal DT, Chode S, Parimi N, Sinacore DR, Hilton T, Armamento-Villareal R, et al. Weight Loss, Exercise, or Both and Physical Function in Obese Older Adults. *N Engl J Med* (2011) 364:1218–29. doi: 10.1056/NEJMoa1008234
248. Muschitz C, Kocijan R, Haschka J, Zendeli A, Pirker T, Geiger C, et al. The Impact of Calcium, Protein Supplementation, and Physical Exercise on Bone Metabolism After Bariatric Surgery: The BABS Study. *J Bone Miner Res* (2016) 31:672–82. doi: 10.1002/jbmr.2707
249. Campanha-Versiani L, Pereira DAG, Ribeiro-Samora GA, Ramos AV, de Sander Diniz MFH, De Marco LA, et al. The Effect of a Muscle Weight-Bearing and Aerobic Exercise Program on the Body Composition, Muscular Strength, Biochemical Markers, and Bone Mass of Obese Patients Who Have Undergone Gastric Bypass Surgery. *Obes Surg* (2017) 27:2129–37. doi: 10.1007/s11695-017-2618-5
250. Diniz-Sousa F, Boppre G, Veras L, Hernández-Martínez A, Oliveira J, Fonseca H. The Effect of Exercise for the Prevention of Bone Mass After Bariatric Surgery: A Systematic Review and Meta-Analysis. *Obes Surg* (2022) 32:912–23. doi: 10.1007/s11695-021-05873-1
251. Sherf Dagan S, Tovim TB, Keidar A, Raziel A, Shibolet O, Zelber-Sagi S. Inadequate Protein Intake After Laparoscopic Sleeve Gastrectomy Surgery is Associated With a Greater Fat Free Mass Loss. *Surg Obes Relat Dis* (2017) 13:101–09. doi: 10.1016/j.soard.2016.05.026
252. Hunter GR, Plaisance EP, Fisher G. Weight Loss and Bone Mineral Density. *Curr Opin Endocrinol Diabetes Obes* (2014) 21:358–62. doi: 10.1097/MED.0000000000000087
253. Hosny IA, Elghawabi HS, Younan WB, Sabbour AA, Gobrial MA. Beneficial Impact of Aerobic Exercises on Bone Mineral Density in Obese Premenopausal Women Under Caloric Restriction. *Skeletal Radiol* (2012) 41:423–7. doi: 10.1007/s00256-011-1196-1
254. Suhaimi A, Yuliawiratman BS. From Prehab to Rehab: The Functional Restoration of a Bariatric Individual. In: N Saiz-Sapena and JM Oviedo, editors. *Bariatric Surgery. From the Non-Surgical Approach to the Post-Surgery Individual Care*. IntechOpen (2020). doi: 10.5772/intechopen.94418
255. Capodaglio P, Brunani A, Giustini A, Negrini S, Saraceni VM, Akyüz G, et al. Disability in Obesity With Comorbidities. A Perspective From the PRM Societies. *Eur J Phys Rehabil Med* (2014) 50:129–32.

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Polyunsaturated Fatty Acids Level and Bone Mineral Density: A Two-Sample Mendelian Randomization Study

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Background: This Mendelian randomization (MR) study aimed to explore the causal relationship between polyunsaturated fatty acids (PUFAs) and bone mineral density (BMD).

Methods: We conducted a two-sample MR analysis to figure out if there is any causal effect of PUFAs on BMD through the summary data from the genome-wide association study (GWAS). Relationships were evaluated through inverse variance weighted (IVW), MR-Egger, weighted median, and maximum likelihood methods. The MR Pleiotropy RESidual Sum and Outlier (MR-PRESSO) test was performed to detect the horizontal pleiotropy.

Results: Our findings revealed that omega-6 fatty acids were negatively related to the TB-BMD (beta-estimate: –0.0515; 95% confidence interval [CI]: –0.0911 to –0.0119; standard error [SE]: 0.0201; p-value: 0.0106). The reverse direction MR analysis showed that TB-BMD was linked to the omega-6 FAs (beta-estimate: –0.0699; 95% CI: –0.1304 to –0.0095; SE: 0.0308; p-value: 0.0265). No statistically significant correlations between PUFAs and BMD were observed after adjusting the interactions between metabolites.

Conclusion: This two-sample MR analyses produced strong and new genomic evidence that there was a causal relationship between omega-6 FAs and BMD. Further investigations are still required to elucidate the potential mechanism.

Keywords: polyunsaturated fatty acids - PUFA, bone mineral density—BMD, mendelian randomization, osteoporosis, omega - 3 fatty acids

INTRODUCTION

Osteoporosis is defined as a systematic musculoskeletal disease featured as the loss of bone mass and the degradation of the micro-architecture of the bone tissue, which is invariably predisposed to the increased fragility of bones and incidence of fractures (1, 2). As the global population is aging, it has been considered one of the most pressing public health concerns. According to the statistics, over 9 million osteoporosis-related fractures were confirmed worldwide annually, in which the direct financial losses incurred were estimated at a 17 billion dollars (3). Therefore, osteoporosis now imposes a major economic and clinical burden on society, in addition to inflicting pain and suffering to patients, especially the elderly (4). Nowadays, clinical diagnosis and assessment of osteoporosis rely heavily on bone mineral density (BMD) measurements, which have been proven to be reliable and effective (5, 6). Notably, both osteoporosis and BMD were demonstrated to be highly heritable and polygenic (7–9).

Optimal intake of certain nutrients is proven to participate in the regulation of BMD and is associated with the progress of osteoporosis (10), such as calcium (11) and retinol (12). Of these nutrients, dietary fats were thought to be critical to maintain normal musculoskeletal structure and functions (13–15). As an important component in our dietary fats, fatty acids (FAs) are mainly categorized as long-chain fatty acids including polyunsaturated fatty acids (PUFAs), monounsaturated fatty acids (MUFAs), saturated fatty acids (SFAs), medium-/short-chain fatty acids (MCFAs/SCFAs), and their metabolites (14). According to previous research, PUFAs may have a dual effect on bone metabolism depending on their structure, origin, relative concentration, and metabolic environment (13). In light of this, numerous studies had indicated that omega-6 promotes bone loss, whereas omega-3 favors bone remodeling. Several possible mechanisms had been proposed and clarified (16–18), which include calcium metabolism modulation (19), synthesis of prostaglandin (17), oxidation of fatty acids, genesis of osteoblast (20), and osteoclastogenesis.

Recently, numerous observational studies had indicated a link between PUFAs and BMD, although the findings remain controversial and conflicting. Besides that, observational studies have inherent limitations to infer causal association, such as reverse causality and confounding risk factors.

Mendelian randomization (MR) analyses, which use single nucleotide polymorphisms (SNPs) associated with exposure as instrumental variables (IVs) to evaluate the potentially causal effect between risk factors and outcomes, have evident advantages over conventional observation studies, according to this rationale (21). It is not affected by traditional confounders (environmental exposure and behaviors) and meets the plausibility of causal effect by time order (causes precede effects). A two-sample MR analysis means that IVs associated with exposure and those associated with outcome were obtained from different datasets of population, which could raise the statistical power.

METHODS

To investigate the causal effect of PUFAs on BMD values, we conducted a two-sample MR study that highly relied upon the summary level GWAS data for analysis (22, 23). In a Mendelian randomization analysis, three core assumptions about instrumental variables must be fulfilled: (1) IVs must be strongly related to the exposure, (2) association with the outcome was solely due to the exposure, and (3) independent of any other confounding variables (21). First, we extracted SNPs strongly associated with each PUFAs as instrument values ($p<5E-08$). To ease the bias due to linkage disequilibrium (LD), we performed the clumping method ($R^2<0.001$, window size=10,000 kb). To estimate the degree of LD, the individuals with European ancestors from the 1000 Genomes Project were used as a reference sample (24).

Second, the summary level data of SNPs related to exposure were retrieved in the outcome data.

Third, harmonization of the chosen SNP effect with the risk factors and outcomes was performed to align the palindromic SNPs (with A/T or G/C pairs). Possible palindromic SNPs were excluded.

Following that, we utilized PhenoScanner, a database of human genotype–phenotype associations, to see if any of the chosen SNPs were correlated with the potential confounders for BMD (25, 26). The threshold was set below: genome-wide significance ($p<1E-5$) and $R^2<0.8$. Moreover, F-statistics was used to assess the strength of IVs, and an F-value >10 indicated strong instruments (27). The strength of each instrument was measured by calculating the F-statistic using the following formula: $F = R^2(N-2)/(1-R^2)$, where R^2 was the proportion of variance in the phenotype explained by the genetic variants, and N was the sample size (28).

Genetic Association With PUFAs

The SNP summary data associated with PUFAs were derived from the Nightingale Health UK Biobank Initiative. The UK Biobank recruited 502,639 European participants aged 37–70 years in 22 assessment centers across the UK. All study participants reached the assessment centers by their own means, and enrollment was not performed at nursing homes (29). The biomarker profiles of 500,000 blood samples from UK Biobank were analyzed in Nightingale Health by utilizing nuclear magnetic resonance (NMR) and proprietary software, which could provide over 200 metabolic biomarkers in a single blood test including fatty acids (30). This first release covers biomarker data from approximately 118,000 EDTA plasma samples from baseline recruitment and 5,000 samples from repeat assessment (with 1,500 participants having both baseline and repeat-visit sample in the first data release). The metabolic biomarker dataset was open to any research institutions or individuals *via* the IEU GWAS database, which was a publicly accessible database of genetic correlation from GWAS summary datasets (23).

We only focused on some particular datasets of PUFAs, and three exposure data were selected, namely, omega-3 FAs, omega-6 FAs, and the ratio of omega-6 FAs to omega-3 FAs.

Genetic Association With BMD

A big GWAS meta-analysis of BMD enrolled 53,236 participants of European origin from GEnetic Factors for Osteoporosis Consortium (31). The femoral neck (FN), the lumbar spine

(LS) (L1–L4), and the forearm (FA) were all measured for BMD by dual-energy X-ray absorptiometry (DXA) machines. Each variant with a minor allele frequency (MAF) >0.5% was checked for its effect on BMD, adjusting for sex, age, age^2, and weight, and standardized to have a mean of zero and standard deviation of one to avoid the potential systematic differences caused by different measuring machines (31). In addition, the summary level data of total body (TB) BMD was employed from one large GWAS meta-analyses comprised of 30 studies and 66,628 individuals from America, Europe, and Australia, in which the majority of the participants came from population-based cohorts of European ancestry (86%) (32). TB-BMD (g/cm^2) was measured by DXA according to the standard manufacturer protocols. Moreover, its value was corrected for age, weight, height, and genomic principal components (derived from GWAS data), and any additional study-specific covariates (e.g., recruiting center) (32). A detailed information related to the GWAS data is provided and shown in **Supplementary Table S1**.

Statistical Analyses

We performed the two-sample MR analysis with the inverse variance weighted (IVW) method (23, 33), MR-Egger method (34, 35), weighted median method (36), and maximum likelihood (33) method to estimate the effect of PUFAs for BMD. In algorithm principle, the IVW method might generate the most precise estimate by integrating the Wald ratios of each SNP's causal effect through meta-analysis (23, 33). To avoid the bias caused by the horizontal pleiotropic effects, we conducted the MR-Egger method and weighted median method to analyze and test the potential directional bias caused by pleiotropy. When no <50% of the weight in the analysis is accounted for by the effective IVs, the weighted median method could offer a plausible estimate of the causal relationships (36). The MR-Egger method, which generated a weighted linear regression between exposure and outcome coefficients, was conducted to evaluate the pleiotropy better. Under the premise of meeting the basic assumption of Instrument Strength Independent of Direct Effect (InSIDE), the slope of the regression line could represent the asymptotically unbiased causal estimate. Apart from this, the horizontal pleiotropy in the average data of the whole genetic instruments could be quantified and presented by the intercept of the MR-Egger regression line (34, 35). Under the condition that the intercept of the regression line is not equal to 0, the intercept of the MR-Egger method can be applied to detect the horizontal pleiotropy. $p<0.05$ was considered to be statistically significant. Moreover, we also performed multivariable MR (MVMR) analysis to control potential interactions between metabolites. The bidirectional Mendelian randomization was also conducted to explore the reverse causation. All the MR analyses were conducted in R statistical software (Version 4.1.1) by utilizing the "TwoSampleMR" package (https://github.com/MRCIEU/TwoSampleMR) (23).

Sensitivity Analyses

For sensitivity analysis, several statistics approaches were applied. Cochran Q statistic was tested to assess and quantity heterogeneity (37). Depending on the degree of heterogeneities ($Q>0.05$ fixed-effect model; $Q<0.05$ random-effect model), the fixed- or random-effect model was used for further analysis. For quantitative analysis of heterogeneities, we also used I^2 to evaluate the magnitude. It is generally accepted that $I^2>50\%$ indicates significant heterogeneity. The directional pleiotropy was assessed through the intercept of the MR-Egger method. As a further step, we also conducted the MR Pleiotropy RESidual Sum and Outlier (MR-PRESSO) test to detect the horizontal pleiotropy and remove the outlier SNPs to reassess the cause estimate (38, 39). The "leave-one-out" sensitivity test was applied to figure out the potentially influential single SNP (**Supplementary Figures S1–12**).

RESULTS

Causal Effect of PUFA on BMD

After verification, the final data of SNPs enrolled in our analysis are shown in **Supplementary Tables S2–8**. We evaluated the causal effect of PUFA, which includes omega-3 FAs, omega-6 FAs, and the ratio of omega-6 FAs to omega-3 FAs on LS-BMD, FN-BMD, FA-BMD, and TB-BMD in the two-sample MR analysis. The scatter plots are displayed in **Supplementary Figures S13–24**. The results are displayed in **Table 1** and **Figure 1**. Based on the IVW analysis, omega-6 fatty acids were proven to be negatively related to the TB-BMD (beta-estimate: −0.0515; 95% confidence interval [CI]: −0.0911 to −0.0119; standard error [SE]: 0.0201; p-value: 0.0106), which indicated that a 1-SD decrease in omega-6 fatty acids was associated with the improvement in TB-BMD levels by 0.0515 g/cm^2. The result was further validated by maximum likelihood method (beta-estimate: −0.0517; 95% CI: −0.0915 to −0.0120; SE: 0.0202; p-value: 0.0106). Moreover, no significant correlations were found between omega-6 FAs and site-specific BMD (LS-BMD, FN-BMD, and FA-BMD) according to the statistical analysis results of IVW method, MR-Egger regression, weighted median method, and maximum likelihood analysis.

A higher ratio of omega-6 FAs to omega-3 FAs was proven to be poorly related to the improved BMD of the lumbar spine (beta-estimate: 0.0726; 95% CI: −0.0376 to 0.1829; SE: 0.0562; p-value: 0.1966) (**Table 1**) according to the result of the IVW analysis. Furthermore, this conclusion from IVW approach was in accordance with those of the other three statistical models. Moreover, no significant correlations were found between the ratio of FAs and FN-BMD or FA-BMD according to the statistical analysis results of IVW method, MR-Egger regression, weighted median method, and maximum likelihood analysis.

Analogously, omega-3 fatty acids also demonstrated no positive correlation to LS-BMD (beta-estimate: −0.0671; 95% CI: −0.1650 to 0.0307; SE: 0.0499; p-value: 0.1789), FN-BMD (beta-estimate: 0.0041; 95% CI: −0.0802 to 0.0885; SE: 0.0430; p-value: 0.9237), FA-BMD (beta-estimate: −0.0722; 95% CI: −0.2408 to 0.0963; SE: 0.0860; p-value: 0.4011), and TB-BMD (beta-estimate: −0.0438; 95% CI: −0.0958 to 0.0082; SE: 0.0265; p-value: 0.0989) (**Table 1**). These conclusions above were also further validated by the MR-Egger analysis, weighted median analysis, and maximum likelihood analysis.

TABLE 1 | MR estimates of the causal effects of PUFAs on BMD using various analysis methods.

Exposures	Outcomes	Number of SNPs	IVW							MR-Egger					
			Estimate	SE	95%CI	MR p-value	Q-value	Heterogeneity p-value	I^2	Estimate	SE	95%CI	MR p-value	Intercept	Intercept P-value
Omega3	LS-BMD	22	−0.0671	0.0499	−0.1650,0.0307	0.1789	20.5023	0.4896	5%	0.0370	0.0984	−0.1558,0.2299	0.7105	−0.0057	0.2334
	FN-BMD	22	0.0041	0.0430	−0.0802,0.0885	0.9237	15.1264	0.8165	5%	−0.0379	0.0849	−0.2044,0.1285	0.6600	0.0023	0.5722
	FA-BMD	24	−0.0722	0.0860	−0.2408,0.0963	0.4011	18.2438	0.7441	4.76%	−0.0500	0.1655	−0.3746,0.2744	0.7651	−0.0012	0.8769
	TB-BMD	36	−0.0438	0.0265	−0.0958,0.0082	0.0989	45.0938	0.1180	2.94%	−0.0817	0.0541	−0.1878,0.0243	0.1403	0.0023	0.4040
Omega6	LS-BMD	29	−0.0247	0.0371	−0.0975,0.0481	0.5058	38.9516	0.0817	3.70%	−0.0753	0.0835	−0.2390,0.0884	0.3752	0.0033	0.4814
	FN-BMD	29	0.0008	0.0319	−0.0618,0.0633	0.9812	12.8553	0.9935	3.70%	−0.0116	0.0602	−0.1296,0.1064	0.8482	0.0008	0.8101
	FA-BMD	30	0.0646	0.0650	−0.0628,0.1919	0.3205	22.8406	0.7838	3.57%	0.0476	0.1229	−0.1933,0.2885	0.7015	0.0011	0.8722
	TB-BMD	45	−0.0515	0.0201	−0.0911, −0.0119	0.0106	41.8432	0.5644	2.33%	−0.0660	0.0376	−0.1398,0.0077	0.0864	0.0010	0.6503
Ratio of Omega6 to Omega3	LS-BMD	18	0.0726	0.0562	−0.0376,0.1829	0.1966	14.2175	0.6516	6.25%	0.0566	0.1156	−0.1701,0.2833	0.6312	0.0009	0.8759
	FN-BMD	18	0.0395	0.0482	−0.0550,0.1342	0.4124	13.4092	0.7083	6.25%	0.0933	0.0994	−0.1014,0.2882	0.3616	−0.0030	0.5446
	FA-BMD	20	−0.0243	0.0965	−0.2134,0.1648	0.8010	17.1364	0.5806	5.56%	0.2241	0.1925	−0.1532,0.6015	0.2595	−0.0144	0.1532
	TB-BMD	26	0.0283	0.0318	−0.0341,0.0909	0.3734	27.3838	0.3369	4.17%	0.0895	0.0633	−0.0345,0.2136	0.1699	−0.0039	−0.2676

Exposures	Outcomes	Number of SNPs	Weighted median				Maximum likelihood		
			Estimate	SE	95%CI	MR p-value	Estimate	SE	95%CI
Omega3	LS-BMD	22	−0.0539	0.0743	−0.1996,0.0917	0.4680	−0.0675	0.0502	−0.1659,0.0309
	FN-BMD	22	0.0367	0.0622	−0.0851,0.1587	0.5544	0.0041	0.0432	−0.0805,0.0888
	FA-BMD	24	0.1477	0.1248	−0.0970,0.3924	0.2369	−0.0726	0.0864	−0.2420,0.0966
	TB-BMD	36	−0.0727	0.0414	−0.1540,0.0085	0.0795	−0.0444	0.0267	−0.0968,0.0079
Omega6	LS-BMD	29	−0.0639	0.0552	−0.1721,0.0444	0.2475	−0.0246	0.0373	−0.0978,0.0486
	FN-BMD	29	0.0156	0.0445	−0.0716,0.1027	0.7261	0.0008	0.0320	−0.0619,0.0635
	FA-BMD	30	0.1069	0.0946	−0.0785,0.2922	0.2584	0.0651	0.0652	−0.0626,0.1928
	TB-BMD	45	−0.0229	0.0293	−0.0804,0.0345	0.4338	−0.0517	0.0202	−0.0915, −0.0120
Ratio of Omega6 to Omega3	LS-BMD	18	0.1160	0.0836	−0.0479,0.2799	0.1654	0.0736	0.0565	−0.0372,0.1844
	FN-BMD	18	0.0692	0.0694	−0.0668,0.2054	0.3184	0.0399	0.0484	−0.0550,0.1350
	FA-BMD	20	−0.0520	0.1420	−0.3305,0.2263	0.7138	−0.0245	0.0970	−0.2146,0.1656
	TB-BMD	26	0.0260	0.0436	−0.0595,0.1115	0.5510	0.0288	0.0321	−0.0341,0.0917

MR, Mendelian randomization; BMD, bone mineral density; FN-BMD, femoral neck BMD; LS-BMD, lumbar spine BMD; FA-BMD, forearm BMD; TB-BMD, total body BMD; SNPs, single nucleotide polymorphisms; IVW, inverse variance weighted; SE, standard error; CI, confidence interval.

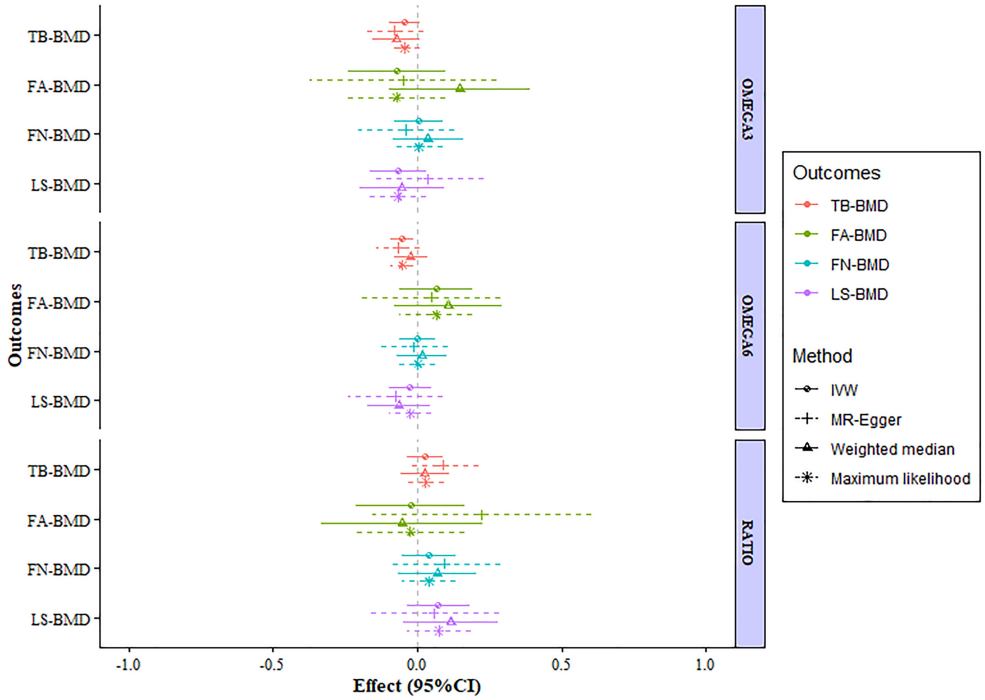

FIGURE 1 | MR estimates of the associations between PUFAs and BMD. The x-axis is the effects of PUFAs on BMD values. The vertical dashed line is the reference at effect = 0. The y-axis presents different BMD types, which are highlighted in different colors. Different MR methods are displayed with different line types. MR, Mendelian randomization; BMD, bone mineral density; FN-BMD, femoral neck BMD; LS-BMD, lumbar spine BMD; FA-BMD, forearm BMD; TB-BMD, total body BMD; SNPs, single nucleotide polymorphisms; IVW, inverse variance weighted; SE, standard error; CI, confidence interval.

Heterogeneity and Sensitivity Analyses

We analyzed heterogeneity through IVW analysis and applied the MR-Egger regression to analyze the pleiotropy. No heterogeneity for the causal effect of PUFAs on BMD was found in our statistical analysis (e.g., as for the causal effect of omega-3 FAs on LS-BMD: Q=20.5023; heterogeneity p-value=0.4896) (**Table 1**). According to the intercept values from the MR-Egger regression, no directional pleiotropy was detected for the causal effect of PUFAs on BMD (e.g., omega-6 FAs to LS-BMD: intercept=0.0033, intercept p-value=0.4814; for FN-BMD: intercept=0.0008, intercept p-value=0.8101; for FA-BMD: intercept=0.0011, intercept p-value=0.8722) (**Table 1**). The MR-PRESSO global test further validated that both outlier and horizontal pleiotropy were not observed in our MR analyses (e.g., omega-6 FAs: p-value=0.083 to LS-BMD; p-value=0.992 to FN-BMD; p-value=0.783 to FA-BMD) (**Table 2**).

Among the instrumental variables, MR-PRESSO did not identify any outlier for the causal effect between PUFAs and BMD.

MVMR and Bidirectional MR

As shown in the result of MVMR (**Table 3**), no statistically significant correlations between PUFAs and BMD were observed after adjusting the interactions between metabolites.

As shown in **Tables 4**, **5**, TB-BMD was proven to be negatively related to the omega-6 fatty acids based on the MR-Egger method (beta-estimate: −0.0699; 95% CI: −0.1304 to −0.0095; SE: 0.0308; p-value: 0.0265). No other reverse causations were observed between BMD and PUFAs.

DISCUSSION

The PUFAs contain two main acid types: omega-3 and omega-6 FAs. Omega-3 PUFAs are a group of fatty acids mainly synthesized in the body and maintained through diet, which predominantly include eicosapentaenoic acid (EPA), alpha-linolenic acid (ALA), and docosahexaenoic acid (DHA). Correspondingly, omega-6 fatty acids that are mainly found in various vegetable oils always come from linoleic acid (LA).Recently, several observational studies reported conflicting and discrepant conclusions on the association between PUFAs and BMD. Therein, omega-3 FAs were validated to positively affect bone remodeling *via* many different processes, including inhibiting osteoclast and promoting osteoblast activities. On the contrary, omega-6 FAs were always thought to be proinflammatory and pernicious to the maintenance of bone health. Accumulating animal experiments have revealed that supplementation of omega-3 FAs could enhance bone density and improve bone quality by various mechanisms. Acting as the specific ligand of peroxisome proliferator-activated receptor γ (PPAR γ), PUFAs could bind to

TABLE 2 | MR-PRESSO estimates of the causal effects of PUFAs on BMD.

Exposures	Outcomes	Number of SNPs	Effect	MR p-value	MR-PRESSO Global test p-value
Omega3	**LS-BMD**	22	–0.079	0.118	0.499
	FN-BMD	22	–0.0002	0.995	0.828
	FA-BMD	24	–0.075	0.309	0.791
	TB-BMD	36	–0.042	0.146	0.174
Omega6	**LS-BMD**	29	–0.025	0.577	0.083
	FN-BMD	29	0.001	0.972	0.992
	FA-BMD	30	0.065	0.272	0.783
	TB-BMD	45	–0.0515	0.012	0.541
Ratio of Omega6 to Omega3	**LS-BMD**	18	0.05	0.414	0.293
	FN-BMD	18	0.029	0.507	0.643
	FA-BMD	20	–0.032	0.716	0.638
	TB-BMD	26	0.032	0.352	0.317

the PPAR γ and induce the differentiation of adipocytes and fatty acids metabolism, which in turn affect the metabolism of the bone tissue (13). In addition, PUFAs could also modulate the formation of inflammatory cytokines to regulate the balance between formation and resorption of the bone *via* acting on the biosynthetic pathway of prostaglandin E2 (PGE2). Previous studies revealed that PUFAs could regulate the expression or the enzyme activity of cyclooxygenase (COX)-2, which is a rate-limiting enzyme in the synthesis of PGE2. The dual effect of PUFAs is that omega-3 FAs favor the downregulation of COX-2, which leads to the decrease in the production of PGE2 and, furthermore, enhance the formation of the bone. As for omega-6 FAs, it produced the entire opposite effect. PUFAs could also affect the bone marrow microcirculation to reduce the metabolic capacity of the bone. The effect of promoting uptaking of calcium from diet has also been reported. Inconsistent with the conclusion drawn from animal models, the observations from clinical trials still remain controversial.

To the best of our knowledge, this is the first time that the causal association between PUFAs and BMD through a two-sample MR analysis is investigated. Our analysis involved 53,236 individuals of European decent for the association with site-specific BMD, 66,628 individuals for TB-BMD, and 114,999 individuals for PUFAs. Our analytical studies demonstrated that omega-6 fatty acids were proven to be negatively related to the TB-BMD. Moreover, reverse causation was also observed between them. However, after adjusting the interactions between metabolites, no cause and effect association was shown based on the MVMR result. This may suggest that the associations between PUFAs and BMD are likely contributed by other confounding risk factors or the interactions between FAs. To ensure the consistency and reliability of the analysis, our research employed multiple statistical process to check the heterogeneity and control the pleiotropy. We also selected the IVs (F-statistics>10) from the large GWAS data to better represent PUFAs and BMD. In general, our two-sample MR study possessed adequate precision and stability to support the conclusion.

As far as we know, the previous observational studies were always limited to the effect of some specific types of PUFAs on bone health or some particular subtypes of the population, such as post-menopausal women and older people. Furthermore, the intake of dietary fatty acids was usually retrospectively estimated using some questionnaires (40, 41). Thus, the inherent methodological limitation of evaluating the supplementation of fatty acids is unavoidable (42). Due to this, it is not surprising that the previous studies are controversial while still puzzling. Most of the observational studies found that BMD was positively

TABLE 3 | MVMR result after adjusting the interactions between FAs.

Outcome	Exposures	NumberofSNPs	Effect	SE	MVMR P Value
LS-BMD	**Omega-3 fatty acids**	44	–4.612	5.0848	0.3643
	Omega-6 fatty acids	47	1.6421	1.8519	0.3752
	Ratio of omega6/omega3	28	–4.0504	4.5302	0.3712
FN-BMD	**Omega-3 fatty acids**	44	2.3857	3.5399	0.5003
	Omega-6 fatty acids	47	–0.8907	1.2893	0.4896
	Ratio of omega6/omega3	28	2.1271	3.1539	0.5000
FA-BMD	**Omega-3 fatty acids**	44	–6.9028	6.9061	0.3175
	Omega-6 fatty acids	48	2.5533	2.5146	0.3099
	Ratio of omega6/omega3	28	–6.0379	6.1529	0.3264
TB-BMD	**Omega-3 fatty acids**	49	1.1004	3.3400	0.7418
	Omega-6 fatty acids	57	–0.4604	1.2164	0.7050
	Ratio of omega6/omega3	31	0.9961	2.9757	0.7378

MR, Mendelian randomization; BMD, bone mineral density; FN-BMD, femoral neck BMD; LS-BMD, lumbar spine BMD; FA-BMD, forearm BMD; TB-BMD, total body BMD; SNPs, single nucleotide polymorphisms; IVW, inverse variance weighted; SE, standard error; CI, confidence interval.

TABLE 4 | MR estimates of the causal effects of PUFAs on BMD using various analysis methods.

Exposures	Outcomes	Number of SNPs	IVW							MR-Egger				
			Estimate	SE	95%CI	MR p-value	Q-value	Heterogeneity p-value	I^2	Estimate	SE	95%CI	MR p-value	Intercept
LS-BMD	Omega-3 fatty acids	20	0.0045	0.0151	−0.0251,0.0342	0.7623	24.4995	0.1776	5.56%	−0.0802	0.0635	−0.2048,0.0444	0.2232	0.0062
	Omega-6 fatty acids	19	0.0084	0.0201	−0.0311,0.0479	0.6764	29.3822	0.0439	5.88%	−0.0892	0.0723	−0.2310,0.0526	0.2344	0.0071
	Ratio of omega6/omega3	20	0.0041	0.0152	−0.0256,0.0341	0.7836	14.7783	0.7365	5.56%	0.0667	0.0576	−0.0463,0.1797	0.2624	−0.0046
FN-BMD	Omega-3 fatty acids	17	−0.0080	0.0234	−0.0544,0.0373	0.7148	27.9430	0.0321	6.67%	−0.1761	0.1192	−0.4099,0.0576	0.1603	0.0108
	Omega-6 fatty acids	15	0.0343	0.0251	−0.0150,0.0837	0.1726	25.4820	0.0301	7.69%	0.2845	0.1355	0.0188,0.5501	0.0559	−0.0161
	Ratio of omega6/omega3	17	0.0224	0.0178	−0.0125,0.0573	0.2082	17.8123	0.3349	6.67%	0.1857	0.0937	0.0020,0.3694	0.0661	−0.0105
TB-BMD	Omega-3 fatty acids	68	−0.0018	0.0128	−0.0269,0.0233	0.8874	87.3676	0.0481	1.52%	−0.0638	0.0356	−0.1337,0.0060	0.0779	0.0034
	Omega-6 fatty acids	68	−0.0007	0.0126	−0.0255,0.0240	0.9515	94.0726	0.0162	1.52%	−0.0699	0.0308	−0.1304,−0.0095	0.0265	0.0041
	Ratio of omega6/omega3	68	0.0069	0.0112	−0.0151,0.0291	0.5372	71.5785	0.3284	1.52%	0.0634	0.0324	−0.0002,0.1270	0.0551	−0.0031

Exposures	Outcomes	Number of SNPs	Weighted median				Maximum likelihood		
			Estimate	SE	95%CI	MR p-value	Estimate	SE	95%CI
LS-BMD	Omega-3 fatty acids	20	0.0271	0.0225	−0.0171,0.0713	0.2303	0.0046	0.0153	−0.0254,0.0347
	Omega-6 fatty acids	19	0.0231	0.0239	−0.0238,0.0701	0.3343	0.0086	0.0161	−0.0228,0.0402
	Ratio of omega6/omega3	20	0.0035	0.0217	−0.0391,0.0461	0.8712	0.0042	0.0153	−0.0258,0.0343
FN-BMD	Omega-3 fatty acids	17	−0.0341	0.0262	−0.0856,0.0172	0.1928	−0.0088	0.0179	−0.0441,0.0264
	Omega-6 fatty acids	15	0.0261	0.0284	−0.0297,0.0819	0.3594	0.0355	0.0189	−0.0016,0.0727
	Ratio of omega6/omega3	17	0.0338	0.0254	−0.0159,0.0837	0.1831	0.0231	0.0180	−0.0122,0.0584
TB-BMD	Omega-3 fatty acids	68	0.0106	0.0178	−0.0243,0.0455	0.5524	−0.0018	0.0113	−0.0241,0.0204
	Omega-6 fatty acids	68	−0.0055	0.0172	−0.0393,0.0281	0.7456	−0.0007	0.0108	−0.0219,0.0203
	Ratio of omega6/omega3	68	0.0115	0.0171	−0.0221,0.0452	0.5001	0.0071	0.0114	−0.0152,0.0294

MR, Mendelian randomization; BMD, bone mineral density; FN-BMD, femoral neck BMD; LS-BMD, lumbar spine BMD; FA-BMD, forearm BMD; TB-BMD, total body BMD; SNPs, single nucleotide polymorphisms; IVW, inverse variance weighted; SE, standard error; CI, confidence interval.

TABLE 5 | MR-PRESSO estimates of the causal effects of BMD on PUFAs.

Exposures	Outcomes	Number of SNPs	Effect	MR p-value	MR-PRESSO Global test p-value
LS-BMD	**Omega-3 fatty acids**	20	–0.001	0.933	0.207
	Omega-6 fatty acids	19	0.003	0.845	0.034(no outlier)
	Ratio of omega6/omega3	20	0.008	0.487	0.817
FN-BMD	**Omega-3 fatty acids**	17	–0.008	0.719	0.035(no outlier)
	Omega-6 fatty acids	15	0.034	0.194	0.049(no outlier)
	Ratio of omega6/omega3	17	0.022	0.250	0.389
TB-BMD	**Omega-3 fatty acids**	68	–0.006	0.619	0.075
	Omega-6 fatty acids	68	–0.001	0.882	0.013(no outlier)
	Ratio of omega6/omega3	68	0.011	0.326	0.391

correlated with the supplementation of omega-3 PUFA or fish oil. According to the Women's Health Initiative Study, positive associations between hip fractures and omega-3 FAs were shown; however, inverse associations were observed between omega-6 FAs, MUFAs, and PUFAs (43). Similarly, in a study of 76,000 women and 45,000 men enrolled, the fracture risk was negatively correlated with the consumption of omega-6 FAs and PUFAs (40). In contrast, a few researchers reported no statistically significant relationship between consumption of PUFAs and BMD or the incidence of fracture (44, 45). Other studies observed completely different findings in which a higher intake of PUFAs may deteriorate bone loss (43). In a recent meta-analysis that enrolled 28 RCTs (7,288 participants), the experimenter reported that the increased supplementation of omega-3 FAs may exert a low magnitude to the increase in BMD of the lumbar spine by 2.6% and femoral neck by 4.1%; however, the grade of evidence was insufficient (46). Another interesting finding that emerged from the analysis is that the increasing intake of total PUFAs may have little to no effect on BMD (46). Recently, in a single-center study of postmenopausal Spanish women, a high level of plasma omega-3 FAs was an independent risk factor of bone health (47).

The vast discrepancy of various studies may be attributed to multiple complex confounders such as sex and gender, etc. One notable confounder is that the consumption of cod liver oil rich in vitamins A and D was likely to exert influence to some degree on bone health (48, 49). Some researchers attempt to explain this phenomenon with more objective and more profound mechanisms, such as circulating fatty acids. Based on the Framingham Osteoporosis Study, which included 765 participants, a negative trend was observed between arachidonic acid (AA) and risk of hip fracture (50). Another cross-sectional study indicated that greater red blood cell omega-3 FAs were beneficial to decrease the risk of hip fracture (51). One important finding is that the influence of fatty acids on BMD may vary dynamically over time, beyond possible sex differences (42).

In accumulating animal experiments, the mechanism of PUFAs affecting bone health could be better elaborated. Deep down to the microlevel, the benefits of fish oil is closely linked to the presence of allelic variants in some genes such as PPAR γ, according to a comparative study on mice with polymorphisms in the PPAR γ gene (6T) (52). On the contrary, no effect of consumption of PUFAs on bone structure or metabolism was found in healthy mice. In another study conducted in ovariectomized rats, the level of PUFAs and ratio of omega-6/omega-3 PUFAs could be the essential important factors for maintaining BMD and bone turnover markers (53). The dietary ratio of 5:1 significantly elevated the amount of DHA in the bone tissues of the femur. This conclusion was also supported by some observational population study; in an investigated population with a higher intake ratio of omega-3 FAs to omega-6 FAs, such as the Japanese population, a lower ratio of osteoporosis was reported (54). In addition, different dietary sources of omega-3 FAs exhibited significant disparities in biochemistry and metabolism. Rozner et al. found that flaxseed oil was effective in ameliorating the micro-architecture, and fish oil could improve BMD, in which the core mechanism may be the alteration of peripheral clock in bone cells (55).

Some study limitations should be noted, although the rationale of MR analyses made it superior to conventional observational studies in excluding the existence of confounders. First, we only focused on the causal associations between a specific type of PUFA and BMD and did not take into consideration some other nutrients that might interact with PUFAs and cause bias. The potential limitation might contribute to the implausible casual relationship between PUFAs and BMD to some extent. Therefore, we conducted MR-Egger and MR-PRESSO methods to exclude the potential pleiotropy. Furthermore, the PhenoScanner tool was adopted to screen and remove the SNPs associated with confounders. Hence, the conclusion of this study should be creditable. Second, the samples were not further substratified according to gender and age, which were believed to be important risk factors of BMD based on previous studies. However, the effect on our analyses could be small due to the strength of the large sample size. Lastly, the exact mechanism underlying the causality between them was not explored in-depth. Therefore, a mechanistic research should be carried out in the future.

CONCLUSION

This two-sample MR analysis produced strong and new genomic evidence that there was causal relationship between omega-6 FAs and BMD. However, a further validation by MVMR and

bidirectional MR suggested that the association between them may be caused by the interactions of metabolites and reverse causality. Further investigations are still required to elucidate the potential mechanism.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

LW and XL conducted study design. LW, CZ, and HL conducted data collection and statistical analysis. LW, NZ, TH, and ZZ conducted data interpretation, manuscript preparation, and literature search. LW and XL conducted funds collection. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2022.858851/full#supplementary-material

REFERENCES

1. Compston JE, McClung MR, Leslie WD. Osteoporosis. *Lancet (London England)* (2019) 393:364–76. doi: 10.1016/s0140-6736(18)32112-3
2. Ensrud KE, Crandall CJ. Osteoporosis. *Ann Internal Med* (2017) 167:Itc17–itc32. doi: 10.7326/aitc201708010
3. Burge R, Dawson-Hughes B, Solomon DH, Wong JB, King A, Tosteson A. Incidence and Economic Burden of Osteoporosis-Related Fractures in the United States, 2005-2025. *J Bone Miner Res Off J Am Soc Bone Miner Res* (2007) 22:465–75. doi: 10.1359/jbmr.061113
4. Harvey N, Dennison E, Cooper C. Osteoporosis: Impact on Health and Economics. *Nat Rev Rheumatol* (2010) 6:99–105. doi: 10.1038/nrrheum.2009.260
5. Kanis JA, Melton LJ3rd, Christiansen C, Johnston CC, Khaltaev N. The Diagnosis of Osteoporosis. *J Bone Miner Res Off J Am Soc Bone Miner Res* (1994) 9:1137–41. doi: 10.1002/jbmr.5650090802
6. WHO Study Group*Assessment of Fracture Risk and its Application to Screening for Postmenopausal Osteoporosis. Report of a WHO Study Group* Vol. 843. World Health Organization technical report series (1994) p. 1–129.
7. Morris JA, Kemp JP, Youlten SE, Laurent L, Logan JG, Chai RC, et al. An Atlas of Genetic Influences on Osteoporosis in Humans and Mice. *Nat Genet* (2019) 51:258–66. doi: 10.1038/s41588-018-0302-x
8. Richards JB, Zheng HF, Spector TD. Genetics of Osteoporosis From Genome-Wide Association Studies: Advances and Challenges. *Nat Rev Genet* (2012) 13:576–88. doi: 10.1038/nrg3228
9. Yang TL, Shen H, Liu A, Dong SS, Zhang L, Deng FY, et al. A Road Map for Understanding Molecular and Genetic Determinants of Osteoporosis. *Nat Rev Endocrinol* (2020) 16:91–103. doi: 10.1038/s41574-019-0282-7
10. Alghadir AH, Gabr SA, Al-Eisa ES, Alghadir MH. Correlation Between Bone Mineral Density and Serum Trace Elements in Response to Supervised Aerobic Training in Older Adults. *Clin Interventions Aging* (2016) 11:265–73. doi: 10.2147/cia.S100566
11. Bhattarai T, Bhattacharya K, Chaudhuri P, Sengupta P. Correlation of Common Biochemical Markers for Bone Turnover, Serum Calcium, and Alkaline Phosphatase in Post-Menopausal Women. *Malaysian J Med Sci* (2014) 21(1):58–61.
12. Maggio D, Polidori MC, Barabani M, Tufi A, Ruggiero C, Cecchetti R, et al. Low Levels of Carotenoids and Retinol in Involutional Osteoporosis. *Bone* (2006) 38:244–8. doi: 10.1016/j.bone.2005.08.003
13. Wauquier F, Léotoing L, Philippe C, Spilmont M, Coxam V, Wittrant Y. Pros and Cons of Fatty Acids in Bone Biology. *Prog Lipid Res* (2015) 58:121–45. doi: 10.1016/j.plipres.2015.03.001
14. Bao M, Zhang K, Wei Y, Hua W, Gao Y, Li X, et al. Therapeutic Potentials and Modulatory Mechanisms of Fatty Acids in Bone. *Cell Prolif* (2020) 53:e12735. doi: 10.1111/cpr.12735
15. Longo AB, Ward WE. PUFAs, Bone Mineral Density, and Fragility Fracture: Findings From Human Studies. *Adv Nutr (Bethesda Md.)* (2016) 7:299–312. doi: 10.3945/an.115.009472
16. Lipina C, Hundal HS. Lipid Modulation of Skeletal Muscle Mass and Function. *J Cachexia Sarcopenia Muscle* (2017) 8:190–201. doi: 10.1002/jcsm.12144
17. Mangano KM, Sahni S, Kerstetter JE, Kenny AM, Hannan MT. Polyunsaturated Fatty Acids and Their Relation With Bone and Muscle Health in Adults. *Curr Osteoporosis Rep* (2013) 11:203–12. doi: 10.1007/s11914-013-0149-0
18. Tachtsis B, Camera D, Lacham-Kaplan O. Potential Roles of N-3 PUFAs During Skeletal Muscle Growth and Regeneration. *Nutrients* (2018) 10:309. doi: 10.3390/nu10030309
19. Haag M, Magada ON, Claassen N, Böhmer LH, Kruger MC. Omega-3 Fatty Acids Modulate ATPases Involved in Duodenal Ca Absorption. *Prostaglandins Leukotrienes Essential Fatty Acids* (2003) 68:423–9. doi: 10.1016/s0952-3278(03)00067-x
20. Maurin AC, Chavassieux PM, Meunier PJ. Expression of PPARgamma and Beta/Delta in Human Primary Osteoblastic Cells: Influence of Polyunsaturated Fatty Acids. *Calcified Tissue Int* (2005) 76:385–92. doi: 10.1007/s00223-004-0108-y
21. Emdin C, Khera A, Kathiresan S. Mendelian Randomization. *JAMA* (2017) 318:1925–6. doi: 10.1001/JAMA.2017.17219
22. Lawlor DA, Harbord RM, Sterne JA, Timpson N, Davey Smith G. Mendelian Randomization: Using Genes as Instruments for Making Causal Inferences in Epidemiology. *Stat Med* (2008) 27:1133–63. doi: 10.1002/sim.3034
23. Hemani G, Zheng J, Elsworth B, Wade KH, Haberland V, Baird D, et al. The MR-Base Platform Supports Systematic Causal Inference Across the Human Phenome. *eLife* (2018) 7:e34408. doi: 10.7554/eLife.34408
24. Abecasis GR, Altshuler D, Auton A, Brooks LD, Durbin RM, Gibbs RA, et al. Map of Human Genome Variation From Population-Scale Sequencing. *Nature* (2010) 467:1061–73. doi: 10.1038/nature09534
25. Kamat MA, Blackshaw JA, Young R, Surendran P, Burgess S, Danesh J, et al. PhenoScanner V2: An Expanded Tool for Searching Human Genotype-Phenotype Associations. *Bioinf (Oxford England)* (2019) 35:4851–3. doi: 10.1093/bioinformatics/btz469
26. Staley JR, Blackshaw J, Kamat MA, Ellis S, Surendran P, Sun BB, et al. PhenoScanner: A Database of Human Genotype-Phenotype Associations. *Bioinf (Oxford England)* (2016) 32:3207–9. doi: 10.1093/bioinformatics/btw373
27. Burgess S, Small DS, Thompson SGA. Review of Instrumental Variable Estimators for Mendelian Randomization. *Stat Methods Med Res* (2017) 26:2333–55. doi: 10.1177/0962280215597579
28. Burgess S, Thompson SG. Avoiding Bias From Weak Instruments in Mendelian Randomization Studies. *Int J Epidemiol* (2011) 40:755–64. doi: 10.1093/ije/dyr036

29. Sudlow C, Gallacher J, Allen N, Beral V, Burton P, Danesh J, et al. UK Biobank: An Open Access Resource for Identifying the Causes of a Wide Range of Complex Diseases of Middle and Old Age. *PLoS Med* (2015) 12: e1001779. doi: 10.1371/journal.pmed.1001779
30. Julkunen H, Cichońska A, Slagboom PE, Würtz P. Metabolic Biomarker Profiling for Identification of Susceptibility to Severe Pneumonia and COVID-19 in the General Population. *eLife* (2021) 10:e63033. doi: 10.7554/eLife.63033
31. Zheng HF, Forgetta V, Hsu YH, Estrada K, Rosello-Diez A, Leo PJ, et al. Whole-Genome Sequencing Identifies EN1 as a Determinant of Bone Density and Fracture. *Nature* (2015) 526:112–7. doi: 10.1038/nature14878
32. Medina-Gomez C, Kemp JP, Trajanoska K, Luan J, Chesi A, Ahluwalia TS, et al. Life-Course Genome-Wide Association Study Meta-Analysis of Total Body BMD and Assessment of Age-Specific Effects. *Am J Hum Genet* (2018) 102:88–102. doi: 10.1016/j.ajhg.2017.12.005
33. Burgess S, Butterworth A, Thompson SG. Mendelian Randomization Analysis With Multiple Genetic Variants Using Summarized Data. *Genet Epidemiol* (2013) 37:658–65. doi: 10.1002/gepi.21758
34. Bowden J, Del Greco MF, Minelli C, Davey Smith G, Sheehan NA, Thompson JR. Assessing the Suitability of Summary Data for Two-Sample Mendelian Randomization Analyses Using MR-Egger Regression: The Role of the I2 Statistic. *Int J Epidemiol* (2016) 45:1961–74. doi: 10.1093/ije/dyw220
35. Burgess S, Thompson SG. Interpreting Findings From Mendelian Randomization Using the MR-Egger Method. *Eur J Epidemiol* (2017) 32:377–89. doi: 10.1007/s10654-017-0255-x
36. Bowden J, Davey Smith G, Haycock PC, Burgess S. Consistent Estimation in Mendelian Randomization With Some Invalid Instruments Using a Weighted Median Estimator. *Genet Epidemiol* (2016) 40:304–14. doi: 10.1002/gepi.21965
37. Pierce BL, Burgess S. Efficient Design for Mendelian Randomization Studies: Subsample and 2-Sample Instrumental Variable Estimators. *Am J Epidemiol* (2013) 178:1177–84. doi: 10.1093/aje/kwt084
38. Verbanck M, Chen CY, Neale B, Do R. Detection of Widespread Horizontal Pleiotropy in Causal Relationships Inferred From Mendelian Randomization Between Complex Traits and Diseases. *Nat Genet* (2018) 50:693–8. doi: 10.1038/s41588-018-0099-7
39. Hemani G, Tilling K, Davey Smith G. Orienting the Causal Relationship Between Imprecisely Measured Traits Using GWAS Summary Data. *PLoS Genet* (2017) 13:e1007081. doi: 10.1371/journal.pgen.1007081
40. Virtanen JK, Mozaffarian D, Willett WC, Feskanich D. Dietary Intake of Polyunsaturated Fatty Acids and Risk of Hip Fracture in Men and Women. *Osteoporosis Int J Established as Result Cooperation Between Eur Foundation Osteoporosis Natl Osteoporosis Foundation USA* (2012) 23:2615–24. doi: 10.1007/s00198-012-1903-3
41. Järvinen R, Tuppurainen M, Erkkilä AT, Penttinen P, Kärkkäinen M, Salovaara K, et al. Associations of Dietary Polyunsaturated Fatty Acids With Bone Mineral Density in Elderly Women. *Eur J Clin Nutr* (2012) 66:496–503. doi: 10.1038/ejcn.2011.188
42. Harris TB, Song X, Reinders I, Lang TF, Garcia ME, Siggeirsdottir K, et al. Plasma Phospholipid Fatty Acids and Fish-Oil Consumption in Relation to Osteoporotic Fracture Risk in Older Adults: The Age, Gene/Environment Susceptibility Study. *Am J Clin Nutr* (2015) 101:947–55. doi: 10.3945/ajcn.114.087502
43. Orchard TS, Cauley JA, Frank GC, Neuhouser ML, Robinson JG, Snetselaar L, et al. Fatty Acid Consumption and Risk of Fracture in the Women's Health Initiative. *Am J Clin Nutr* (2010) 92:1452–60. doi: 10.3945/ajcn.2010.29955
44. Salari Sharif P, Asalforoush M, Ameri F, Larijani B, Abdollahi M. The Effect of N-3 Fatty Acids on Bone Biomarkers in Iranian Postmenopausal Osteoporotic Women: A Randomized Clinical Trial. *Age (Dordrecht Netherlands)* (2010) 32:179–86. doi: 10.1007/s11357-009-9122-3
45. Virtanen JK, Mozaffarian D, Cauley JA, Mukamal KJ, Robbins J, Siscovick DS. Fish Consumption, Bone Mineral Density, and Risk of Hip Fracture Among Older Adults: The Cardiovascular Health Study. *J Bone Miner Res Off J Am Soc Bone Miner Res* (2010) 25:1972–9. doi: 10.1002/jbmr.87
46. Abdelhamid A, Hooper L, Sivakaran R, Hayhoe RPG, Welch A. The Relationship Between Omega-3, Omega-6 and Total Polyunsaturated Fat and Musculoskeletal Health and Functional Status in Adults: A Systematic Review and Meta-Analysis of RCTs. *Calcified Tissue Int* (2019) 105:353–72. doi: 10.1007/s00223-019-00584-3
47. Roncero-Martín R, Aliaga I, Moran JM, Puerto-Parejo LM, Rey-Sánchez P, de la Luz Canal-Macías M, et al. Plasma Fatty Acids and Quantitative Ultrasound, DXA and pQCT Derived Parameters in Postmenopausal Spanish Women. *Nutrients* (2021) 13:1454. doi: 10.3390/nu13051454
48. Bischoff-Ferrari HA, Willett WC, Wong JB, Giovannucci E, Dietrich T, Dawson-Hughes B. Fracture Prevention With Vitamin D Supplementation: A Meta-Analysis of Randomized Controlled Trials. *JAMA* (2005) 293:2257–64. doi: 10.1001/JAMA.293.18.2257
49. Feskanich D, Singh V, Willett WC, Colditz GA. Vitamin A Intake and Hip Fractures Among Postmenopausal Women. *JAMA* (2002) 287:47–54. doi: 10.1001/JAMA.287.1.47
50. Farina EK, Kiel DP, Roubenoff R, Schaefer EJ, Cupples LA, Tucker KL. Plasma Phosphatidylcholine Concentrations of Polyunsaturated Fatty Acids are Differentially Associated With Hip Bone Mineral Density and Hip Fracture in Older Adults: The Framingham Osteoporosis Study. *J Bone Miner Res Off J Am Soc Bone Miner Res* (2012) 27:1222–30. doi: 10.1002/jbmr.1581
51. Orchard TS, Ing SW, Lu B, Belury MA, Johnson K, Wactawski-Wende J, et al. The Association of Red Blood Cell N-3 and N-6 Fatty Acids With Bone Mineral Density and Hip Fracture Risk in the Women's Health Initiative. *J Bone Miner Res Off J Am Soc Bone Miner Res* (2013) 28:505–15. doi: 10.1002/jbmr.1772
52. Bonnet N, Somm E, Rosen CJ. Diet and Gene Interactions Influence the Skeletal Response to Polyunsaturated Fatty Acids. *Bone* (2014) 68:100–7. doi: 10.1016/j.bone.2014.07.024
53. Watkins BA, Li Y, Seifert MF. Dietary Ratio of N-6/N-3 PUFAs and Docosahexaenoic Acid: Actions on Bone Mineral and Serum Biomarkers in Ovariectomized Rats. *J Nutr Biochem* (2006) 17:282–9. doi: 10.1016/j.jnutbio.2005.05.012
54. Paunescu AC, Dewailly E, Dodin S, Nieboer E, Ayotte P. Dioxin-Like Compounds and Bone Quality in Cree Women of Eastern James Bay (Canada): A Cross-Sectional Study. *Environ Health Global Access Sci Source* (2013) 12:54. doi: 10.1186/1476-069x-12-54
55. Rozner R, Vernikov J, Griess-Fishheimer S, Travinsky T, Penn S, Schwartz B, et al. The Role of Omega-3 Polyunsaturated Fatty Acids From Different Sources in Bone Development. *Nutrients* (2020) 12:3494. doi: 10.3390/nu12113494

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REVIEW
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Common Dietary Modifications in Preclinical Models to Study Skeletal Health

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Bone is a highly dynamic tissue that undergoes continuous remodeling by bone resorbing osteoclasts and bone forming osteoblasts, a process regulated in large part by osteocytes. Dysregulation of these coupled catabolic and anabolic processes as in the case of menopause, type 2 diabetes mellitus, anorexia nervosa, and chronic kidney disease is known to increase fracture risk. Recent advances in the field of bone cell metabolism and bioenergetics have revealed that maintenance of the skeleton places a high energy demand on these cells involved in bone remodeling. These new insights highlight the reason that bone tissue is the beneficiary of a substantial proportion of cardiac output and post-prandial chylomicron remnants and requires a rich supply of nutrients. Studies designed for the specific purpose of investigating the impact of dietary modifications on bone homeostasis or that alter diet composition and food intake to produce the model can be found throughout the literature; however, confounding dietary factors are often overlooked in some of the preclinical models. This review will examine some of the common pre-clinical models used to study skeletal biology and its pathologies and the subsequent impact of various dietary factors on these model systems. Furthermore, the review will include how inadvertent effects of some of these dietary components can influence bone cell function and study outcomes.

Keywords: nutrition, metabolism, diets, bone, fracture

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GENERAL INTRODUCTION

Skeletal Health and Background

The human skeleton represents a major organ system that undergoes continuous breakdown and rebuilding, a process referred to as bone remodeling. In fact, it has been estimated that the adult skeleton turns over or is replaced once every ~10 years. In support of such a dynamic tissue, bone is the beneficiary of a substantial proportion of the body's cardiac output and post-prandial chylomicron remnants, which presumably supply it with a rich source of nutrients (1). Of course, the rate of bone turnover is influenced by a multitude of factors, including age, sex, genetics, hormonal status and lifestyle factors (2, 3). It is this dynamic nature that gives bone tissue

the ability to adapt to different forces, which will determine its tensile strength and elastic characteristics. These structural and material properties in turn confer the bone's ability to resist fracture. Our appreciation of the cellular players involved in bone remodeling has become quite extensive, including the role of the bone resorbing osteoclasts, bone forming osteoblasts, and mechano-sensing osteocytes that regulated bone turnover (3). However, disruptions in these tightly processes can result in an uncoupling or imbalance in their activity that leads to skeletal pathologies.

A classic example of a clinically relevant skeletal disease is osteoporosis, frequently diagnosed based on a low bone mineral density (BMD). This can be a result of increased bone resorption and/or reduced bone formation, which ultimately results in a structural deficit of bone, leading to increased fracture risk. Osteoporosis and low bone mass (i.e., osteopenia) represent major public health problems, affecting ~54 million people in the U.S. and nearly half of all adults aged 50 and older (4, 5). Perhaps even more alarming is the recent report describing osteoporosis-related fractures as being responsible for more hospitalizations than heart attacks, strokes and breast cancer combined (6). Along with the substantial financial burden (~$19 billion/year), osteoporosis-related fractures often lead to multiple comorbidities (i.e., hypertension, infections, fluid and electrolyte imbalance), and patients frequently experience diminished quality of life due to immobility, pain, and isolation (7–9). While therapeutic options have significantly aided in the management of osteoporosis, some patients still experience undesirable, adverse side-effects, and overall patient compliance to these drug regimens is low (6, 10–12). Therefore, continued investigation into the molecular mechanisms regulating skeletal homeostasis and search for alternative prevention and treatment strategies is necessary.

Although clinical randomized control trials are the gold standard for the study of osteoporosis, they are limited by the time required to detect significant treatment effects in BMD and fracture risk, and the ability to study mechanistic alterations occurring at the tissue and cellular levels. Given these limitations of human-based research, rodent models have proved to be an invaluable tool when studying skeletal health. Rodent models, particularly mice, are an ideal system as they are relatively cheap, their genome has been sequenced, short lifespan for aged studies, and perhaps most importantly, their regulation of bone is like that of humans. In this regard, mice and rats both experience bone turnover by osteoclasts, osteoblasts, and osteocytes, albeit this remodeling unit is much faster than humans with a total remodeling unit occurring in ~1 month in mice (3). While this could be a limitation is some instances, it is also advantageous as structural changes can be observed in mice ~4-6 weeks in response to treatments. Therefore, mouse models have been widely used to study skeletal pathologies, including age-related osteoporosis, disuse osteoporosis, post-menopausal osteoporosis, secondary osteoporosis including glucocorticoid treatment, anorexia nervosa, and chronic kidney disease, as well as diabetes-related bone fragility. However, while using such models' investigators should exercise caution as to control for, or at the very least consider, potentially confounding variables to yield reproducible, reliable data that supports major conclusions. This seems intuitive, but diet for example, is sometimes overlooked and/or not taken into full consideration. It's true that calcium and vitamin D have long been considered key nutrients in bone health, however, even these micronutrients are sometimes underappreciated in the field. Additionally, macro- and micro-nutrient (i.e., vitamins and minerals) dietary composition along with non-nutrient dietary components such as phytochemicals are not always accounted for in the diets fed to laboratory animals. Therefore, it is the aim of this review to bring attention to commonly used preclinical, rodent models to study bone diseases and how dietary components can impact study design and/or confound results.

History of Rodent Diet Formulations

To begin, it's important to revisit the historical perspective of rodent diets. Unbeknownst to some scientists, nutritional status of laboratory rodents was an active and heavily discussed topic in the 1970's. In fact, in 1973 an *ad hoc* committee was formed by the American Institute of Nutrition (AIN) to identify dietary standards for laboratory rodents, which aimed to assist scientists by providing a nutritionally adequate diet that could be standardized among studies (13). This need grew out of concern from commonly used cereal or grain-based diets, referred to as "chow", which are suspect to inherent variation (**Figure 1A**). While sufficient to sustain rodent life, chow diets are rudimentary in their nutritional composition and vary greatly depending on the manufacturer, season, and harvest location, introducing experimental variability (14). Thus, the AIN committee formulated a purified diet (**Figure 1B**), termed AIN-76, in which all components were 'purified' thus allowing for precise ingredient formulation and subsequent standardization. Once the AIN-76 diet started to be used, some important concerns arose, namely nephrocalcinosis or kidney calcification and insufficient blood clotting (15),. These concerns along with the fact that the diet had been based on studies with a maximum of 6 months duration and the need to periodically revisit the dietary requirements prompted an 'AIN-76 Workshop' to convene in 1989, which addressed these concerns, along with refining the diet to thoughtfully establish the AIN-93 diets (16, 17). After almost 3 decades of testing, the AIN-93 diets currently stand today and are available in a growth formula (AIN-93G) and adult maintenance formula (AIN-93M) (**Table 1**); however, recent discussions have highlighted the need to periodically review the diet formulation. Nonetheless, given the purified nature of this diet it establishes specified amounts and proportions of macro- and micronutrients, ultra-trace elements, along with ensuring the diet palatability and stability. Therefore, in addition to providing researchers a reproducible diet, this diet establishes a "base", which can be easily manipulated to test scientific research questions related to nutritional aspects and model systems. In the sections to follow, rodent models commonly used for bone-related research will be highlighted, along with dietary factors that need to be considered during study design.

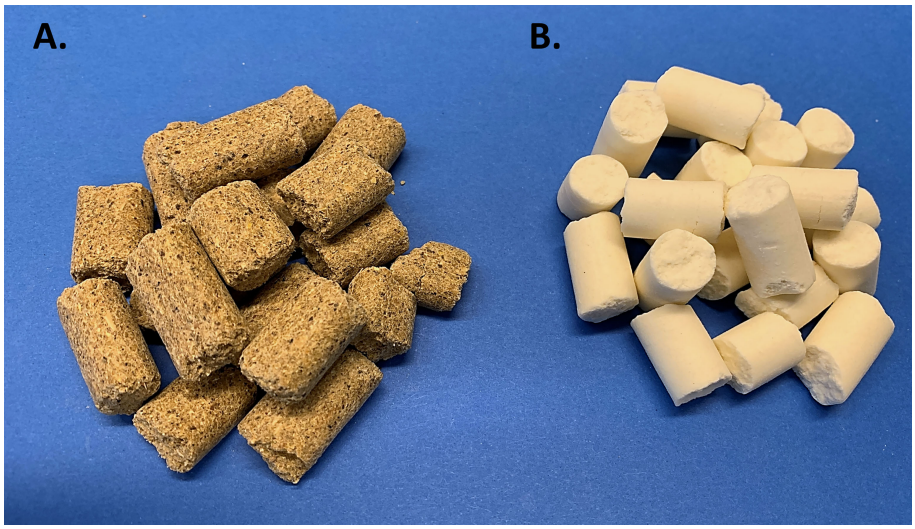

FIGURE 1 | Commonly Used Rodent Diets. **(A)** Grain-based 'chow' diet is often closed label and varies based on environment, location, and season. **(B)** Purified diet (AIN-93M) which has documented nutritional composition and formula.

PRECLINICAL RODENT MODELS TO STUDY BONE AND RELEVANT NUTRITIONAL ASPECTS

Ovariectomy (OVX) Model of Post-Menopausal Osteoporosis

The lack of estrogen that occurs post-menopause is a significant risk factor for osteoporosis. In fact, one in two postmenopausal women will experience osteoporosis, and most will suffer a fracture during their lifetime (10, 19). As such, bilateral oophorectomy or ovariectomy (OVX) of rodents is a commonly used model, which mimics significant bone loss associated with the early post-menopausal period. While the use of this model is common in bone research, the nutritional nuances associated with this model are not always appreciated. For example, use of chow diet can be particularly concerning. As described above, this grain-based diet fluctuates in its components, but these diets are also formulated with soy protein/soybean meal, which contain high amounts of phytoestrogens (i.e., isoflavones). Noteworthy, many of these isoflavones such as daidzein and genistein can act by binding to estrogen receptors (ER), namely ERβ and to a lesser extent ERα, eliciting either pro- or anti- estrogenic effects (20, 21). To give context, oral administration of daidzein and/or genistein to OVX Sprague-Dawley rats has been demonstrated to reduce femoral bone loss, prevent bone loss, and even increase bone density (22, 23). Although the doses of these compounds in chow are not likely to be as high as those reported here, it is evident that these compounds, which are found in many chow diets can have a direct impact on the OVX-model unbeknownst to the researcher. Furthermore, given the variability of these compounds across chow diets, it is expected the use of these diets could yield inconsistent skeletal outcomes. Conversely, the AIN-93 diet does contain soybean oil; however, no phytoestrogens have been detected in these purified diets (24).

Another nutritional aspect to note when using the OVX model to study bone, is that OVX often results in hyperphagia or increased food intake (25–27). This is in addition to global metabolic alterations associated with the model and menopause. The increased food intake is important because many researchers have previously highlighted the complex association between adiposity and skeletal homeostasis. Therefore, alterations occurring in the skeletal could be as simple as increased weight gain and body weight. For that matter, arguably these OVX animals could demonstrate various skeletal phenotypes due to altered nutrient intake. A technique often used to control for OVX-induced increases in food intake is to match- or pair-feed OVX group to that of Sham controls. In the case of pair feeding, the amount fed to the OVX group is based on the food intake of the Sham group the prior day so that feeding is adjusted daily. In contrast match feeding, the amount of food consumed by the Sham control over a few days, (e.g., a week) would then be fed to the OVX-group the following week. Our labs and others have demonstrated that regardless of this matched diet, OVX animals gain more weight relative to Sham, but in this scenario this observation is not due simply to increased food intake and reflect alterations occurring in systemic metabolism.

High-Fat Diet Induced Obesity (DIO) Models

One of the most striking health consequences related to the prevalence of obesity has been the staggering increase in cases of type 2 diabetes mellitus (T2DM). Over the last two decades, studies designed to determine whether T2DM influenced fracture risk

TABLE 1 | Key nutritional components and dietary formulation of the AIN-93M diet.

Nutrient	kcal (%)	Ingredients	g/kg	Notes
Protein	14.7			
		Casein	140	Casein provides >85% protein. While multiple protein sources exist, casein was selected as it provides an adequate amino acid composition and is readily available. The major limitation is that casein contains a low amount of cystine, therefore, L-cystine is added to the diet. Casein also contains significant amount of phosphorous.
		L-Cystine	1.8	
Carbohydrate	75.9			
		Cornstarch	495.69	Starch was selected as the carbohydrate source to replace the high amounts of sucrose in the AIN76 diet, which caused many off-target effects. A diet high in starch will not pellet properly, therefore, dextrinized starch (maltodextrin) is added. A small amount of sucrose is added to provide sweetness and improve palatability.
		Maltodextrin	125	
		Sucrose	100	
Fat	9.4	Soybean Oil	40	Soybean oil provides the essential fatty acids, linoleic and linolenic acid. The amount for AIN93M diet was selected to provide an (n-6):(n-3) ratio of 7 and a polyunsaturated: saturate ratio of 4. An additional margin of safety was added for the AIN93G diet.
Fiber		Cellulose	50	Cellulose is wood-fiber, and while fiber is not considered a 'nutrient' it provides beneficial regulation of the gut microflora populations. Of the various fiber sources, iron seems to be the largest mineral contaminant.
Minerals		Mineral Mix*	35	Mineral mix contains essential minerals and ultra-trace elements such as calcium, potassium, phosphorous, sodium, chloride, sulfur, magnesium, iron, zinc, copper, selenium, chromium, manganese, fluoride, nickel, iodine, molybdenum, and vanadium. Mineral mix also contains powered sucrose as a dispersal medium for vitamins.
		Choline bitartrate	2.5	
Vitamins		Vitamin Mix*	10	This vitamin mix provides the known essential vitamins for laboratory rodents including, nicotinic acid, pantothenate, pyridoxine, thiamin, riboflavin, folic acid, biotin, vitamin B12, vitamin E, vitamin A, vitamin D3, vitamin K1. Vitamin mix also contains powered sucrose as a dispersal medium for vitamins. These vitamins are especially sensitive to light degradation.
Anti-oxidant		tert-Butylhydroquinone (TBHQ)	0.01	Oxidation of highly polyunsaturated oils/fats are likely and therefore, TBHQ is added to effectively prevent from oxidation. Of note, when fats are altered in diets, it is likely that additional considerations should be taken including storage temperatures and frequency of food replacement.

Each component was thoughtfully considered during the formulation process (17, 18). (*) Mineral mix and vitamin mix are specific to the AIN-93 diets.

based solely on the assessment of BMD revealed mixed results, with the preponderance of the evidence indicating that patients were not at increased risk (28–30). However, subsequent studies with fracture as the primary outcome variable have challenged these initial findings and the clinical evidence now indicates that patients with T2DM have an increased risk of fracture, independent of BMD (31–34). This likely results in the fracture risk of patients with T2DM being underestimated when using BMD. The apparent disconnect between BMD and fracture risk in T2DM has perplexed researchers and clinicians alike, however, a consistent finding appears to be that diabetic patients (32, 35–37) and obese animal models of T2DM (38, 39) demonstrate impaired bone turnover, particularly reduced bone formation. Therefore, these animal models provide a valuable tool for the continued investigation behind molecular mechanisms contributing to fragility fractures in T2DM.

Regarding preclinical animal models to study skeletal related outcomes associated with T2DM some debate exists relative to the "best" model system. While some investigators rely on genetically modified transgenic and congenic mouse models, others utilize nutritional interventions in the form of high fat diets. We have previously provided an in-depth review regarding this model system (40). When performing these studies some key considerations include percent fat of diet, fat source, compensatory carbohydrate source of the control diet (i.e., added fat decreases the proportion of carbohydrate and/or protein), feeding schedule, and food/calorie intake. It is important to appreciate that the term, "high fat" is relative to the standard AIN diet. Since the AIN-93M diet contains ~10% kilocalories (kcal) derived from fat (soybean oil), anything above this amount would constitute as "high" fat. Two of the more commonly used commercially available high fat diets used to induce obesity are a 45% and 60% kcal from fat diets. These high fat diets are typically formulated with less soybean oil but use lard or beef tallow to increase fat content. These lard-based high fat diets are high in saturated (myristic, palmitate, and stearic) and unsaturated (oleic and palmitoleic) fatty acids relative to the AIN-93 diets. Conversely, some labs have used the commercially available Surwit diet to induce obesity and/or glucose intolerance (41). A major difference between this diet and the previously described high fat diets is that while fat is comparable at 58% kcal, the primary fat source in the Surwit diet is hydrogenated coconut oil, which contains a high amount of medium chain, saturated fatty acids (i.e., lauric acid and myristic acid). Additionally, the major carbohydrate source in the Surwit diet is sucrose, as opposed to cornstarch in the other high fat diets. This large amount of sucrose, which is digested to yield glucose and fructose, can have a profound effect on systemic metabolism aside from the high fat content (42). As such, the carbohydrate source, along with the full dietary formula, should be considered. Another example of dietary carbohydrate modifications impacting study related outcomes is that control diets relative to the experimental selected high fat diets, must be formulated with higher carbohydrates. This can be accomplished by increasing purified carbohydrate sources such as cornstarch, sucrose, or maltodextrin. Of these ingredients, cornstarch appears to impact metabolic response the least and is generally comparable to the AIN-93 diet (18). Therefore, it's likely that increasing the amount of sucrose to account for carbohydrates can impair glucose tolerance in the absence of weight gain (42). Therefore, if obesity is the required outcome this may not be of concern, but if obesity-related metabolic perturbations such as

impaired glucose tolerance, control mice could exhibit a similar phenotype compared to experimental high fat group. It is also worth noting that high sucrose content in the diet will also produce a sweeter, more palatable food, that could impact food intake. These details again underscore the importance and care which should be taken when dietary modifications *are the* model and the need to include the details of these modifications in published reports.

Calorie Restriction Model of Anorexia Nervosa

Anorexia nervosa is an eating disorder characterized by the severe restriction of food/nutrient intake, which results in abnormally low bodyweight and an intense fear of gaining weight. This disorder is associated with a significant reduction is BMD accounting for ~40% of patients being diagnosed with osteoporosis (92% osteopenia) and a 3-fold increase in lifetime fracture risk (43). Another striking skeletal phenotype associated with anorexia is despite the lipodystrophic response, bone marrow adipocytes increase in both their number and size (44). While the precise function of bone marrow adipocytes remains unclear, a general inverse association exists between bone marrow adipose tissue (BMAT) and BMD clinically (45). Therefore, this unique adipose depot has been of particular interest in clinical pathologies such as anorexia.

Relative to preclinical modeling, dietary manipulation in the form of calorie restriction is often used to mimic anorexia as it results in reduced BMD and expansion of BMAT. While it remains somewhat debated, a 30% reduction in total calories is often used in this model as it produces the desired outcome of reduced skeletal parameters and increases marrow adiposity (46, 47). Of note, investigators should consider formulating the diet such that a 30% reduction in calories does not result in micronutrient deficiencies. For example, we have previously used a formula which resulted in a 30% reduction in total kilocalories, but calcium and phosphate were matched to that of controls. In this regard, bone loss was evident and our ability to control these variables allowed us to determine that mineral deficiency was not the sole culprit (48). Similarly, phosphate restriction has also been shown to increase bone marrow adiposity and given the matched diet in our experiments, the same can be said for calorie restricted expansion of BMAT (49).

Other major nutritional and metabolic considerations using the calorie restriction model of anorexia nervosa involves the feast-famine feeding schedule and individual housing. Relative to the feeding schedule, animals are often food restricted during their active or dark cycle, only to be fed during the day, at which time they often consume most of their food. Therefore, the precision of the model relative to anorexia remains somewhat under debate. Additionally, animals are often individually housed to ensure each animal is consuming a known amount of food and to avoid a dominate animal from ingesting most of the food, thereby restricted others further. While controls should also be individually housed, this does introduce metabolic and behavioral disruption to the animals. Individual housing of mice has been shown to reduce growth rate while increasing energy intake and expenditure, due in part to maintain thermal neutrality without huddling of litter (50). Behavioral and endocrine alterations are also noted during individual housing and vary in degree amongst different mouse strains (e.g., BALB/c demonstrate increased anxiety-like behavior versus Swiss Webster which are considered 'normal'); however, they should be considered when using this model (51).

Dietary Models of Chronic Kidney Disease

The chemical composition for the mineral portion of bone, or the hydroxyapatite [$Ca_{10}(PO_4)_6(OH)_2$], inherently emphasizes the importance of mineral metabolism on skeletal health. Therefore, mineral imbalances that occur during chronic kidney diseases (CKD) significantly impact bone resulting in reduced BMD, promoting skeletal fragility and increased incidence of fracture (52). Traditional techniques for inducing renal failure have included surgical methods and currently, the most widespread methods are unilateral ureteral obstruction and 5/6 nephrectomy. Both methods lead to interstitial fibrosis by infiltration of macrophages and tubular cell death by apoptosis and necrosis, thus causes significant renal dysfunction (53). Limitations of these surgical models include the dependence on surgical skills, demand of post-operative care, high mortality rates, and reduced flexibility of dynamic urea alterations which results in the inability to study graduate disease progression (53). Additionally, dietary modification by means of using an adenine supplemented diet, commonly 0.2-0.25% adenine, has been shown to induce phenotypic CKD in rats (54). This method takes advantage of a mechanism by which adenine is oxidized *via* xanthine dehydrogenase, which yields 2,8-dihydroxyadenine. Given the low solubility of 2,8-dihydroxyadenine, stones are precipitated in the kidney tubules resulting in nephrolithiasis with extensive tubular dilation, necrosis, and fibrosis, accompanied by renal dysfunction. The adenine model has the advantage of sharing similar pathological features with human CKD (e.g., tubulointerstitial fibrosis, inflammation, glomerulosclerosis and moderate vascular calcification), with little variation between animals and develops over a relatively short period of time (54, 55). Up until recently, this model was exclusive to rats, as mice were reluctant to consume the adenine-based diets, which resulted in high morbidity and mortality due to starvation and malnutrition rather than renal failure. This limitation was recently circumvented by mixing the adenine in a chow-based diet supplemented with casein (56). In this capacity the casein effectively removed the inherent smell and taste of adenine, which resulted in mice sufficiently consuming the diet to replicate the renal dysfunction noted in rats (56).

Based on reports in the literature, some investigators supplement adenine into a purified casein-based diet, but many incorporate adenine into chow-based diets, with or without the addition of casein (56–59). The practice of incorporating nutrients into chow-based diets can still result in closed label formulations that leave researchers with little control over ingredient variability. Particularly noteworthy when incorporating the adenine into chow-based diets, these diets fluctuate in their mineral content which could significantly impact study-related outcomes. Despite this CKD model being driven by dietary alterations, its striking how few publications provide nutritional or dietary information. Additionally, some reports mention a gradual reduction in food

intake with the adenine supplemented diet, but given the chow component, it remains unclear what this "reduction" accounts for in terms of actual kcal and other nutritional components. It is noteworthy that along with the CKD phenotype induced by adenine supplementation, mice also demonstrate reduced bone parameters to include expansion of BMAT. It is of particular interest whether this phenotype is directly driven by the CKD, by a relative decrease in food intake or both. Thus, monitoring of food intake is warranted. In the same vein, phosphate restriction alone has been shown to exert a similar outcome by decreasing bone formation and increasing BMAT. Therefore, when using these models, it is important to consider variables such as dietary modifications and components to fully scrutinize molecular mechanisms.

Other Nutritional Considerations When Studying Skeletal Metabolism

In addition to the issues highlighted above, other considerations and limitations exist relative to dietary influence on preclinical rodent models while studying skeletal-related outcomes. A topic that has attracted much attention from the scientific community is that of the gut-microbiome and its influence on health and disease, to include bone homeostasis (60, 61). In fact, the gut microbiome has been shown to influence all the of the preclinical models previously discussed (62–66). Because the study of the gut microbiome often requires the use of germ-free or immune-compromised models, several dietary factors should be considered. To start, diet sterilization is required common practices include ɣ-irradiation and high-vacuum autoclaving of the diets, both of which can have profound effects on diet integrity. For example, ɣ-irradiation can result in profound losses of vitamins C, B_1, and A, in addition to destruction of unsaturated fatty acids (67, 68). Furthermore, autoclaving rodent diet has recently been shown to increase (~3x) dietary advanced glycation end-products (AGEs) which impacts the progression of CKD (69). Therefore, the potential loss of nutrients along with potential dietary modifications should be considered when using diets that have been irradiated and/or autoclaved. Another diet issue that should be considered relative to the gut microbiome is the fiber content of diets. Grain-based chow and purified diets can vary greatly in their fiber content. Chow diets typically contain very high levels of soluble and insoluble fiber (~20% of total composition) compared to purified diets, which historically contain ~5% total fiber (14, 63). This is critical as bacteria residing in the gut produce short-chain fatty acids upon fermentation as well as other secondary metabolites which can affect bone (70). It is also worth noting, the coprophagic behavior of both rats and mice, is an important behavioral and nutritional habit used to supply essential nutrients upon a "second digestion" of fecal content. This practice results in substantially higher microbial loads in the large intestine, some 100 times higher than if coprophagia was deterred (71). Therefore, diet handling along with rodent behavior can directly impact the bioavailability of nutrients which may not have been accounted for based on original composition of the diet.

As much of this review has focused on rodent diets, another important nutritional consideration is that of food intake. This issue has been raised in conjunction with several of the models: 1) the OVX model often consumes more food relative to Sham mice; 2) the high-fat diet model of obesity, typically consume less food compared to control, but greater calories per gram; 3) the calorie restricted model relies of restriction based on ad libitum feeding group; and 4) the adenine diet of CKD often consume less due to reduced palatability/preference. However, other preclinical models used to study bone could also impact food intake including dental defects and hormonal status, and as such, care should be taken if nutritional intervention is used in the study design. Additionally, many studies within the field of bone and mineral metabolism use optical imaging of live mice fluorescent reporter mice, as in the case of multiple cancer models and with the Thermo-UCP1 promoter (72, 73). In this case, it has recently been reported that the alfalfa meal from chow diets produces a great amount of autofluorescence in the abdominal region due to the chlorophyll using the far-red and near-infrared filters (74). Therefore, its plausible that this autofluorescence can skew results, especially if time of diet ingestion is different (i.e., treatment alters food intake and/or scans done at differing times of the day).

As a final cautionary note, aside from 'diet', water consumed by rodent models can be a source of experimental confounders. Firstly, water can be considered a source of nutrients, namely minerals, especially in regions where the water is 'hard'. While many animal facilities provide water that is often purified of minerals to some degree using deionization (DI) or reverse osmosis (RO), only RO can remove protozoa, viruses, and bacterium from the water. Therefore, the use of tap water is highly discouraged. Another consideration relative to the source of water is that some transgenic-mouse models use a tetracycline (Tet)/doxycycline (Dox)-inducible Cre recombination system (e.g., osteoprogenitor targeted promoters such as osterix (*Sp7*) and chondrocyte targets under the type II collagen promoter (*Col2A1*) (75, 76). While these inducible model systems provide a valuable tool to time the manipulation of gene expression, the Tet/Dox treatments are often delivered in the water and could impact bone-related study outcomes as they fluorescently label bone surface (77, 78), alter gut microbiome, and have been noted to taste bitter (79–81). This taste-aversion has even been combatted in some studies by the addition of sucrose in the water, however, this modification should be considered.

CONCLUSION

When using some of the preclinical rodent models for osteoporosis research in many ways researchers assume a part of the role of 'nutritionist'. In this capacity, researchers can ask their scientific question while controlling for potential study-confounders introduced *via* dietary sources. This review has aimed to highlight some key dietary considerations of dietary modifications when studying skeletal outcomes, **Figure 2**, however, it is not exhaustive. At a minimum, care should be taken to provide adequate dietary information when reporting results and detailing methodology, especially when the experimental model involves dietary modifications. Arguably, failure to do so is comparable to using a genetic mouse model

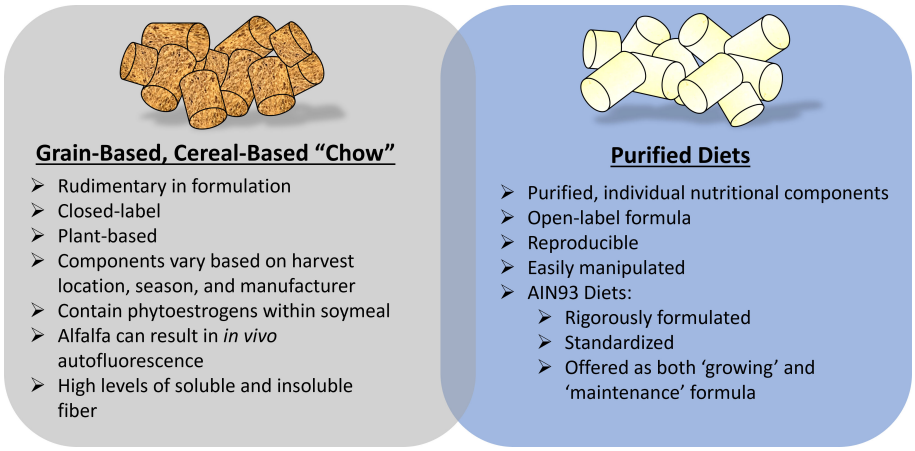

FIGURE 2 | Key dietary considerations when using preclinical rodent models to study osteoporosis-related research. Fundamentally, considerations related to grain-based, chow diets versus a purified diet are important. A hybrid of these diets (chow mixed with purified ingredients) is often discouraged unless critical for study design.

without providing the field of 'osteodietology' has the exciting potential to use dietary modifications to better understand and enhance skeletal health, beyond calcium and vitamin D, key nutritional considerations must be included.

AUTHOR CONTRIBUTIONS

ER-R and BJS contributed to the conception of the review. ER-R wrote the first draft of the review. Both authors contributed to manuscript revision, read, and approved the submitted version.

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REFERENCES

1. Niemeier A, Niedzielska D, Secer R, Schilling A, Merkel M, Enrich C, et al. Uptake of Postprandial Lipoproteins Into Bone *In Vivo*: Impact on Osteoblast Function. *Bone* (2008) 43(2):230–7. doi: 10.1016/j.bone.2008.03.022
2. Tritos NA, Klibanski A. Effects of Growth Hormone on Bone. *Prog Mol Biol Transl Sci* (2016) 138:193–211. doi: 10.1016/bs.pmbts.2015.10.008
3. Kenkre JS, Bassett J. The Bone Remodelling Cycle. *Ann Clin Biochem* (2018) 55(3):308–27. doi: 10.1177/0004563218759371
4. Wright NC, Saag KG, Dawson-Hughes B, Khosla S, Siris ES. The Impact of the New National Bone Health Alliance (NBHA) Diagnostic Criteria on the Prevalence of Osteoporosis in the USA. *Osteoporos Int* (2017) 28(4):1225–32. doi: 10.1007/s00198-016-3865-3
5. Aspray TJ, Hill TR. Osteoporosis and the Ageing Skeleton. *Subcell Biochem* (2019) 91:453–76. doi: 10.1007/978-981-13-3681-2_16
6. Lewiecki EM, Ortendahl JD, Vanderpuye-Orgle J, Grauer A, Arellano J, Lemay J, et al. Healthcare Policy Changes in Osteoporosis Can Improve Outcomes and Reduce Costs in the United States. *JBMR Plus* (2019) 3(9):e10192. doi: 10.1002/jbm4.10192
7. Ensrud KE, Kats AM, Boyd CM, Diem SJ, Schousboe JT, Taylor BC, et al. Association of Disease Definition, Comorbidity Burden, and Prognosis With Hip Fracture Probability Among Late-Life Women. *JAMA Intern Med* (2019) 179(8):1095–103. doi: 10.1001/jamainternmed.2019.0682
8. Ferrari SL, Abrahamsen B, Napoli N, Akesson K, Chandran M, Eastell R, et al. Diagnosis and Management of Bone Fragility in Diabetes: An Emerging Challenge. *Osteoporos Int* (2018) 29(12):2585–96. doi: 10.1007/s00198-018-4650-2
9. Bauer DC. Osteoporosis Treatment After Hip Fracture: Bad News and Getting Worse. *JAMA Netw Open* (2018) 1(3):e180844. doi: 10.1001/jamanetworkopen.2018.0844
10. Yedavally-Yellayi S, Ho AM, Patalinghug EM. Update on Osteoporosis. *Prim Care* (2019) 46(1):175–90. doi: 10.1016/j.pop.2018.10.014
11. Nogues X, Martinez-Laguna D. Update on Osteoporosis Treatment. *Med Clin (Barc)* (2018) 150(12):479–86. doi: 10.1016/j.medcli.2017.10.019
12. Khosla S, Cauley JA, Compston J, Kiel DP, Rosen C, Saag KG, et al. Addressing the Crisis in the Treatment of Osteoporosis: A Path Forward. *J Bone Miner Res* (2017) 32(3):424–30. doi: 10.1002/jbmr.3074
13. Bieri JG. AIN-76 Diet. *J Nutr* (1979) 109(5):925–6. doi: 10.1093/jn/109.5.925
14. Pellizzon MA, Ricci MR. Choice of Laboratory Rodent Diet May Confound Data Interpretation and Reproducibility. *Curr Dev Nutr* (2020) 4(4):nzaa031. doi: 10.1093/cdn/nzaa031
15. Nielsen FH. 90th Anniversary Commentary: The AIN-93 Purified Diets for Laboratory Rodents-The Development of a Landmark Article in The Journal of Nutrition and Its Impact on Health and Disease Research Using Rodent Models. *J Nutr* (2018) 148(10):1667–70. doi: 10.1093/jn/nxy121
16. Reeves PG. AIN-76 Diet: Should We Change the Formulation? *J Nutr* (1989) 119(8):1081–2. doi: 10.1093/jn/119.8.1081
17. Reeves PG, Nielsen FH, Fahey GCJr. AIN-93 Purified Diets for Laboratory Rodents: Final Report of the American Institute of Nutrition *Ad Hoc* Writing

Committee on the Reformulation of the AIN-76A Rodent Diet. *J Nutr* (1993) 123(11):1939–51. doi: 10.1093/jn/123.11.1939
18. Reeves PG. Components of the AIN-93 Diets as Improvements in the AIN-76A Diet. *J Nutr* (1997) 127(5 Suppl):838s–41s. doi: 10.1093/jn/127.5.838S
19. Bijelic R, Milicevic S, Balaban J. Risk Factors for Osteoporosis in Postmenopausal Women. *Med Arch* (2017) 71(1):25–8. doi: 10.5455/medarh.2017.71.25-28
20. Poschner S, Maier-Salamon A, Zehl M, Wackerlig J, Dobusch D, Pachmann B, et al. The Impacts of Genistein and Daidzein on Estrogen Conjugations in Human Breast Cancer Cells: A Targeted Metabolomics Approach. *Front Pharmacol* (2017) 8:699. doi: 10.3389/fphar.2017.00699
21. Paterni I, Granchi C, Katzenellenbogen JA, Minutolo F. Estrogen Receptors Alpha (Erα) and Beta (Erβ): Subtype-Selective Ligands and Clinical Potential. *Steroids* (2014) 90:13–29. doi: 10.1016/j.steroids.2014.06.012
22. Picherit C, Coxam V, Bennetau-Pelissero C, Kati-Coulibaly S, Davicco MJ, Lebecque P, et al. Daidzein is More Efficient Than Genistein in Preventing Ovariectomy-Induced Bone Loss in Rats. *J Nutr* (2000) 130(7):1675–81. doi: 10.1093/jn/130.7.1675
23. Fanti P, Monier-Faugere MC, Geng Z, Schmidt J, Morris PE, Cohen D, et al. The Phytoestrogen Genistein Reduces Bone Loss in Short-Term Ovariectomized Rats. *Osteoporos Int* (1998) 8(3):274–81. doi: 10.1007/s001980050065
24. Brown NM, Setchell KD. Animal Models Impacted by Phytoestrogens in Commercial Chow: Implications for Pathways Influenced by Hormones. *Lab Invest* (2001) 81(5):735–47. doi: 10.1038/labinvest.3780282
25. Larco DO, Cruthirds DF, Weiser MJ, Handa RJ, Wu TJ. The Effect of Chronic Immobilization Stress on Leptin Signaling in the Ovariectomized (OVX) Rat. *Endocrine* (2012) 42(3):717–25. doi: 10.1007/s12020-012-9716-x
26. Witte MM, Resuehr D, Chandler AR, Mehle AK, Overton JM. Female Mice and Rats Exhibit Species-Specific Metabolic and Behavioral Responses to Ovariectomy. *Gen Comp Endocrinol* (2010) 166(3):520–8. doi: 10.1016/j.ygcen.2010.01.006
27. DeMambro VE, Le PT, Guntur AR, Maridas DE, Canalis E, Nagano K, et al. Igfbp2 Deletion in Ovariectomized Mice Enhances Energy Expenditure But Accelerates Bone Loss. *Endocrinology* (2015) 156(11):4129–40. doi: 10.1210/en.2014-1452
28. van Daele PL, Stolk RP, Burger H, Algra D, Grobbee DE, Hofman A, et al. Bone Density in non-Insulin-Dependent Diabetes Mellitus. *Rotterdam Study Ann Intern Med* (1995) 122(6):409–14. doi: 10.7326/0003-4819-122-6-199503150-00002
29. Stolk RP, Van Daele PL, Pols HA, Burger H, Hofman A, Birkenhager JC, et al. Hyperinsulinemia and Bone Mineral Density in an Elderly Population: The Rotterdam Study. *Bone* (1996) 18(6):545–9. doi: 10.1016/8756-3282(96)00079-8
30. Piepkorn B, Kann P, Forst T, Andreas J, Pfutzner A, Beyer J. Bone Mineral Density and Bone Metabolism in Diabetes Mellitus. *Horm Metab Res* (1997) 29(11):584–91. doi: 10.1055/s-2007-979106
31. Schwartz AV, Vittinghoff E, Bauer DC, Hillier TA, Strotmeyer ES, Ensrud KE, et al. Association of BMD and FRAX Score With Risk of Fracture in Older Adults With Type 2 Diabetes. *Jama* (2011) 305(21):2184–92. doi: 10.1001/jama.2011.715
32. Dobnig H, Piswanger-Solkner JC, Roth M, Obermayer-Pietsch B, Tiran A, Strele A, et al. Type 2 Diabetes Mellitus in Nursing Home Patients: Effects on Bone Turnover, Bone Mass, and Fracture Risk. *J Clin Endocrinol Metab* (2006) 91(9):3355–63. doi: 10.1210/jc.2006-0460
33. Nicodemus KK, Folsom AR. Type 1 and Type 2 Diabetes and Incident Hip Fractures in Postmenopausal Women. *Diabetes Care* (2001) 24(7):1192–7. doi: 10.2337/diacare.24.7.1192
34. Schwartz AV, Sellmeyer DE, Ensrud KE, Cauley JA, Tabor HK, Schreiner PJ, et al. Older Women With Diabetes Have an Increased Risk of Fracture: A Prospective Study. *J Clin Endocrinol Metab* (2001) 86(1):32–8. doi: 10.1210/jcem.86.1.7139
35. Farr JN, Drake MT, Amin S, Melton LJ, McCready LK 3rd, Khosla S. *In Vivo* Assessment of Bone Quality in Postmenopausal Women With Type 2 Diabetes. *J Bone Miner Res* (2014) 29(4):787–95. doi: 10.1002/jbmr.2106
36. Krakauer JC, McKenna MJ, Buderer NF, Rao DS, Whitehouse FW, Parfitt AM. Bone Loss and Bone Turnover in Diabetes. *Diabetes* (1995) 44(7):775–82. doi: 10.2337/diab.44.7.775
37. Gerdhem P, Isaksson A, Akesson K, Obrant KJ. Increased Bone Density and Decreased Bone Turnover, But No Evident Alteration of Fracture Susceptibility in Elderly Women With Diabetes Mellitus. *Osteoporos Int* (2005) 16(12):1506–12. doi: 10.1007/s00198-005-1877-5
38. Lecka-Czernik B, Stechschulte LA, Czernik PJ, Dowling AR. High Bone Mass in Adult Mice With Diet-Induced Obesity Results From a Combination of Initial Increase in Bone Mass Followed by Attenuation in Bone Formation; Implications for High Bone Mass and Decreased Bone Quality in Obesity. *Mol Cell Endocrinol* (2015) 410:35–41. doi: 10.1016/j.mce.2015.01.001
39. Rendina-Ruedy E, Graef JL, Davis MR, Hembree KD, Gimble JM, Clarke SL, et al. Strain Differences in the Attenuation of Bone Accrual in a Young Growing Mouse Model of Insulin Resistance. *J Bone Miner Metab* (2016) 34(4):380–94. doi: 10.1007/s00774-015-0685-z
40. Rendina-Ruedy E, Smith BJ. Methodological Considerations When Studying the Skeletal Response to Glucose Intolerance Using the Diet-Induced Obesity Model. *Bonekey Rep* (2016) 5:845. doi: 10.1038/bonekey.2016.71
41. Winzell MS, Ahrén B. The High-Fat Diet-Fed Mouse: A Model for Studying Mechanisms and Treatment of Impaired Glucose Tolerance and Type 2 Diabetes. *Diabetes* (2004) 53 (Suppl 3):S215–9. doi: 10.2337/diabetes.53.suppl_3.s215
42. Sumiyoshi M, Sakanaka M, Kimura Y. Chronic Intake of High-Fat and High-Sucrose Diets Differentially Affects Glucose Intolerance in Mice. *J Nutr* (2006) 136(3):582–7. doi: 10.1093/jn/136.3.582
43. Fazeli PK, Klibanski A. Effects of Anorexia Nervosa on Bone Metabolism. *Endocr Rev* (2018) 39(6):895–910. doi: 10.1210/er.2018-00063
44. Fazeli PK, Klibanski A. The Paradox of Marrow Adipose Tissue in Anorexia Nervosa. *Bone* (2019) 118:47–52. doi: 10.1016/j.bone.2018.02.013
45. Rendina-Ruedy E, Rosen CJ. Bone-Fat Interaction. *Endocrinol Metab Clin North Am* (2017) 46(1):41–50. doi: 10.1016/j.ecl.2016.09.004
46. Cawthorn WP, Scheller EL, Learman BS, Parlee SD, Simon BR, Mori H, et al. Bone Marrow Adipose Tissue is an Endocrine Organ That Contributes to Increased Circulating Adiponectin During Caloric Restriction. *Cell Metab* (2014) 20(2):368–75. doi: 10.1016/j.cmet.2014.06.003
47. Devlin MJ, Cloutier AM, Thomas NA, Panus DA, Lotinun S, Pinz I, et al. Caloric Restriction Leads to High Marrow Adiposity and Low Bone Mass in Growing Mice. *J Bone Miner Res* (2010) 25(9):2078–88. doi: 10.1002/jbmr.82
48. Maridas DE, Rendina-Ruedy E, Helderman RC, DeMambro VE, Brooks D, Guntur AR, et al. Progenitor Recruitment and Adipogenic Lipolysis Contribute to the Anabolic Actions of Parathyroid Hormone on the Skeleton. *FASEB J* (2019) 33(2):2885–98. doi: 10.1096/fj.201800948RR
49. Ko FC, Martins JS, Reddy P, Bragdon B, Hussein AI, Gerstenfeld LC, et al. Acute Phosphate Restriction Impairs Bone Formation and Increases Marrow Adipose Tissue in Growing Mice. *J Bone Miner Res* (2016) 31(12):2204–14. doi: 10.1002/jbmr.2891
50. Schipper L, van Heijningen S, Karapetsas G, van der Beek EM, van Dijk G. Individual Housing of Male C57BL/6J Mice After Weaning Impairs Growth and Predisposes for Obesity. *PloS One* (2020) 15(5):e0225488. doi: 10.1371/journal.pone.0225488
51. McVey Neufeld KA, Kay S, Bienenstock J. Mouse Strain Affects Behavioral and Neuroendocrine Stress Responses Following Administration of Probiotic Lactobacillus Rhamnosus JB-1 or Traditional Antidepressant Fluoxetine. *Front Neurosci* (2018) 12:294. doi: 10.3389/fnins.2018.00294
52. Nickolas TL, Leonard MB, Shane E. Chronic Kidney Disease and Bone Fracture: A Growing Concern. *Kidney Int* (2008) 74(6):721–31. doi: 10.1038/ki.2008.264
53. Yang HC, Zuo Y, Fogo AB. Models of Chronic Kidney Disease. *Drug Discovery Today Dis Models* (2010) 7(1-2):13–9. doi: 10.1016/j.ddmod.2010.08.002
54. Diwan V, Mistry A, Gobe G, Brown L. Adenine-Induced Chronic Kidney and Cardiovascular Damage in Rats. *J Pharmacol Toxicol Methods* (2013) 68(2):197–207. doi: 10.1016/j.vascn.2013.05.006
55. Rahman A, Yamazaki D, Sufiun A, Kitada K, Hitomi H, Nakano D, et al. A Novel Approach to Adenine-Induced Chronic Kidney Disease Associated Anemia in Rodents. *PloS One* (2018) 13(2):e0192531. doi: 10.1371/journal.pone.0192531
56. Jia T, Olauson H, Lindberg K, Amin R, Edvardsson K, Lindholm B, et al. A Novel Model of Adenine-Induced Tubulointerstitial Nephropathy in Mice. *BMC Nephrol* (2013) 14:116. doi: 10.1186/1471-2369-14-116

57. Metzger CE, Swallow EA, Stacy AJ, Tippen SP, Hammond MA, Chen NX, et al. Reversing Cortical Porosity: Cortical Pore Infilling in Preclinical Models of Chronic Kidney Disease. *Bone* (2021) 143:115632. doi: 10.1016/j.bone.2020.115632
58. Claramunt D, Gil-Peña H, Fuente R, García-López E, Loredo V, Hernández-Frías O, et al. Chronic Kidney Disease Induced by Adenine: A Suitable Model of Growth Retardation in Uremia. *Am J Physiol Renal Physiol* (2015) 309(1): F57–62. doi: 10.1152/ajprenal.00051.2015
59. de Frutos S, Luengo A, García-Jérez A, Hatem-Vaquero M, Griera M, O'Valle F, et al. Chronic Kidney Disease Induced by an Adenine Rich Diet Upregulates Integrin Linked Kinase (ILK) and its Depletion Prevents the Disease Progression. *Biochim Biophys Acta Mol Basis Dis* (2019) 1865(6):1284–97. doi: 10.1016/j.bbadis.2019.01.024
60. Tyagi AM, Darby TM, Hsu E, Yu M, Pal S, Dar H, et al. The Gut Microbiota is a Transmissible Determinant of Skeletal Maturation. *Elife* (2021) 10. doi: 10.7554/eLife.64237
61. Zaiss MM, Jones RM, Schett G, Pacifici R. The Gut-Bone Axis: How Bacterial Metabolites Bridge the Distance. *J Clin Invest* (2019) 129(8):3018–28. doi: 10.1172/JCI128521
62. Yu M, Pal S, Paterson CW, Li JY, Tyagi AM, Adams J, et al. Ovariectomy Induces Bone Loss *via* Microbial-Dependent Trafficking of Intestinal TNF+ T Cells and Th17 Cells. *J Clin Invest* (2021) 131(4). doi: 10.1172/JCI143137
63. Pellizzon MA, Ricci MR. The Common Use of Improper Control Diets in Diet-Induced Metabolic Disease Research Confounds Data Interpretation: The Fiber Factor. *Nutr Metab (Lond)* (2018) 15:3. doi: 10.1186/s12986-018-0243-5
64. Dalby MJ, Ross AW, Walker AW, Morgan PJ. Dietary Uncoupling of Gut Microbiota and Energy Harvesting From Obesity and Glucose Tolerance in Mice. *Cell Rep* (2017) 21(6):1521–33. doi: 10.1016/j.celrep.2017.10.056
65. Zheng X, Wang S, Jia W. Calorie Restriction and its Impact on Gut Microbial Composition and Global Metabolism. *Front Med* (2018) 12(6):634–44. doi: 10.1007/s11684-018-0670-8
66. Mo Y, Sun H, Zhang L, Geng W, Wang L, Zou C, et al. Microbiome-Metabolomics Analysis Reveals the Protection Mechanism of α-Ketoacid on Adenine-Induced Chronic Kidney Disease in Rats. *Front Pharmacol* (2021) 12:657827. doi: 10.3389/fphar.2021.657827
67. Ramírez-Cahero HF, Valdivia-López MA. Effect of Gamma Radiation on Sugars and Vitamin C: Radiolytic Pathways. *Food Chem* (2018) 245:1131–40. doi: 10.1016/j.foodchem.2017.11.057
68. Hrubša M, Siatka T, Nejmanová I, Vopršalová M, Kujovská Krčmová L, Matoušová K, et al. Biological Properties of Vitamins of the B-Complex, Part 1: Vitamins B(1), B(2), B(3), and B(5). *Nutrients* (2022) 14(3). doi: 10.3390/nu14030484
69. Biruete A, Srinivasan S, O'Neill KD, Vorland CJ, Hill Gallant KM, Cai W, et al. Adverse Effects of Autoclaved Diets on the Progression of Chronic Kidney Disease and Chronic Kidney Disease-Mineral Bone Disorder in Rats. *Am J Nephrol* (2020) 51(5):381–9. doi: 10.1159/000506729
70. Lucas S, Omata Y, Hofmann J, Böttcher M, Iljazovic A, Sarter K, et al. Short-Chain Fatty Acids Regulate Systemic Bone Mass and Protect From Pathological Bone Loss. *Nat Commun* (2018) 9(1):55. doi: 10.1038/s41467-017-02490-4
71. Bogatyrev SR, Rolando JC, Ismagilov RF. Self-Reinoculation With Fecal Flora Changes Microbiota Density and Composition Leading to an Altered Bile-Acid Profile in the Mouse Small Intestine. *Microbiome* (2020) 8(1):19. doi: 10.1186/s40168-020-0785-4
72. Liu P, Ji Y, Yuen T, Rendina-Ruedy E, DeMambro VE, Dhawan S, et al. Blocking FSH Induces Thermogenic Adipose Tissue and Reduces Body Fat. *Nature* (2017) 546(7656):107–12. doi: 10.1038/nature22342
73. Haltalli MLR, Lo Celso C. Intravital Imaging of Bone Marrow Niches. *Methods Mol Biol* (2021) 2308:203–22. doi: 10.1007/978-1-0716-1425-9_16
74. Bhaumik S, DePuy J, Klimash J. Strategies to Minimize Background Autofluorescence in Live Mice During Noninvasive Fluorescence Optical Imaging. *Lab Anim (NY)* (2007) 36(8):40–3. doi: 10.1038/laban0907-40
75. Rodda SJ, McMahon AP. Distinct Roles for Hedgehog and Canonical Wnt Signaling in Specification, Differentiation and Maintenance of Osteoblast Progenitors. *Development* (2006) 133(16):3231–44. doi: 10.1242/dev.02480
76. Grover J, Roughley PJ. Generation of a Transgenic Mouse in Which Cre Recombinase is Expressed Under Control of the Type II Collagen Promoter and Doxycycline Administration. *Matrix Biol* (2006) 25(3):158–65. doi: 10.1016/j.matbio.2005.11.003
77. van Gaalen SM, Kruyt MC, Geuze RE, de Bruijn JD, Alblas J, Dhert WJ. Use of Fluorochrome Labels in *In Vivo* Bone Tissue Engineering Research. *Tissue Eng Part B Rev* (2010) 16(2):209–17. doi: 10.1089/ten.teb.2009.0503
78. Warner AJ, Hathaway-Schrader JD, Lubker R, Davies C, Novince CM. Tetracyclines and Bone: Unclear Actions With Potentially Lasting Effects. *Bone* (2022) 159:116377. doi: 10.1016/j.bone.2022.116377
79. Redelsperger IM, Taldone T, Riedel ER, Lepherd ML, Lipman NS, Wolf FR. Stability of Doxycycline in Feed and Water and Minimal Effective Doses in Tetracycline-Inducible Systems. *J Am Assoc Lab Anim Sci* (2016) 55(4):467–74.
80. Stavroulaki EM, Suchodolski JS, Pilla R, Fosgate GT, Sung CH, Lidbury JA, et al. Short- and Long-Term Effects of Amoxicillin/Clavulanic Acid or Doxycycline on the Gastrointestinal Microbiome of Growing Cats. *PloS One* (2021) 16(12):e0253031. doi: 10.1371/journal.pone.0253031
81. Hill EM, Howard CD, Bale TL, Jašarević E. Perinatal Exposure to Tetracycline Contributes to Lasting Developmental Effects on Offspring. *Anim Microbiome* (2021) 3(1):37. doi: 10.1186/s42523-021-00099-z

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The effects of vegetarian diets on bone health: A literature review

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In these recent years many people are adopting a vegetarian type diet due to the numerous positive health effects of this regimen such as the reduction of the incidence of many chronic disorders like diabetes, hypertension, obesity and cancer. However this diet is quite restrictive and so it could be possible to have a deficiency in some specific nutrients, increasing the risk of osteoporosis and fractures. Although there are conflicting results on the effects of the vegetarian diet on bone health and fracture incidence, it is always recommendable in vegetarian people to have an adequate intake of calcium and vitamin D, through an increased intake of supplements, natural and fortified foods, an adequate intake of protein, fruit, vegetables, as well as vitamin B12. The aim of this literature review is to revise the actual knowledge of the effect of some nutrients and vegetarian diets on bone health.

KEYWORDS

vegetarian diets, bone, fracture, bone density, nutrients, adults, elderly, review

Introduction

In these recent years many people are adopting a vegetarian type diet due to the numerous positive health effects of this regimen such as the reduction of the incidence of many chronic disorders like diabetes, hypertension, obesity and cancer (1–3). However this diet is quite restrictive and so it could be possible to have a deficiency in some specific nutrients such as calcium and vitamin D, thus leading to bone loss, osteoporosis and an increased risk of fracture (4). Vegetarians subjects exclude from their diet fish, meat and all

their derivatives (5). Generally the classification of the vegetarian diet is based on the type of foods included or excluded. We talk about the lacto-ovo-vegetarian diet when dairy products and eggs are included, while a lacto-vegetarian diet includes only dairy products and finally the vegan diet which excludes all animal derivatives. However, we can find numerous heterogeneities among these diets also related to the personal choice of each individual. Vegetarian people usually have lower BMI, lower blood pressure and reduced serum levels of total and low density lipoprotein cholesterol (6). In this respect, vegetarian foods are healthy and seem to be able to reduce the incidence of obesity, hypertension, diabetes, ischemic heart disease, metabolic syndrome, CVD, and some types of cancers, due to the reduction of BMI values (3, 7–9). Moreover vegetarian diet confers a high fiber intake, which is associated with a decreased incidence of pancreatic cancer, and with a reduction of all cause of mortality, especially CVD mortality (10). Generally most vegetarians have a healthy lifestyle, but various studies indicate that vegetarian diets can also have a negative impact on bone health, partly related to a low BMI, but also to reduced intakes of vitamin B12, calcium and vitamin D (11–14).

It is well known that dietary habits may have implications also on muscle function (2). The different nutrient composition of vegetarian diet compared to an omnivorous diet, may alter physiological responses to physical exercise and influence physical performance. In particular, nutrient composition might alter the responses to physical exercise because the different macro- and micronutrient intake may alter cardiac output, mitochondrial function, substrate availability and oxygen carrying capacity (15). These effects, especially when they occur in elderly people, may impact on the age-related loss of muscle mass and strength that may result in sarcopenia. Sarcopenia is a muscle disorder characterized by low muscle strength and mass which increases the risk for frailty, falls, hospitalization, impaired recovery, and mortality (16). The possible effect of diet on frailty is still controversial but recent studies seem to indicate that adherence to diets characterized by high consumption of plant-derived foods and lower consumption of animal-derived foods could be able to reduce the risk of frailty in community–dwelling older adults (17). Nutrition play an important role for maintaining bone health through the life and to reach an adequate peak of bone mass during growth which may impact on bone strength along with other lifestyle factors and physical activity, reducing bone loss or fracture risk (18).

Nutrients variability in vegetarian diets and their role on bone health

Bone is an active and dynamic tissue that needs sufficient nutrients for the processes of remodeling and mineralization (19).

Dietary intake of some nutrients such as protein, Vitamin D, calcium, alcohol, or caffeine influences the regulation of bone remodeling (20). Vegetarian and vegan regimen diets have a reduced intake of calcium and proteins. Both these nutrients are essential for the maintenance but also for the development of bone mass and density. Therefore osteoporosis may affects both vegetarians and vegans more often than omnivores, which diet includes both vegetal and animal products. Additionally, bone health in vegetarians may be negatively influenced by other nutritional factors. Vegetarians often have lower consumption of zinc, phosphorus, vitamin B12, copper, which all have an effect on bone homeostasis (21). On the other hand, high quality vegetarian diet may include intakes of nutrients which protect bones, such as potassium (which lead to much lower acid load), Vitamin K, magnesium, some antioxidants such as Vitamins C and E and carotenoids, some anti inflammatory phyto-nutrients found in vegetables, fruits, legumes, nuts, tea, and herbs (22). The increased intake of fruits and vegetables leads to an increased amount of magnesium and potassium with positive effects on calcium and bone metabolism (23). Magnesium increases bone strength and influences calcium transport in the intestine (23). Vitamin K has been also associated with a protective affect on fracture risk (24). Nutrients and food in vegetarian diets and nutrients deficiency compared to omnivore are shown in Tables 1, 2. The different effects of nutrients variability in vegetarian and vegan diet on bone health are showed in Figure 1.

Calcium and proteins

Calcium and proteins are generally consumed in form of dairy products and meat. Consequently, lacto-vegetarians do not have risk of calcium deficiency (21), while vegans consume substantially less calcium than other vegetarians and omnivores (1). Generally vegetarians should respect the same dietary indications and the same dietary intake references as omnivores to maintain bone homeostasis. As already mentioned,

TABLE 1 Nutrients and food in vegetarian diet.

Nutrients	Food
Protein	eggs, soy milk, soybean, soy products, tofu
Calcium	milk, cheese and yogurt, cabbage, mustard greens, broccoli, okra, legumes
Magnesium	tomatoes, spinach, legumes, beet, potatoes, raisins
Potassium	bananas, tomatoes, raisins, potatoes, spinach, papaya, oranges
Zinc	whole grains, beans, nuts
Vitamin C	broccoli, papaya, grapefruits, pineapple, oranges, strawberries
Vitamin K	collard greens, spinach, mustard greens
Vitamin B12	shiitake mushroom, yogurt, eggs, milk, cheese, nori

TABLE 2 Nutrient deficiency of different vegetarian diets (excluding fortified foods) compared to omnivores.

	Lacto-ovo-vegetarian	Lacto-vegetarian	Vegan
Calcium	No difference	No difference	Severe deficiency
Protein	No difference	No difference *	No difference *
Vitamin D	Mild deficiency	Mild deficiency	Severe deficiency
Iron	No difference **	No difference **	No difference **
Zinc	No difference	No difference	No difference
Vitamin B12	Mild deficiency	Mild deficiency	Severe deficiency

*different balance animal vs vegetal protein intake.
**despite a similar iron intake, vegetarian diet is associated with increased prevalence of anemia.

dairy products represent an important source of calcium from diet. However also various plant-based foods contain a good amount of absorbable calcium. These include broccoli, bok choy, tofu, kale and calcium-fortified foods such as fruit juices, energy bars and vegetable milks (25). The amount of oxalic acid and phytic acid, which could be found in some plant foods, is able to influence the levels of bioavailable calcium. Examples of foods rich in oxalic acid are rhubarb, spinach and chard; in these cases the absorption of calcium is highly reduced, equal to 5%, while for vegetables with reduced oxalic acid, such as broccoli and bok choy, the calcium absorption is 50% (26). Many studies have indicated that an increased dietary protein intake may increase the excretion of urinary calcium (27). Consequently, vegetarians, whose diet includes a reduced protein intake, should have reduced urinary losses of calcium and therefore need less calcium. Recently, several studies have indicated that the relationship between calcium balance and protein intake is much more complex and generally a diet rich in proteins is associated with positive effects on bone health, with an improvement of calcium absorption, especially in case of diets with a reduced calcium content (28, 29). Furthermore, proteins help to maintain bone structure and homeostasis by improving muscle strength and suppressing parathyroid hormone (PTH) (30). Vegetarians can found proteins in corn, soy, rice and wheat, which contain amounts of sulfates similar to what present in milk, meat and eggs (31). Moreover, the vegetarian diet involves a high intake of fruits and vegetables which are a good sources of potassium, calcium, magnesium, vitamin K and vitamin C; these nutrients are rich in antioxidants which could reduce the oxidative stress and bone resorption (32). The nutrients intake of vegetarians can vary according to food choices. Generally, the protein intake of non vegetarians is about 1% to 18% of the energy intake, whereas the protein intake in lacto-ovo-vegetarians and vegans is approximately 12-14% and 10-12%, respectively. Moreover, the type of diet, of course, influences the sources and the type of protein. A recent study on a population of Seventh Day Adventists showed that animal proteins is about 6.3% in non-vegetarians, 2.4% in lacto-ovo-vegetarians and 0.6% in vegans (33). Two recent studies have also compared nutrient intake between vegetarians and non vegetarians. The first study, named the European Investigation into Cancer and Nutrition (EPIC) 4- Oxford, evaluated the

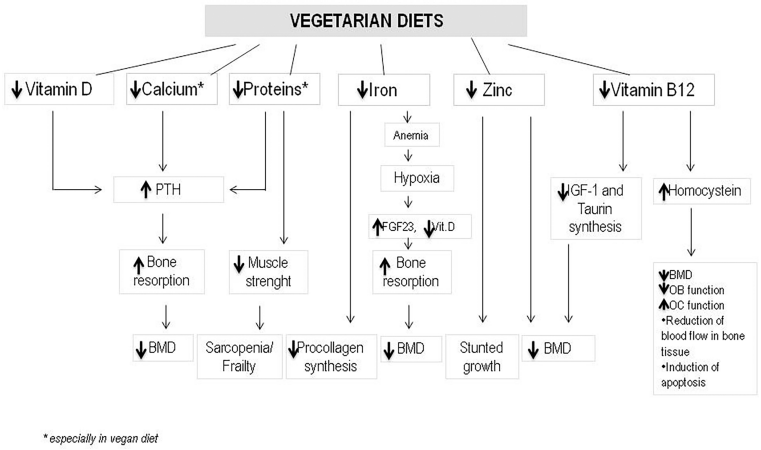

FIGURE 1
The different effects of nutrients variability in vegetarian and vegan diet on bone health. (PTH, Parathyroid hormone; BMD, bone mineral density; FGF23, fibroblast growth factor 23; IGF-1, insulin growth factor-1; OB, osteoblast; OC, osteoclast).

dietary intake of 29,913 meat consumers, 16,095 lacto-ovo-vegetarians and 2,112 vegans in the UK (34). The average percentage of protein energy was 16% in male meat consumers, 13.1% in male vegetarians, 12.9% in male vegans, 17.3% in meat users, 13.8% in vegetarians females and 13.5% in female vegans. Moreover, vegans showed higher intakes of magnesium and vitamin C but lower intakes of vitamins B12, D, calcium and zinc. The second study instead compared the intake of nutrients in different groups of adults belonging to the seventh day Adventist sect in the United States and Canada; 33,634 meat consumers, 21,799 lacto-ovo-vegetarians and 5,694 vegans were considered (33). The average protein intake, calcium, phosphorus, vitamin B12, sodium and zinc did not differ between the two groups. Furthermore, lacto-ovo-vegetarians and vegans had a significant higher intake of fiber; however vegans had a significant reduced intake of vitamin D and magnesium when compared with non vegetarians ($p < 0.05$). A recent systematic review confirms that average protein intake is lower in vegetarians (13.4%) and vegans (12.9%) compared to meat eaters (16.0%), independently from the intake of supplements (14). Also calcium intake tends to be reduced in vegans with respect to vegetarians and meat eaters (14). Importantly a reduced intake of animal proteins could represent a big issue in specific populations, like patients with cancer, in which a balanced combination of animal and plant derived proteins is essential for supporting bone and muscle health and avoiding malnutrition in active cancer and during chemotherapy albeit a plant derived diet could be recommended in cancer prevention (2).

Vitamin D

Vitamin D is able to modulate bone homeostasis by stimulating intestinal calcium absorption, promoting bone mineralization and maintaining muscle mass and strength (35). Vitamin D sources for vegetarians are represented by breakfast cereals, fortified plant-based beverages, fortified orange juice and fortified margarines. However, modest levels of vitamin D can be found also in mushrooms after exposure to ultraviolet light (36). Generally, dairy products are often fortified with vitamin D and they represent a good food source of this nutrient for lacto-ovo-vegetarians and lacto-vegetarians, while vegetable milk fortified with vitamin D, provides a source of this nutrient for vegans. However, these types of fortified foods are not easily available in Europe and elsewhere. For example, in Finland, during winter period, the dietary intake of vitamin D in lacto-ovo-vegetarians and vegans seems to be not sufficient to maintain both 25OH vitamin D and PTH levels in the normal range with possible negative effects on bone mineral density (BMD) (37). Various studies have also analyzed vitamin D status in vegetarians. The EPIC-Oxford study found significantly reduced levels of 25OH-vitamin D in vegetarian subjects compared to those eating meat while vegans had the lowest levels. Serum levels of 25OH-vitamin D equal to 25 nmol/l were found in 8% of vegans and 3% of vegetarians (13). The identification of an adequate dietary source of vitamin D is therefore necessary in vegetarians and vegans to maintain bone health and homeostasis. Although both fortified foods and UV-exposed mushrooms are widely used by vegetarian as plant sources of vitamin D, the amount they provide is not sufficient to guarantee the currently recommended RDAs of 600 IU/day for subjects from 19 to 70 years of age and 800 IU/day for subjects over 70 years of age, thus indicating that vitamin D supplementation in vegetarians is necessary. For low daily doses, both vitamin D2 and Vitamin D3 seems to be equally effective in maintaining circulating levels of serum 25OH-vitamin D (38). However, when given as a single dose, vitamin D3 appears to be more effective than vitamin D2 for increasing vitamin D levels (39). A recent meta-analysis on the effects of vitamin D-fortified foods on serum 25OH-vitamin D levels, markers of bone turnover (BTM) and BMD showed a significant increase in serum 25OH-vitamin D and BMD and a decrease in PTH levels (40). Thus, a vegetarian diet with appropriate food and supplements may provide a sufficient vitamin D intake and maintain a normal BMD (22).

Vitamin B12

Vitamin B12 is essential for DNA synthesis, red blood cell formation, the myelination and function of the central nervous system and homocysteine metabolism (41). Vitamin B12 deficiency is quite common especially among elderly subjects and vegans who do not take supplements due to a reduced dietary intake of foods of animal origin. Generally the vegan dietary intake of vitamin B12 is below the daily recommended intake (DRI), while in lacto-ovo-vegetarians it can be variable according the use of dairy products (41). Vegans must obtain their vitamin B12 either from supplements or regular use of vitamin B12-fortified foods, such as breakfast cereals, vegetarian meat analogs, plant-based beverages. The introduction of unfortified plant foods such as leafy vegetables, algae (spirulina), fermented soy foods, mushrooms, and seaweeds, is not able to guarantee the daily recommended intake (DRI) of vitamin B12 (42). Other non animal sources of this vitamin are represented by fortified products like soy products, cereals and yeast. A deficiency of vitamin B12 may develop slowly in adult individuals. An adequate intake of vitamin B12 is important to prevent a sub-clinical deficiency that may go undetected along time. Generally, vitamin B12 deficiency is indicated by elevated serum levels of methylmalonic acid (MMA), while the serum vitamin B12 level is not a reliable indicator of vitamin B12 status (1). Vegetarians have reduced vitamin B12 levels and increased homocysteine levels compared to non vegetarians.

Recently a European study showed that vitamin B12 deficiency was present in 11% of omnivores, in 77% of lacto-ovo-vegetarians and in 92% of vegans when compared with omnivores (43). Moreover, to confirm this vitamin deficiency 5% of omnivores, 68% of lacto-ovo-vegetarians and 67% of vegans showed elevated serum levels of methylmalonic acid. Importantly, the negative effect of vitamin B12 deficiency on bone homeostasis may be complex. As first vitamin B12 deficiency may impact directly on taurine synthesis and insulin-like growth factor 1(IGF-1) production. However, vitamin B12 may also act through different mechanisms: 1) the reduction of bone mineral density and content 2) the reduction of osteoblasts function along with an increase of osteoclasts activity, 3) the reduction of blood flow in bone, 4) apoptosis induction through molecular pathways mediated by reactive oxygen-species (44). The relation between vitamin B12 deficiency and bone has not been deeply investigated to date. However, a recent observational study found that serum concentrations increase the risk of bone loss in patients with reduced levels of folate and vitamin B12 (45). Moreover a recent review indicates that average vitamin B12 intake is higher in meat eaters compared to vegetarians and vegans independently from supplements and similar results has been observed for what concern Vitamin B status (14).

Iron and zinc

Iron may act as a co-factor for different enzymes involved in immune function processes, such as myeloperoxidase, and play an important role in amino acid metabolism and thyroid hormone synthesis (46). Generally omnivores have better iron status, with elevated concentrations of heme iron which is generally better absorbed. However, vegetarians, especially those with a well balanced dietetic regimen, are not at risk of iron deficiency. A correct iron intake is provided by a diet rich in seeds, wholegrain, legumes, green leafy vegetables, dried fruits, nuts and iron fortified cereal products. Generally, vegetarian diets may contain the same amount of iron than omnivore diets (34). However, anemia related to iron deficiency is more frequent in vegetarians than in omnivores (46). Iron plays an important function in many enzymatic pathways, including those involved in the process of collagen synthesis. Moreover, iron is able to regulate bone metabolism through the modulation of vitamin D functions. Concerning this point, the cytochrome P450 super family, which are monooxygenases containing heme, plays an important role (47). The relationship between iron and bone health derives from clinical studies in patients with iron overload associated with bone loss. The hypothesis of a possible relationship between bone and iron metabolism was described in some studies in which patients with disorders of iron metabolism, such as sickle cell disease, thalassemia, and hereditary hemochromatosis, showed an increased incidence of fractures and osteoporosis (48). In healthy populations, however, the relationship between bone metabolism and iron status is more controversial. Some studies indicated a positive correlation between BMD and serum ferritin in elderly men but not in women (49). In contrast, other studies found a negative association in women older than 45 years of age between BMD and either ferritin saturation or transferrin and no association in male subjects (50, 51). Thus, several mechanisms by which bone metabolism may be affected by iron deficiency, have been supposed. As we already mentioned iron is fundamental in vitamin D metabolism because it is an essential cofactor in the processes of hydroxylation of lysil and prolyl residues of procollagen. Another mechanism that can be involved is hypoxia, which is very frequent in anemic subjects in which oxygen supply to tissues is generally markedly reduced. It has been described that hypoxia is able to induce bone resorption, because it may increase osteoclasts activity which subsequently induces an increase in osteoblasts activation and function (52). Thus, it has been hypothesized that chronic iron deficiency may induce increased bone resorption and increase the risk of osteoporosis (47). Finally recent findings suggest that iron is able to regulate fibroblast growth factor 23 (FGF-23), a bone-derived hormone which plays an important role in phosphate homeostasis (53).

Zinc may act as a coenzyme for several enzymes which are involved in different processes like immunity, growth, bone function, regulation of gene expression and cognitive function (54). Zinc deficiency may cause stunted growth, reduced appetite, alopecia, dermatitis, impaired immunity and endocrine dysfunction (54). Zinc deficiency may be present both in vegetarians and in non-vegetarians (55). Phytates contained in cereals and legumes are able to reduce zinc absorption, while sprouting fermenting, or soaking reduce the levels of phytate making zinc more bioavailable (56). Vegetarian dietary sources of zinc include wholegrain, seeds, nuts, legumes, dairy products, tempeh, and tofu (57). The use of supplements and fortified breakfast cereals and foods may be essential for vegans (6). Some studies in postmenopausal women indicated that Zinc could be a possible marker of bone resorption (58). In fact urinary loss of zinc correlates with decreased bone mass and increased bone resorption (58). In addition, other studies in postmenopausal women showed significant associations between reduced concentrations of zinc, magnesium, iron and copper with reduced BMD (59). *In vivo* studies suggested that zinc may have a positive effect on the process of fracture healing after trauma both in animal models and in patients with fractures (60). Therefore, the importance of zinc supplementation for the maintenance of bone health has emerged. A recent observational study in elderly patients with osteoporosis and zinc deficiency showed that zinc supplementation may increase BMD and prevent fracture occurrence (61).

Effect of vegetarian and vegan diet on bone mineral density (BMD) and fracture risk

Two important indicators of bone health are BMD, as assessed by dual x-ray absorptiometry (DXA) and impaired bone quality, which are responsible for the bone fragility and the risk of fracture. Various studies have examined these factors in vegetarians. It is well known that BMD is a good predictor of osteoporotic fracture risk (62). Various studies of BMD in vegetarians have reported conflicting and inconsistent results; some found no significant difference in terms of BMD, others reported reduced BMD values in vegetarians versus non vegetarians (63). These discrepancies may be attributable to the scarce number of cases examined, the differences between the types of vegetarian subjects studied and the lack of data on some factors such as physical activity, BMI, and nutritional intake. To better clarify these contrasting aspects, Ho-Pham et al. conducted a Bayesian meta-analysis to evaluate the effects of vegetarian diet on BMD (63). Nine different BMD studies in vegetarian subjects were considered, more than half in women. BMD in both the lumbar spine and the femoral neck was reduced by 4% in vegetarians (including both lacto-ovo-vegetarians and vegans) with respect to omnivores. Furthermore, the BMD was reduced by 6% at the femoral neck in vegans compared to non vegetarians with similar results also in the lumbar spine; however these differences were considered not clinically relevant in terms of fracture risk. We already mentioned that protein intake may be very variable in vegetarian and vegan diet according the food choice. Although various data confirm the negative role of protein deficiency on bone metabolism, a meta-analysis has shown that only 1-2% of BMD can be attributable to protein intake which can have both positive and neutral effects on BMD itself (64). Moreover another recent meta-analysis showed no difference between animal protein and soy on bone mineral density (BMD) and some markers of bone turnover (65). However, few studies have specifically examined the role of proteins in bone homeostasis in vegetarians. Recently, a cohort study of 1,865 peri- and postmenopausal women followed longitudinally for 25 years, evaluated the effects of eating meat or a vegetarian diet on wrist fracture risk (66). Vegetarian female subjects with the lowest intakes of vegetable proteins (beans, soy, soy milk, nuts and meat analogs) presented the highest risk of a wrist fracture. Moreover, a 68% reduction in this risk (HR: 0.32; 95% CI: 0.13, 0.79) was observed in vegetarian women who ate plant proteins more than once daily compared to those who ate 3 times per week; similar results were also present in those who consumed large quantities of beans, cheeses and meat analogs. A larger study of more than 17,000 vegetarian men and women showed that those with high intakes of meat analogs have a similar reduction in hip fracture risk to those with low intakes (HR: 0.34; 95% CI: 0.12, 0.95) (67). Recently, a cross-sectional study investigated the associations of veganism with BMD measured with calcaneal quantitative ultrasound (QUS), and also investigated the differences in the concentrations of different nutritional factors and bone related biomarkers between omnivores and vegans. This study showed lower levels of the QUS parameters in vegans compared to omnivores, with reduced levels of zinc, lysine, vitamin A, B2, selenium, protein P, urinary iodine, n-3 fatty acids and calcium levels, providing evidence of impaired bone homeostasis in vegans compared to omnivores, suggesting a relationship between different nutrition-related biomarkers and bone health (68). The EPIC-Oxford study instead examined the risk of fracture in consumers of fish, meat, vegetarians (who also ate eggs and dairy products) and vegans (69). In this study, 34,000 subjects aged between 20 and 89 were examined, followed for an average of 5.2 years and were asked whether there had been any previous fractures or not. The fracture risk was higher in vegans, although this association was partly reduced when the finding was corrected for non dietary factors such as alcohol and smoking. When, however, only subjects with reduced calcium intake were considered, there was no longer any difference between the various groups in terms of fracture incidence, thus suggesting that a correct calcium intake is fundamental for bone health regardless of other dietary habits (68). Recently the prospective EPIC-Oxford cohort study evaluated the fracture risk between vegetarians, non vegetarians and vegans (70). When compared with meat eaters and after adjustment for body mass index (BMI), socio-economic factors and lifestyle confounders, the risks of hip fracture were higher in fish consumers (hazard ratio 1.26; 95% CI 1.02–1.54), in vegetarians (1.25; 1.04–1.50), and in vegans (2.31; 1.66–3.22). Moreover, vegan subjects also showed higher risks of total fractures (1.43; 1.20–1.70), leg fractures (2.05; 1.23–3.41), and other main fractures (1.59; 1.02–2.50) than meat eaters. These risk differences were partly related to lower BMI, and presumably lower intakes of proteins and calcium (70). Other studies on postmenopausal Vietnamese women did not find significant differences in the risk of fracture at the vertebral level, comparing vegans with non vegetarians, while a greater risk of fracture at the wrist level was highlighted in a series of women. Therefore, vegetarians generally show a BMD similar to non vegetarians; likewise fracture risk does not differ if the calcium intake is adequate and the diet provides a correct protein intake. Furthermore, vitamin B12 deficiency, often present in vegetarians and particularly in vegans, has also been associated with reduced BMD and increased risk of fracture. Vitamin B12 deficiency, both mild and moderate, causes an increase in circulating levels of homocysteine, which is able to stimulate osteoclasts, inhibit osteoblasts and alter collagen crosslinks (71).

Conclusions

The effects of a vegetarian diet on bone homeostasis have many implications. Reports and results may vary in different points such as populations size, study design and conclusions. Some studies showed significantly lower BMD in vegetarian subjects, especially vegans, which may explain the increased fracture risk, while other studies did not find any difference in bone health, suggesting that calcium and vitamin D intake is adequate for maintaining healthy bones and preventing fractures (72–74). In conclusion, although there are conflicting data on the effects of the vegetarian diet on bone health and fracture risk, in vegetarians it is always reasonable to follow some nutritional and dietary recommendations such as an adequate intake of calcium and vitamin D (through the intake of natural, fortified foods and supplements), an adequate intake of proteins and an abundant intake of fruit, vegetables, and vitamin B12.

Author contributions

Conceptualization: DM and AF. Methodology: LG and RV. Data curation: DM, RV, GC, RC, CM, LG, IC, and AF. Writing—original draft preparation: DM, AF, and LG. Writing—review and editing: LG, IC. Supervision, DM, RV, AF and LG. All authors have read and agreed to the published version of the manuscript. All authors contributed to the article and approved the submitted version.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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References

1. Craig WJ, Mangels AR, Fresán U, Marsh K, Miles FL, Saunders AV, et al. The safe and effective use of plant-based diets with guidelines for health professionals. *Nutrients* (2021) 13:4144. doi: 10.3390/nu13114144

2. Ford KL, Arends J, Atherton PJ, Engelen MPKJ, Gonçalves TJM, Laviano A, et al. The importance of protein sources to support muscle anabolism in cancer: An expert group opinion. *Clin Nutr* (2022) 41:192–201. doi: 10.1016/j.clnu.2021.11.032

3. Dewell A, Weidner G, Sumner MD, Chi CS, Ornish D. A very-Low-Fat vegan diet increases intake of protective dietary factors and decreases intake of pathogenic dietary factors. *J Am Diet Assoc* (2008) 108:347–56. doi: 10.1016/j.jada.2007.10.044

4. New SA. Do vegetarians have a normal bone mass? *Osteoporosis Int* (2004) 15:679–88. doi: 10.1007/s00198-004-1647-9

5. Craig WJ, Mangels ARAmerican Dietetic Association. Position of the American dietetic association: vegetarian diets. *J Am Diet Assoc* (2009) 109:1266–82. doi: 10.1016/j.jada.2009.05.027

6. Craig WJ. Nutrition concerns and health effects of vegetarian diets. *Nutr Clin Pract* (2010) 25:613–20. doi: 10.1177/0884533610385707

7. Key TJ, Appleby PN, Spencer EA, Travis RC, Allen NE, Thorogood M, et al. Cancer incidence in British vegetarians. *Br J Cancer* (2009) 101:192–7. doi: 10.1038/sj.bjc.6605098

8. Rizzo NS, Sabaté J, Jaceldo–Siegl K, Fraser GE. Vegetarian dietary patterns are associated with a lower risk of metabolic syndrome: The adventist health study 2. *Diabetes Care* (2011) 34:1225–7. doi: 10.2337/dc10-1221

9. Fraser GE. Associations between diet and cancer, ischemic heart disease, and all–cause mortality in non–Hispanic white California seventh–day adventists. *Am J Clin Nutr* (1999) 70:532S–538S. doi: 10.1093/ajcn/70.3.532s

10. Veronese N, Solmi M, Caruso MG, Giannelli G, Osella AR, Evangelou E, et al. Dietary fiber and health outcomes: An umbrella review of systematic reviews and meta–analyses. *Am J Clin Nutr* (2018) 107:436–44. doi: 10.1093/ajcn/nqx082

11. Spencer EA, Appleby PN, Davies GK, Key TJ. Diet and body-mass index in 38000 EPICOxford meat-eaters, fish-eaters, vegetarians, and vegans. *Int J Obes Relat Metab Disord* (2003) 27:728–34. doi: 10.1038/sj.ijo.0802300

12. Pawlak R, Parrott SJ, Raj S, Cullum-Dugan D, Lucus D. How prevalent is vitamin B(12) deficiency among vegetarians? *Nutr Rev* (2013) 71:110–7. doi: 10.1111/nure.12001

13. Crowe FL, Steur M, Allen NE, Appleby PN, Travis RC, Key TJ. Plasma concentrations of 25-hydroxyvitamin d in meat eaters, fish eaters, vegetarians and vegans: results from the EPIC-Oxford study. *Public Health Nutr* (2011) 14:340–6. doi: 10.1017/S1368980010002454

14. Neufingerl N, Eilander A. Nutrient intake and status in adults consuming plant-based diets compared to meat-eaters: A systematic review. *Nutrients* (2021) 14:29. doi: 10.3390/nu14010029

15. Pohl A, Schünemann F, Bersiner K, Gehlert S. The impact of vegan and vegetarian diets on physical performance and molecular signaling in skeletal muscle. *Nutrients* (2021) 13:3884. doi: 10.3390/nu13113884

16. Domić J, Grootswagers P, van Loon LJC, de Groot LCPGM. Perspective: Vegan diets for older adults? a perspective on the potential impact on muscle mass and strength. *Adv Nutr* (2022) 13:712–25. doi: 10.1093/advances/nmac009

17. Maroto-Rodriguez J, Delgado-Velandia M, Ortolá R, Carballo-Casla A, García-Esquinas E, Rodríguez-Artalejo F, et al. Plant-based diets and risk of frailty in community-dwelling older adults: the seniors-ENRICA-1 cohort. *Geroscience* (2022). doi: 10.1007/s11357-022-00614-3

18. Moran JM. Nutrition and women's bone health. *Nutrients* (2022) 14:763. doi: 10.3390/nu14040763

19. Knurick JR, Johnston CS, Wherry SJ, Aguayo I. Comparison of correlates of bone mineral density in individuals adhering to lacto–ovo, vegan, or omnivore diets: A cross–sectional investigation. *Nutrients* (2015) 7:3416–26. doi: 10.3390/nu7053416

20. Reed JA, Anderson JJ, Tylavsky FA, Gallagher PNJr. Comparative changes in radial–bone density of elderly female lacto–ovovegetarians and omnivores. *Am J Clin Nutr* (1994) 59:1197S–202S. doi: 10.1093/ajcn/59.5.1197S

21. Mangels AR. Bone nutrients for vegetarians. *Am J Clin Nutr* (2014) 100:469S–75S. doi: 10.3945/ajcn.113.071423

22. Tucker KL. Vegetarian diets and bone status. *Am J Clin Nutr* (2014) 100:329S–335S. doi: 10.3945/ajcn.113.071621

23. New SA. Intake of fruit and vegetables: Implications for bone health. *Proc Nutr Soc* (2003) 62:889–899. doi: 10.1079/PNS2003310

24. Booth SL, Tucker KL, Chen H, Hannan MT, Gagnon DR, Cupples LA, et al. Dietary vitamin K intakes are associated with hip fracture but not with bone mineral density in elderly men and women. *Am J Clin Nutr* (2000) 71:1201–1208. doi: 10.1093/ajcn/71.5.1201

25. Food and Nutrition Board, Institute of Medicine. *Dietary reference intakes for calcium and vitamin d*. Washington, DC: National Academies Press (2011).

26. Weaver CM, Proulx WR, Heaney R. Choices for achieving adequate dietary calcium with a vegetarian diet. *Am J Clin Nutr* (1999) 70:543S–8S. doi: 10.1093/ajcn/70.3.543s

27. Kerstetter JE, Allen LH. Dietary protein increases urinary calcium. *J Nutr* (1990) 120:134–6. doi: 10.1093/jn/120.1.134

28. Thorpe MP, Evans EM. Dietary protein and bone health: harmonizing conflicting theories. *Nutr Rev* (2011) 69:215–30. doi: 10.1111/j.1753-4887.2011.00379.x

29. Kerstetter JE, Kenny AM, Insogna KL. Dietary protein and skeletal health: a review of recent human research. *Curr Opin Lipidol* (2011) 22:16–20. doi: 10.1097/MOL.0b013e3283419441

30. Genaro PS, Martini LA. Effect of protein intake on bone and muscle mass in the elderly. *Nutr Rev* (2010) 68:616–23. doi: 10.1111/j.1753-4887.2010.00321.x

31. Massey LK. Dietary animal and plant protein and human bone health: a whole foods approach. *J Nutr* (2003) 133:862S–55S. doi: 10.1093/jn/133.3.862S

32. Hamidi M, Boucher BA, Cheung AM, Beyene J, Shah PS. Fruit and vegetable intake and bone health in women aged 45 years and over: a systematic review. *Osteoporos Int* (2011) 22:1681–93. doi: 10.1007/s00198-010-1510-0

33. Rizzo NS, Jaceldo-Siegl K, Sabate J, Fraser GL. Nutrient profiles of vegetarian and nonvegetarian dietary patterns. *J Acad Nutr Diet* (2013) 113:1610–9. doi: 10.1016/j.jand.2013.06.349

34. Davey GK, Spencer EA, Appleby PN, Allen NE, Knox KH, Key TJ. EPIC-Oxford: Lifestyle characteristics and nutrient intakes in a cohort of 33,883 meat-eaters and 31,546 non meat-eaters in the UK. *Public Health Nutr* (2003) 6:259–69. doi: 10.1079/PHN2002430

35. Bouillon R, Marcocci C, Carmeliet G, Bikle D, White JH, Dawson-Hughes B, et al. Skeletal and extraskeletal actions of vitamin d: Current evidence and outstanding questions. *Endocr Rev* (2019) 40:1109–51. doi: 10.1210/er.2018-00126

36. Kamweru PK, Tindibale EL. Vitamin d and vitamin d from ultraviolet-irradiated mushrooms. *Int J Med Mushrooms* (2016) 18:205–14. doi: 10.1615/IntJMedMushrooms.v18.i3.30

37. Outila TA, Kärkkäinen MU, Seppänen RH, Lamberg-Allardt CJ. Dietary intake of vitamin d in premenopausal, healthy vegans was insufficient to maintain concentrations of serum 25-hydroxyvitamin d and intact parathyroid hormone within normal ranges during the winter in Finland. *J Am Diet Assoc* (2000) 100:434–41. doi: 10.1016/S0002-8223(00)00134-6

38. Holick MF, Biancuzzo RM, Chen TC, Klein EK, Young A, Bibuld D, et al. Vitamin D2 is as effective as vitamin D3 in maintaining circulating concentrations of 25-hydroxyvitamin d. *J Clin Endocrinol Metab* (2008) 93:677–81. doi: 10.1210/jc.2007-2308

39. Tripkovic L, Lambert H, Hart K, Smith CP, Bucca G, Penson S, et al. Comparison of vitamin D2 and vitamin D3 supplementation in raising serum 25-hydroxyvitamin d status: A systematic review and meta-analysis. *Am J Clin Nutr* (2012) 95:1357–64. doi: 10.3945/ajcn.111.031070

40. Tangestani H, Djafarian K, Emamat H, Arabzadegan N, Shab-Bidar S. Efficacy of vitamin d fortified foods on bone mineral density and serum bone biomarkers: A systematic review and meta-analysis of interventional studies. *Crit Rev Food Sci Nutr* (2020) 60:1094–103. doi: 10.1080/10408398.2018.1558172

41. Rizzo G, Laganà AS, Rapisarda AM, La Ferrera GM, Buscema M, Rossetti P, et al. Vitamin B12 among vegetarians: Status, assessment and supplementation. *Nutrients* (2016) 8:767. doi: 10.3390/nu8120767

42. Watanabe F, Yabuta Y, Bito T, Teng F. Vitamin B12 containing plant food sources for vegetarians. *Nutrients* (2014) 6:1861–73. doi: 10.3390/nu6051861

43. Herrmann W, Schorr H, Obeid R, Geisel J. Vitamin b-12 status, particularly holotranscobalamin II and methylmalonic acid concentrations, and hyperhomocysteinemia in vegetarians. *Am J Clin Nutr* (2003) 78:131–6. doi: 10.1093/ajcn/78.1.131

44. Pawlak R. Vitamin B12 status is a risk factor for bone fractures among vegans. *Med Hypotheses* (2021) 153:110625. doi: 10.1016/j.mehy.2021.110625

45. Hsieh RL, Huang YL, Chen WJ, Chen HH, Shiue HS, Lin YC, et al. Associations between plasma folate and vitamin B12, blood lead, and bone mineral density among adults and elderly who received a health examination. *Nutrients* (2022) 14:911. doi: 10.3390/nu14040911

46. Craig WJ. Iron status of vegetarians. *Am J Clin Nutr* (1994) 59:1233S–7S. doi: 10.1093/ajcn/59.5.1233S

47. Toxqui L, Vaquero MP. Chronic iron deficiency as an emerging risk factor for osteoporosis: a hypothesis. *Nutrients* (2015) 7:2324–44. doi: 10.3390/nu7042324

48. Weinberg ED. Role of iron in osteoporosis. *Pediatr Endocrinol Rev* (2008) 6:81–5.

49. Lee KS, Jang JS, Lee DR, Kim YH, Nam GE, Han BD, et al. Serum ferritin levels are positively associated with bone mineral density in elderly Korean men: The 2008–2010 Korea national health and nutrition examination surveys. *J Bone Miner Metab* (2014) 32:683–90. doi: 10.1007/s00774-013-0540-z

50. Kim BJ, Ahn H, Bae SJ, Kim EH, Lee SH, Kim HK, et al. Iron overload accelerates bone loss in healthy postmenopausal women and middle-aged men: A 3-year retrospective longitudinal study. *J Bone Miner Res* (2012) 27:2279–90. doi: 10.1002/jbmr.1692

51. Guggenbuhl P, Brissot P, Loreal O. Miscellaneous non-inflammatory musculoskeletal conditions. haemochromatosis: The bone and the joint. *Best Pract Res Clin Rheumatol* (2011) 25:649–64. doi: 10.1016/j.berh.2011.10.014

52. Arnett TR, Gibbons DC, Utting JC, Orriss IR, Hoebertz A, Rosendaal M, et al. Hypoxia is a major stimulator of osteoclast formation and bone resorption. *J Cell Physiol* (2003) 196:2–8. doi: 10.1002/jcp.10321

53. Simic P, Babitt JL. Regulation of FGF23: Beyond bone. *Curr Osteoporos Rep* (2021) 19:563–73. doi: 10.1007/s11914-021-00703-w

54. King JC. Zinc: An essential but elusive nutrient. *Am J Clin Nutr* (2011) 94:679S–84S. doi: 10.3945/ajcn.110.005744

55. Hunt JR. Bioavailability of iron, zinc, and other trace minerals from vegetarian diets. *Am J Clin Nutr* (2003) 78:633S–9S. doi: 10.1093/ajcn/78.3.633S

56. Gibson RS, Perlas L, Hotz C. Improving the bioavailability of nutrients in plant foods at the household level. *Proc Nutr Soc* (2006) 65:160–8. doi: 10.1079/pns2006489

57. Saunders AV, Craig WJ, Baines SK. Zinc and vegetarian diets. *Med J Aust* (2013) 199:S17–21. doi: 10.5694/mja11.11493

58. Relea P, Revilla M, Ripoll E, Arribas I, Villa LF, Rico H. Zinc, biochemical markers of nutrition, and type I osteoporosis. *Age Ageing* (1995) 24:303–7. doi: 10.1093/ageing/24.4.303

59. Zheng J, Mao X, Ling J, He Q, Quan J. Low serum levels of zinc, copper, and iron as risk factors for osteoporosis: A meta-analysis. *Biol Trace Elem Res* (2014) 160:15–23. doi: 10.1007/s12011-014-0031-7

60. Sadighi A, Roshan MM, Moradi A, Ostadrahimi A. The effects of zinc supplementation on serum zinc, alkaline phosphatase activity and fracture healing of bones. *Saudi Med J* (2008) 29:1276–9.

61. Nakano M, Nakamura Y, Miyazaki A, Takahashi J. Zinc pharmacotherapy for elderly osteoporotic patients with zinc deficiency in a clinical setting. *Nutrients* (2021) 13:1814. doi: 10.3390/nu13061814

62. Bagger YZ, TankÓ LB, Alexandersen P, Hansen HB, Qin G, Christiansen C. The long-term predictive value of bone mineral density measurements for fracture risk is independent of the site of measurement and the age at diagnosis: results from the prospective epidemiological risk factors study. *Osteoporos Int* (2006) 17:471–7. doi: 10.1007/s00198-005-0009-6

63. Ho-Pham LT, Nguyen ND, Nguyen TV. Effect of vegetarian diets on bone mineral density: a Bayesian meta-analysis. *Am J Clin Nutr* (2009) 90:943–50. doi: 10.3945/ajcn.2009.27521

64. Darling AL, Millward DJ, Torgerson DJ, Hewitt CE, Lanham-New SA. Dietary protein and bone health: a systematic review and meta-analysis. *Am J Clin Nutr* (2009) 90:1674–92. doi: 10.3945/ajcn.2009.27799

65. Shams-White MM, Chung M, Fu Z, Insogna KL, Karlsen MC, LeBoff MS, et al. Animal versus plant protein and adult bone health: A systematic review and meta-analysis from the national osteoporosis foundation. *PLoS One* (2018) 13:e0192459. doi: 10.1371/journal.pone.0192459

66. Thorpe DL, Knutsen SF, Beeson WL, Rajaram S, Fraser GE. Effects of meat consumption and vegetarian diet on risk of wrist fracture over 25 years in a cohort of peri- and postmenopausal women. *Public Health Nutr* (2008) 11:564–72. doi: 10.1017/S1368980007000808

67. Lousuebsakul-Matthews V, Thorpe DL, Knutsen R, Beeson WL, Fraser GE, Knutsen SF. Legumes and meat analogues consumption are associated with hip fracture risk independently of meat intake among Caucasian men and women: the adventist health study-2. *Public Health Nutr* (2014) 17:2333–43. doi: 10.1017/S1368980013002693

68. Menzel J, Abraham K, Stangl GI, Ueland PM, Obeid R, Schulze MB, et al. Vegan diet and bone health-results from the cross-sectional RBVD study. *Nutrients* (2021) 13:685. doi: 10.3390/nu13020685

69. Appleby P, Roddam A, Allen N, Key T. Comparative fracture risk in vegetarians and non vegetarians in EPIC-Oxford. *Eur J Clin Nutr* (2007) 61:1400–6. doi: 10.1038/sj.ejcn.1602659

70. Tong TYN, Appleby PN, Armstrong MEG, Fensom GK, Knuppel A, Papier K, et al. Vegetarian and vegan diets and risks of total and site-specific fractures: results from the prospective EPIC-Oxford study. *BMC Med* (2020) 18:353. doi: 10.1186/s12916-020-01815-3

71. Ho-Pham LT, Vu BQ, Lai TQ, Nguyen ND, Nguyen TV. Vegetarianism, bone loss, fracture and vitamin d: a longitudinal study in Asian vegans and non-vegans. *Eur J Clin Nutr* (2012) 66:75–82. doi: 10.1038/ejcn.2011.131

72. Veronese N, Reginster JY. The effects of calorie restriction, intermittent fasting and vegetarian diets on bone health. *Aging Clin Exp Res* (2019) 31:753–8. doi: 10.1007/s40520-019-01174-x

73. Hsu E. Plant-based diets and bone health: Sorting through the evidence. *Curr Opin Endocrinol Diabetes Obes* (2020) 27:248–52. doi: 10.1097/MED.0000000000000552

74. Chuang TL, Lin CH, Wang YF. Effects of vegetarian diet on bone mineral density. *Tzu Chi Med J* (2021) 33:128–34. doi: 10.4103/tcmj.tcmj_84_20

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Dietary organosulfur compounds: Emerging players in the regulation of bone homeostasis by plant-derived molecules

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The progressive decline of bone mass and the deterioration of bone microarchitecture are hallmarks of the bone aging. The resulting increase in bone fragility is the leading cause of bone fractures, a major cause of disability. As the frontline pharmacological treatments for osteoporosis suffer from low patients' adherence and occasional side effects, the importance of diet regimens for the prevention of excessive bone fragility has been increasingly recognized. Indeed, certain diet components have been already associated to a reduced fracture risk. Organosulfur compounds are a broad class of molecules containing sulfur. Among them, several molecules of potential therapeutic interest are found in edible plants belonging to the *Allium* and *Brassica* botanical genera. Polysulfides derived from *Alliaceae* and isothiocyanates derived from *Brassicaceae* hold remarkable nutraceutical potential as anti-inflammatory, antioxidants, vasorelaxant and hypolipemic. Some of these effects are linked to the ability to release the gasotrasmitter hydrogen sulfide (H_2S). Recent preclinical studies have investigated the effect of organosulfur compounds in bone wasting and metabolic bone diseases, revealing a strong potential to preserve skeletal health by exerting cytoprotection and stimulating the bone forming activity by osteoblasts and attenuating bone resorption by osteoclasts. This review is intended for revising evidence from preclinical and epidemiological studies on the skeletal effects of organosulfur molecules of dietary origin, with emphasis on the direct regulation of bone cells by plant-derived polysulfides, glucosinolates and isothiocyanates. Moreover, we highlight the potential molecular mechanisms underlying the biological role of these compounds and revise the importance of the so-called 'H_2S-system' on the regulation of bone homeostasis.

KEYWORDS

organosulfur compounds (OSCs), osteoporosis, hydrogen sulfide (H_2S), *Brassicaceae*, *Allium*, glucosinolates, isothiocyanates, polysulfides

Highlights

A literature search was conducted using MEDLINE database. Relevant pre-clinical and clinical studies were selected using a combination of keywords including bone, diet and/or organosulfur compounds, *Allium*, *Brassicaceae*, alliin, allicin, garlic, ajoene, diallyl trisulfide, diallyl disulfide, *S*-allylcysteine, diallyl sulfide, glucosinolate, thiosulfinate, sulforaphane, broccoli, methyl sulfide, isothiocyanates. Additional studies were identified by an extensive manual search of bibliographic references in original papers and reviews. Abstracts and non-English papers were not included. This study selected a total of *in vitro* studies (10 *Alliaceae*, 9 *Brassicaceae*); *in vivo* studies (17 *Alliaceae*, 11 *Brassicaceae*) and population-based studies (4 *Alliaceae*, 1 *Brassicaceae*).

Introduction

Osteoporosis (OP) is a chronic metabolic bone disease characterized by the deterioration of bone microarchitecture and a reduction in bone mass, leading to decreased bone strength and increased risk of bone fracture (1). Approximately 6 % of men and 21 % of women aged 50–84 years are diagnosed with OP and the number of fragility fractures in Europe has increased from 3.1 to nearly 4.3 million in 20 years since year 2000 (2); due to the strong correlation with the ageing of the population, the prevalence of OP is projected to further increase over the next decades (3).

At the bone tissue level, OP is characterized by increased bone porosity which results from the loss of balance between bone formation and bone resorption as aging, disuse, inflammatory diseases, hormonal imbalance or the effect of glucocorticoids impair the ability of osteoblast to keep up with the pace of bone resorption by the osteoclasts (4). Importantly, aging is associated with a decreased number of osteoprogenitor cells, inhibited proliferation, decreased mineralizing capacity, and a shift of osteogenic differentiation toward adipogenesis in senescent mesenchymal stromal cells (MSCs) (5–7).

Pharmacotherapy helps patients to prevent the occurrence or recurrence of fragility fractures and to manage symptoms. However, drugs are mostly used in patients who already show severe bone loss, and the existence of side effects, although very limited in prevalence, often leads to low patient's adherence to anti-OP drugs (8, 9). In this context, non-pharmacological strategies aimed at preventing excessive bone loss hold relevance given that OP remains in most cases a subclinical condition until fracture occurs.

One safe way to prevent bone loss and reduce the risk of bone fracture is to positively impact bone mass through healthy lifestyles and nutrition (10, 11). In particular, the importance of defining specific diet regimens for the prevention of excessive bone fragility has been increasingly recognized (12–15). Adherence to Mediterranean diet lowered hip fracture risk (16) and certain micronutrients contained in fruit and vegetables contributed to delay bone fragility in ageing and to decrease the incidence of bone fractures (17–20). Moreover, a dietary pattern consisting of a high consumption of fruits, vegetables and seafood, has been shown to be directly associated with increased bone mineral density (BMD), independent of dietary calcium intake (21, 22).

Phytochemicals are defined as the chemical bioactive components of nutrient plants that may provide desirable health benefits beyond basic nutrition to reduce the risk of major chronic diseases. They include several classes of compounds: terpenoids, polyphenols, alkaloids, organosulfur compounds (OSCs) and phytosterols (23). Concerning OSCs, much of the research on their health benefits has been in the areas of cardiovascular diseases, cancer and neurological disorders (24–26). However, a growing body of scientific evidence supports the idea that dietary OSCs may play an important role for skeletal health by favoring bone anabolism, inhibiting bone catabolism, and preventing pathological bone loss.

This manuscript intends to provide an up-to-date review of the current evidence from preclinical (both *in vitro* and *in vivo*) and clinical studies on the skeletal effects of OSCs of dietary origin, discussing the chemical nature, the mechanism of action and the potential role of hydrogen sulfide (H_2S) in their biological action. A specific focus is given to the pair glucoraphanin (GRA)-sulforaphane (SFN) as a paradigm of OSCs-H_2S system in bone tissue. Finally, implications and future challenges in the field will be discussed considering the potential translation of OSCs-containing dietary components to clinical studies.

Dietary sources and chemical nature of OSCs

Naturally derived OSCs are a broad class of molecules containing sulfur, predominantly found in edible plants belonging to the *Allium* and *Brassica* (also known as cruciferous vegetables) genera. These plants have been widely used throughout the centuries either as vegetables for culinary purposes as well as in folk and traditional medicine, given their renowned medicinal properties and therapeutic effects. *Allium* genus consists of more than 600 species which are among the oldest cultivated vegetables used as food and still represent one of the main components of the Mediterranean diet (27). *Brassica* genus consists of 37 species; among them, several species are known for their nutritional and therapeutic properties (28, 29). A partial list of edible plants belonging to the *Allium* and *Brassica* genera, and their main content in OSCs, is reported in Table 1.

In *Allium*, over half of the total sulfur content within the mature garlic bulb is found in the form of *S*-alk(en)yl cysteine sulfoxides (ASCOs) (69), non-protein sulfur amino acids which are converted to their respective thiosulfinates or propanethial-*S*-oxide upon tissue damage (70).

The synthesis of ASCOs in *Allium* species starts with the transformation of γ-glutamyl peptides (such as γ-L-glutamyl-*S*-methyl-L-cysteine) into sulfur-containing γ-glutamyl-*S*-alk(en)yl-cysteines such as γ-glutamyl-*S*-methyl-cysteines, γ-glutamyl-*S*-allyl-cysteine, γ-glutamyl-propenyl-L-cysteine sulfoxide (PeCSO). These are further deglutamylated and *S*-oxygenated to yield *S*-alk(en)yl-L-cysteine sulfoxides (71, 72). These reactions are catalyzed by γ-glutamyl transpeptidase, L-glutaminases, and oxidase in the cytoplasm of plant cells. The intact garlic bulbs contain alliin, γ-glutamyl-*S*-allyl-L-cysteine (GSAC), methiin, *S*-*trans*-1-propenyl-L-cysteine sulfoxide, *S*-2-carboxypropylglutathione, *S*-allylcysteine (SAC) (37).

When the bulbs are cut, crushed, chopped or chewed, the enzyme alliinase (a vacuolar lyase) is released from vacuoles and catalyzes the formation of sulfenic acids from L-cysteine sulfoxides: *S*-allyl-L-cysteine sulfoxide (alliin); *S*-methyl-L-cysteine sulfoxide (methiin); *S*-propyl-L-cysteine sulfoxide (propiin); *S*-*trans*-1-propenyl-L-cysteine sulfoxide (isoallin) (71, 72). Sulfenic acids spontaneously react with each other to form unstable compounds called thiosulfinates (69): eg. alliin is converted into allicin (alkenyl alkene thiosulfinate - diallyl thiosulfinate). Allicin immediately decomposes into allyl sulfide (AS), diallyl disulfide (DADS), diallyl trisulfide (DATS), diallyl tetrasulfide, dipropyl disulfide (DPDS), ajoenes, and vinyldithiins (72). The direct catabolism of γ-glutamylcysteine by γ-glutamyltranspeptidase leads to the formation of SAC and *S*-allylmercaptocysteine (SAMC). Allicin can react with glutathione and L-cysteine to produce *S*-allylmercaptoglutathione (SAMG) and SAMC, respectively (69, 72).

Among *Allium*, the most common ASCOs are alliin, methiin, propiin and isoalliin (70, 73, 74). However, they are differentially expressed in specific edible plants. The most abundant in garlic is alliin; in onion isoalliin, methiin, propiin are predominantly detected.

In *Brassica* vegetables two different kinds of OSCs are present: methiin, mainly known from *Allium* vegetables, and glucosinolates (S-β-thioglucoside N-hydroxhysulfates, GLS). Methiin is metabolized to (+)-*S*-alk(en)yl-L-cysteine sulfoxides which can degrade to volatile organosulfur compounds (VOSCs) such as *S*-methyl methane thiosulfinate, which is converted to dimethyl trisulfide and dimethyl disulfide.

GLS are sulfur-based compounds that consist of β-thioglycoside N-hydroxysulfates with various side chains and a sulfur-linked β-D-glycopyranose moiety. A very different profile of GLS may be found in different *Brassica* extracts (75). Natural isothiocyanates (ITCs) are bioactive OSCs derived from the hydrolysis of GLS by the enzyme myrosinase. In plant cells, GLS are physically separated from myrosinases and come in contact only upon tissue damage or crushing. Importantly, myrosinase is not expressed by mammalian cells; however, a small proportion is converted in the mouth by action of plant myrosinase released by chewing (76); moreover, the gut microbiota is entailed with myrosinase activity and constitutes the major site in humans where GLS are hydrolyzes to ITCs (77). While GLS are chemically stable and are characterized by a relatively long half-life, ITCs are highly reactive and short-lived *in vivo* (75, 78).

Effect of OSCs on bone tissue: Preclinical evidence

The effect of OSCs in bone tissue has been investigated in several preclinical models, revealing a strong potential to preserve skeletal health by stimulating the bone forming activity of osteoblasts and inhibiting the bone resorbing activity of osteoclasts, two of the key processes of bone remodeling (79).

Figures 1, 2 provide a graphical summary, respectively, of the main biological processes and molecular targets regulated by OSCs within MSCs/osteoblasts and monocytes/osteoclasts. A detailed description of these mechanisms is provided in the next paragraphs.

Tables 2–5 summarize data from preclinical studies showing an effect of extracts rich in OSCs or individual OSCs molecules derived from *Allium* (Tables 2, 3) and *Brassica* vegetables (Tables 4, 5).

Importantly, while data obtained from studies on purified molecules (labeled with * in the tables) clearly attest to the effectiveness of individual OSCs, the effect of OSCs-rich extracts may result from the combined action of other phytochemicals contained in the extracts. Indeed, *Allium* species contains polyphenols, flavonoids, flavanols, anthocyanins, tannins, ascorbic acid, saponins and fructans (109–111); *Brassica* species contains ascorbic acid, phenolics, carotenoids, terpenes, phytoalexins and alkaloids (29, 112).

Regulation of osteogenesis and bone formation

Osteoblasts, the bone forming cells, regulate bone homeostasis by synthesizing a wide variety of extracellular protein of bone matrix. They differentiate from MSCs through the osteogenic differentiation process which is regulated by an orchestrated activation of several pathways. The master regulator of osteogenic differentiation is runt-related transcription factor 2 (RUNX-2), which is expressed in the early stages of differentiation and is at the intersection of several signaling pathways among which growth hormone-janus Kinase 2 (GH-JAK2), bone morphogenetic protein-SMAD (BMP-SMAD), canonical Wingless/Integrated (Wnt) and Notch signaling (113, 114).

TABLE 1 Most common OSCs found in edible *Allium* and *Brassica* vegetables.

Edible plants	Genus	Main OSCs		REF
Garlic (*Allium sativum L.*)	*Allium*	• γ-glutamyl-*S*-allyl-L-cysteine • allicin • alliin • methiin • *S*-*trans*-1-propenylcysteine sulfoxide • *S*-2-carboxypro-pylglutathione • *S*-allylcysteine • ajoene • vinyldithiins • diallyl sulfide • diallyl disulfide • diallyl trisulfide	• *S*-allylcysteine • *S*-allylmercaptocysteine • *S*-allylmercaptoglutathione • methyl allyl disulfide • methyl allyl trisulfide • *S*-allylmercaptocysteine • dipropyl disulfide • dipropyl trisulfide • 1-propenylpropyl disulfide • dimethyl disulfide • allyl mercaptan • propyl propane thiosulfonate	(30–39)
Onion (*Allium cepa L.*)	*Allium*	• isoalliin • methiin • propiin • diallyl disulfide • diallyl trisulfide • γ-L-glutamyl-trans-*S*-1-propenyl-L-cysteine sulfoxide	• onionin A • cycloalliin • *S*-methyl cysteine sulfoxide • *S*-propenyl cysteine sulfoxide • *S*-alk(en)yl cysteine sulfoxides • dipropyl disulfide • cycloalliin	(40–45)
Welsh onion (*Allium fistulosum* L.)	*Allium*	• γ-glutamyl-*S*-allyl-L-cysteine • allicin	• alliin • diallyl disulfide	(46, 47)
Hooker's Onion (*Allium hookeri*)	*Allium*	• alliin • methiin	• cycloalliin • *S*-propyl-L-cysteine sulfoxide	(48–50)
Long-stamen chive (*Allium macrostemon*)	*Allium*	• alliin • methyl alliin		(51)
Leek (*Allium ampeloprasum* var. *porrum*)	*Allium*	• methiin • isoalliin		(52)
Shallot (*Allium ascalonicum*)	*Allium*	• isoalliin • methiin	• propiin • γ-glutamyl-*S*-alk(en)ylcysteines	(53)
Turnip (*Brassica rapa L.*)	*Brassica*	• glucoraphanin & sulforaphane • gluconapin & 3-butenyl isothiocyanate • glucobrassicanapin & 4-pentenyl isothiocyanate/gluconapoleiferin • gluconasturtiin & 2-phenethyl isothiocyanate • goitrin • berteroin	• progoitrin • glucoalyssin • glucoerucin • glucobrassicin & 4-hydroxyglucobrassicin/ 4-methoxyglucobrassicin • glucoberteroin • neoglucobrassicin	(54, 55)
Broccoli (*Brassica oleracea var. italica L.*)	*Brassica*	• sulforaphane • glucoiberin • 3-hydroxy,4(α-L-rhamnopyranosyloxy) benzyl glucosinolate		(56–58)
Water cress (*Lepidum sativum L.*)	*Brassica*	• glucotropaeolin		(59)
Cabbages (*Brassica oleracea var. capitata L.*)	*Brassica*	• glucoraphanin • progoitrin • sinigrin • gluconapin • glucoerucin	• glucobrassicin & 4-hydroxyglucobrassicin/• 4-methoxyglucobrassicin • neoglucobrassicin • glucoiberin	(60, 61)
Rocket (*Eruca sativa*)	*Brassica*	• glucoraphanin • glucoraphenin • glucosativin • glucoerucin • 4-hydroxyglucobrassicin • glucotropaeolin	• glucolepiidin • glucoiberverin • glucoalyssin • diglucothiobeinin • glucoibarin	(62)
Kohlrabi *(Brassica oleracea* var. *gongylodes)*	*Brassica*	• glucoraphanin & sulforaphane • glucoerucin & methylthiobutyl isothiocyanate • benzyl isothiocyanate • gluconasturtiin & phenylethyl isothiocyanate	• sinigrin & allyl isothiocyanate • glucobrassicin & hydroxyglucobrassicin • neoglucobrassicin • methiin	(63, 64)

(Continued)

TABLE 1 Continued

Edible plants	Genus	Main OSCs		REF
Radish *(Raphanus sativus)*	*Brassica*	• 3-butenyl isothiocyanate • glucobrassicin/4-methoxyglucobrassicin/4- hydroxyglucobrassicin/ indole-3-carbinol	• glucodehydroerucin • glucoraphasatin • glucoraphenin/sulforaphene • sulforaphane	(65)
Tuscan black kale *(Brassica oleracea L.)*	*Brassica*	• glucoerucin • glucobrassicin • glucoraphanin		(66)
Rapes (*Brassica napus L.*)	*Brassica*	• glucoalyssin • glucobrassicin & hydroxyglucobrassicin • neoglucobrassicin	• gluconasturtin • gluconapin • glucobrassicanapin • progoitrin	(61, 67)
Arugula (*Eruca Sativa Mill.*)	*Brassica*	• glucoraphanin & sulforaphane	• glucoerucin & erucin	(68)

Among the genes targeted by RUNX-2 are osteocalcin (OCN), collagen I (Col I), bone sialoprotein (BSP), osteopontin (OPN), alkaline phosphatase (ALP). BSP, OPN and ALP are correlated to matrix mineralization; Coll I and OCN are among the major components of bone matrix. Wnts-β-catenin signal activates osteogenic target genes such as distal-less homeobox 5 (Dlx5) and osterix (Osx) (115) and suppresses the transcription of adipogenic transcription factors such as peroxisome proliferator-activated receptor-γ (PPAR-γ) (116). SMAD family number 1 (SMAD-1) is a critical immediate downstream mediator of BMP receptor transduction (117). Among downstream targets of canonical Wnt and BMP signaling is WNT1-inducible signaling pathway protein 1 (WISP-1), which is involved in the positive regulation of osteogenesis and negative regulation of adipogenesis (118). Interestingly, the expression of H_2S generating enzymes, cystathionine-β-synthase (CBS) and cystathionine-γ-lyase (CSE), was found to be transcriptionally up-regulated during osteogenesis and to correlate with the biosynthesis of mineral matrix (119), thus suggesting a role for endogenous H_2S in osteogenic differentiation. Osteogenic differentiation is associated to increased ALP activity and mineralization *in vitro* and increased BMD *in vivo*. Osteoblast finally differentiate toward osteocytes, multifunctional bone cells that are embedded in mineralized bone matrix. Osteocytes act as orchestrators of bone remodeling, through regulation of both osteoclast and osteoblast activity; as regulators of phosphate metabolism and calcium availability, by functioning as an endocrine cell; as mechanosensory cells (120). Key factors produced by osteocytes are sclerostin (a negative regulator of bone mass), FGF-23 (a regulator of phosphate metabolism), and the key regulator of osteoclast differentiation receptor activator of nuclear factor κβ ligand (RANKL), also produced by osteoblasts and MSCs (120, 121).

Most studies investigating OSCs extracts focused on a commonly used human osteoblastic model, the human osteosarcoma cell line (MG-63 cells). They showed increased cell proliferation and increased osteogenesis/mineralization by *Allium Hookeri* roots treatments (48); increased osteogenesis by *Allium fistulosum* (80) and *Brassica Rapa L.* (Jeong); while no effect on proliferation and differentiation was shown by treatment with water solution of onion crude powder (81). However, MG-63 cells are osteoblasts derived from osteosarcoma, a malignant bone tumors, thus are not fully representative of physiological osteoblasts (122). Increased cells proliferation by *Allium* genus was also shown by ginger and garlic extracts released by 3D-printed calcium phosphate scaffolds on human fetal osteoblast cells (82); increased osteogenesis by *Allium fistulosum* was also shown in the mouse C57BL/6 osteoblastic calvaria cell line (MC3T3-E1) (80). Up to date no studies on primary cultures of human MSCs have been performed with extracts derived from *Alliaceae* or *Brassicaceae*.

Treatment with *Alliaceae* extracts improved bone formation in normal control rats (41, 48, 88) and mitigated the bone loss due to several pathological conditions among which osteoporosis (47, 80, 94). Similarly, extracts from *Brassicaceae* induced bone formation in control rats (54) and prevented bone loss in several models of osteoporosis (59, 99, 106–108). Interestingly, treatment with *Lepidium sativum* resulted in improved fracture healing (28, 123).

Notably, several studies focused on purified OSCs molecules, revealing a specific effect of OSCs on proliferation, osteogenic differentiation, and bone formation. Behera et al. showed increased proliferation, ALP activity and mineralization in murine MSCs derived from femur bone marrow (BMMSCs) upon allyl sulfide stimulation, with a mechanism implicating increased RUNX-2 and OCN expression (83). Thaler et al. demonstrated increased mineralization in mouse MSCs and in an *ex vivo* culture of calvariae explants treated with SFN

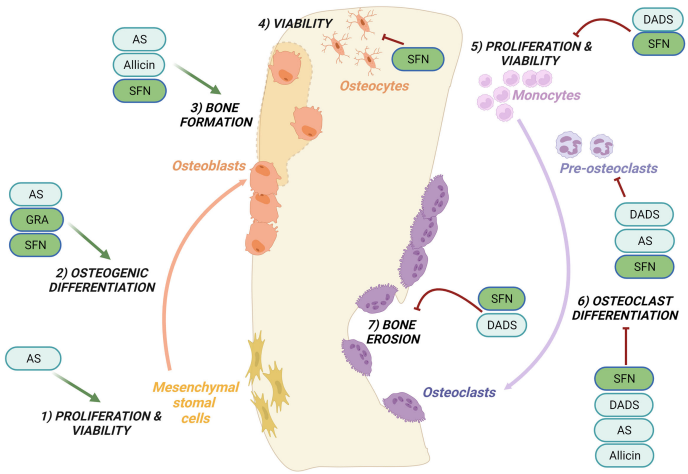

FIGURE 1
Regulation of bone remodeling processes by purified OSCs molecules. Bone remodeling is governed by the balance between bone formation by the osteoblasts (left side) and bone erosion by the osteoclasts (right side). Ancillary processes are shown. OSCs specifically regulate the following processes: promote cells proliferation and viability of mesenchymal stromal cells (1) while inhibit the proliferation and viability of monocytes (5); promote the osteogenic differentiation (2) and bone formation (3); inhibit at different stages osteoclast differentiation (6) and reduce bone erosion (7); inhibit the viability of osteocytes (4). Among the OSCs which modulate bone processes are allicin, allyl sulfide (AS), sulforaphane (SFN), glucoraphanin (GRA), diallyl sulfide (DADS). See the text for details.

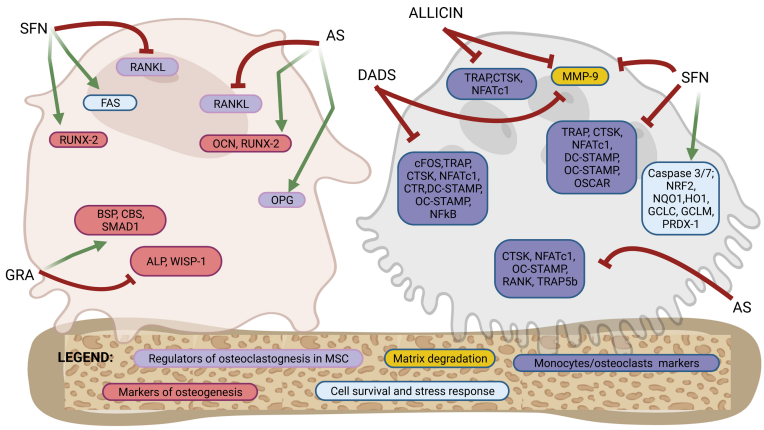

FIGURE 2
Molecular targets of purified OSCs molecules in bone cells. Osteoblastogenesis and osteoclastogenesis are the two key processes of bone remodeling and are regulated by a tightly organized activation of specific molecular targets. This figure shows a schematic representation of a mesenchymal stromal cells/osteoblast and a monocyte/osteoclast to highlight the specific molecular targets regulated by OSCs at different stages of differentiation from precursors to fully differentiated cells. Among the OSCs which drives the modulation of specific molecular targets are allicin, allyl sulfide (AS), sulforaphane (SFN), glucoraphanin (GRA) and diallyl sulfide (DADS). The overall effects are an activation of osteogenic differentiation in mesenchymal stromal cells and both a direct and indirect inhibition of osteoclast differentiation. Follows a list of the molecular targets shown in the figure. Markers of osteoblastogenesis: osteocalcin (OCN), runt-related transcription factor 2 (RUNX-2), alkaline phosphatase (ALP), WNT1-inducible-signaling pathway protein 1 (WISP-1), bone sialoprotein (BSP), cystathionine-β-synthase (CBS), SMAD family member 1 (SMAD-1). Markers of regulators of osteoclastogenesis produced by mesenchymal stromal cells or osteoblasts: receptor activator of nuclear factor-κB ligand (RANKL), osteoprotegerin (OPG). Marker of cells survival and stress response: FAS, caspase 3/7, nuclear factor erythroid-derived 2-related factor 2 (NRF2), NAD(P)H: quinone oxidoreductase 1 (NQO1), heme oxygenase-1 (HO1), glutamate cysteine ligase catalytic subunit (GCLC), glutamate-cysteine ligase modifier subunit (GCLM), peroxiredoxin 1 (PRDX-1). Markers of osteoclasts: nuclear factor of activated T-cells cytoplasmic 1 (NFATc1), cathepsin K (CTSK), receptor activator of NF-KB (RANK), osteoclast stimulatory transmembrane protein (OC-STAMP), dendritic cell specific transmembrane protein (DC-STAMP), osteoclasts-specific activating receptor (OSCAR), tartrate-resistant acid phosphatase (TRAP), calcitonin receptor (CTR), c-fos, tartrate-resistant acid phosphatase 5b (TRAP-5b), matrix metallopeptidase 9 (MMP-9). See the text for details.

TABLE 2 *Alliaceae*-derived OSCs: effects on *in vitro* models of osteoclastogenesis and osteoblastogenesis.

Molecule tested	Experimental *in vitro* model	Concentration	Main effect	Specific outcomes	Authors	Ref
Hot-water extract and ethanol extracts of *Allium hookeri* roots	MG-63 cells line	0.1-0.5-1-5-10-25-50-100 μg/ml	Increased proliferation and osteogenesis	• ↑ viability/proliferation; no cytotoxicity (WST-8 assay) • ↑ ALP activity (pNPP detection) • ↑ collagen (Sirius red assay) • ↑ mineralization (Alizarin Red staining)	Park et al.	(48)
Aqueous and ethanolic extracts of *Allium fistulosum*	MG-63 cell line	1-4-8-10-16-32-50-63-125 μg/ml	Increased osteogenesis	• no cytotoxicity (MTT assay) • ↑ ALP activity (ALP assay kit)	Ryuk et al.	(80)
Water solution of onion crude powder	MG-63 cell line	300 μg/ml	No effect on proliferation or differentiation	• ALP activity similar to control cells (ALP assay kit) • Col I on cell lysate was similar to control cells (4-hydroxyproline quantification) • OCN, OPN in cells surnatants similar to control cells (ELISA)	Tang et al.	(81)
Aqueous and ethanolic extracts of *Allium fistulosum*	MC3T3-E1 cell line	1-4-8-10-16-32-50-63-125 μg/ml	Increased proliferation and osteogenesis	Ethanolic extracts: • ↑ viability/proliferation; no cytotoxicity (MTT assay) • ↑ALP activity (ALP assay kit) Water extracts: • no cytotoxicity (MTT assay) • ↑ALP activity (ALP assay kit)	Ryuk et al.	(80)
Water *Allium sativum L.* extract	Human fetal osteoblast cells	3D-printed calcium phosphate scaffolds releasing ginger and garlic extract	Increased osteoblast proliferation	• ↑ proliferation (MTT assay)	Bose et al.	(82)
Allyl sulfide (AS) *	BMMSCs isolated from Age-associated OP mice's femurs	Mice were fed by oral gavage with AS (200 mg/kg) for 3-months	• Rescue of proliferation and osteogenesis • Indirect inhibition of osteoclastogenesis	• ↑ proliferation as compared to aged mice (MTT assay) • ↑ALP activity (ALP staining), ↑ mineralization (Alizarin red staining), • ↑ RUNX-2 and OCN in cells (western blot) • ↑ OPG and ↓ RANKL in surnatants (ELISA)	Behera et al.	(83)
***Allium cepa* L. extracts**	*In vitro* bioactivity assay (simulated body fluid)	Chitosan + *Allium cepa L.* (ChAC) and Chitosan + *Allium cepa L.* + PLGA (ChPAC)	Improved natural bioactivity of chitosan	• Increased apatite cristals in the surface • Improved Phosphorous/Calcium ratio	Monárrez-Cordero et al.	(84)
Water *Allium sativum L.* extract	Human osteoclast cells from THP1 monocytes	3D-printed calcium phosphate scaffolds releasing ginger and garlic extract	Inhibition of osteoclast activity	• ↓ resorption (pit assay)	Bose et al.	(82)
Ethanolic extract of onion	RAW 264.7 cell line	0.1-0.2-0.4 mg/ml	Inhibition of osteoclastogenesis	• no cytotoxicity (MTT assay)	Law et al.	(85)

(Continued)

TABLE 2 Continued

Molecule tested	Experimental *in vitro* model	Concentration	Main effect	Specific outcomes	Authors	Ref
				• ↓ osteoclasts (TRAP assay)		
Freeze dried onion juice	RAW 264.7 cell line	0.1-0.2-0.4 mg/ml	Inhibition of osteoclastogenesis	• no cytotoxicity (MTT assay) • ↓ osteoclasts (TRAP assay)	Law et al.	(85)
Water solution of onion crude powder	RAW 264.7 cell line	15-50-150-300 µg/ml	Inhibition of osteoclastogenesis	• no cytotoxicity (MTT assay) • ↓ osteoclasts (TRAP assay) • ↓ CD51/61 (vitronectin receptor), MMP-9 and TRAP mRNA (RT-PCR) • ↓ ERK, p38 and NF-κB (western blot)	Tang et al.	(81)
Diallyl disulfide (DADS) *	RAW 264.7 cell line	1-10-100-1000 µg/ml 20-40-60-80-100 µg/ml	Inhibition of osteoclastogenesis and bone resorption	• ↓ cytotoxicity at concentration higher to 100 µg/ml (CCK-8 assay) • ↓ osteoclast and resorption (TRAP assay PIT assay) • ↓ c-fos, NFATc1, TRAP, MMP9, CTR, CTSK, DC-STAMP, OC-STAMP mRNA • ↓ osteoclast fusion (FAK staining) • ↓ NF-κB, p-STAT3, NFATc1, c-FOS (western blot)	Yang et al.	(86)
Alliin *	RAW 264.7 cell line	0.1-0.5-1-5-10-100 µg/ml	Inhibition of osteoclastogenesis	• No cytotoxicity (CCK-8 assay) • ↓ osteoclasts and resorption (TRAP assay and pit assay) • ↓ c-fos, NFATc1, MMP9, DC-STAMP, OC-STAMP, RANK, TRAP (RT-PCR) • ↓ Nox-1, NFATc1, c-fos (western blot) • ↓ ROS (detection by fluorescent probe)	Chen et al.	(87)
Water solution of onion crude powder	Osteoclast derived from bone marrow cells of femurs of 6-8-week-old Sprague–Dawley rats	15-50-150-300 µg/ml	Inhibition of osteoclastogenesis	• no cytotoxicity (MTT assay) • ↓ osteoclasts (TRAP assay)	Tang et al.	(81)
Water solution of onion crude powder	Osteoclast derived from long bones of 6-day-old rabbits	15-50-150-300 µg/ml	Inhibition of bone resorption	• ↓ resorption (pit assay)	Tang et al.	(81)
Commercial onion powder (Chia Hui, Taipei, Taiwan)	Osteoclast derived from bone marrow cells of femurs of 6-8-week-old Sprague–Dawley rats	300 µg/ml	Inhibition of osteoclastogenesis	• ↓ osteoclasts (TRAP assay) • Inhibition of ERK, p38, and NF-κB activation (western blot)	Tang et al.	(81)
GPCS isolated by bioassay-guided fractionation of	Osteoclasts derived from femora and tibiae of 2-days-old Wistar Hanlbm rats	1-10-30 mg/ml 2-4-8 mM	Inhibition of osteoclast differentiation and activity	• ↓ osteoclast differentiation and resorption by GPCS	Wetli et al.	(41)

(Continued)

TABLE 2 Continued

Molecule tested	Experimental *in vitro* model	Concentration	Main effect	Specific outcomes	Authors	Ref
***Allium cepa L.* Bulbs** *				(TRAP staining and pit assays)		
Diallyl disulfide (DADS) *	BMMs obtained from the femur and tibia bone marrow of 6-wk-old C57BL/6 mice	20-40-60-80-100 µg/ml	Inhibition of osteoclastogenesis	• ↓ cytotoxicity at concentration higher to 100 µg/ml (CCK-8 assay) • ↓ osteoclast (TRAP assay)	Yang et al.	(86)
Allyl sulfide (AS)*	BM cells	Cultured under 15% conditioned medium derived from BMMSCs culture of Age-associated OP mouse model (Fed by oral gavage with AS (200 mg/kg) for 3-months)	Inhibition of osteoclastogenesis *via* a paracrine mechanism	• ↓ osteoclasts (TRAP staining) • ↓ TRAP-5b expression in cells lysates (ELISA) • ↓ NFATc1, CTSK, RANK and OC-STAMP mRNA (RT-PCR)	Behera et al.	(83)

Most *in vitro* studies were conducted by using water or ethanol extracts from *Allium* edible plants (4 studies, 13 *in vitro* models; *Allium* hookeri roots, *Allium fistulosum*, *Allium sativum L.*, *Allium cepa L.*); a few used purified OSCs (3 studies, 6 *in vitro* models; diallyl disulfide (DADS), allyl sulfide (AS), γ-glutamyl-*trans*-S-1-propenyl-L-cysteine sulfoxide – GPCS, alliin). Most studies showed an increased osteoblast proliferation and osteogenesis and an inhibited osteoclastogenesis. Notably, only the effects of purified OSCs (labeled with * in the table) can be attributable entirely to OSCs. The concentrations tested ranged from 0.1 to 300 µg/ml. Murine *in vitro* models of osteoclastogenesis: osteoclasts derived from bone marrow of femora and tibiae of rats, rabbits, mice; RAW 264.7 cells. Human *in vitro* models of osteoclastogenesis: osteoclast cells from human THP1 monocytes. Murine *in vitro* models of osteoblastogenesis used: MC3T3-E1 (mouse C57BL/6 calvaria cells line); murine bone marrow (BM) cells; bone marrow-derived mesenchymal stem cells (BMMSCs) isolated from age-associated (AG) osteoporosis (OP) mice's femurs. Murine in vitro models for studying indirect inhibition of osteoclastogenesis: bone marrow-derived mesenchymal stem cells (BMMSCs), bone marrow macrophages (BMM) and murine bone marrow (BM). Human *in vitro* models of osteoblastogenesis: MG-63 cells line (human osteosarcoma cells line), human fetal osteoblast. Functional assays for osteoclastogenesis used: tartrate-resistant acid phosphatase positive (TRAP staining); pit assay. Functional assays for osteoblastogenesis: alizarin red staining (marker of mineralization), sirius red assay (marker of collagen I), *p*-nitrophenyl phosphate (pNPP) measurement. Proliferation/viability assays: 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, cell counting kit-8 (CCK-8) cell viability assay, water-soluble tetrazolium-8 (WST-8) assay. Markers of osteoclasts: nuclear factor of activated T-cells cytoplasmic 1 (NFATc1), cathepsin K (CTSK), receptor activator of NF-KB (RANK), osteoclast stimulatory transmembrane protein (OC-STAMP), tartrate-resistant acid phosphatase (TRAP), tartrate-resistant acid phosphatase 5b (TRAP-5b), receptor activator of nuclear factor-κB ligand (RANKL), dendritic cell specific transmembrane protein (DC-STAMP), reactive oxygen species (ROS), calcitonin receptor (CTR), p-signal transducer and activator of transcription 3 (p-STAT3), NADPH Oxidase 1 (Nox-1), c-fos, nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), p38, extracellular signal-regulated kinase (ERK), matrix metallopeptidase 9 (MMP-9), CD51/61 (vitronectin receptor). Markers of osteoblastogenesis: collagen I (Col I), osteocalcin (OCN), osteopontin (OPN), runt-related transcription factor 2 (RUNX-2), osteoprotegerin (OPG), alkaline phosphatase (ALP). ↑ means up-regulation; ↓ means down-regulation.

TABLE 3 *Alliaceae*-derived OSCs: effects on *in vivo* models of bone loss.

Molecule tested	Experimental *in vivo* model description	Mode of administration, dose and duration	Main effect	Specific outcomes	Authors	Ref
Ethanol extracts of *Allium macrostemon* bulbs	Female, 25-day-old, Sprague–Dawley rats (adolescent mice)	Gavage, 100 and 300 mg/kg, twice daily for 10 days	Increase tibial longitudinal bone growth	• Increase tibial longitudinal bone growth (fluorescence photomicrograph after tetracycline hydrochloride) • ↑ IGF-1 and BMP-2 in the proliferative and hypertrophic zones of growth plate (immunohistochemistry)	Kim et al.	(85)
Hot-water extracts of *Allium hookeri* roots	Female, 3-week-old, Sprague-Dawley rats	Oral treatment, 500 mg/kg, single daily dose, for 6 weeks	Improved bone formation	• ↑ serum levels of OCN (ELISA) • ↑ BMD, BV, BV/TV, Tb.Th, Tb.N; • ↓ Tb.Sp, BS/BV (microCT in proximal tibia)	Park et al.	(48)
Wheat bread added with *Allium sativum L.*	Male weaning Wistar rats	Oral administration, 3 g per 100 g wheat flour, for 60 days	Increase in BMD	• ↑ total skeleton BMC and BMD, femur BMD, tibia BMD • Spine (S-BMD) and proximal tibia (T-BMD) was not affected (DEXA) • ↑ femur calcium	Weisstaub et al.	(88)

(Continued)

TABLE 3 Continued

Molecule tested	Experimental *in vivo* model description	Mode of administration, dose and duration	Main effect	Specific outcomes	Authors	Ref
Ethanolic extracts of *Allium cepa L.* bulbs	Male, 9-week-old, Wistar Hanlbm rats	Orally given, one gram, daily treatment, for 10 days	Inhibition of bone resorption	↓ bone resorption (urinary excretion of tritium)	Wetli et al.	(41)
Homogenized of *Allium sativum L.*	Hypercholesterolemic rat model (Pregnant albinorat Wistar fed with hypercholesterolemic diet, and their offspring)	Intragastrical injection, 100 mg/kg, a week prior to onset of feeding with hypercholesterolemic diet	Improved endochondral ossification	↑ ossification in mandibular, humerus, radio-ulna, femur, tibio-fibula, scapula and ilium (Alizarin red S for ossified skeletal bones in fixed offspring)	El-Sayyad et al.	(89)
Water *Allium sativum L.* extract	*In vivo* implants in bicortical rat distal femur defects (Sprague–Dawley rats)	3D-printed calcium phosphate scaffolds designed with a bimodal pore distribution releasing ginger and garlic extract, implanted for 4-10 weeks	Increase in osteoinductivity	• ↑ osteoid tissue formation, mineralization (masson-goldner trichrome assay) • ↑ bone area, osteocytes (haematoxylin and eosin) • ↑ Col I (Col I staining)	Bose et al.	(82)
Aqueous and ethanolic extracts of *Allium fistulosum*	CDD mice - Mice model of bone loss due to nutritional deficiency (Male, 4-week-old, C57BL/6 mice, fed with a calcium- and vitamin D-deficient diet for 5 weeks)	Oral treatment, 150 and 450 mg/kg, ad libitum feeding for 4 weeks	Prevention nutritional deficiency-induced bone loss and retarded bone growth	• ↑ serum calcium, OC and Col I *vs* CDD mice (ELISA) • ↑ serum ALP, OCN and Col I *vs* normal control mice (ELISA) • ↑ femoral and tibial BMC and BMD *vs* CDD mice and similar to normal control (DEXA) • Thicker growth plates *vs* CDD mice and similar to normal control (measured after hematoxylin and eosin stain)	Ryuk et al.	(80)
Water extract of *Allium fistulosum* root	Rat model of OP and osteoarthritis (Female, 8-week-old, Sprague–Dawley rats, ovariectomy and MIA-induced OA)	Within rice porridge, 250 and 750 mg/kg, food supply was replaced every two days for 8 weeks	Prevention of bone loss	• ↑ BMD in lumbar bone spine, OA leg and control leg (DEXA) • ↓ serum ALP activity (ELISA)	Yang et al.	(47)
Oil extract of *Allium sativum L.* from raw cloves	Rat model of OP (Female albinorats, ovariectomy)	Gavage, 100 mg/kg body wt/day, single evening dose for 30 days	Prevention of bone loss	• ↓ serum ALP activity (pNPP measurements) and TRAP activity (commercial kit) • ↑ BMD of femur, thoracic rib, thoracic vertebra and lumbar vertebra (measured by Archimedes' principle)	Mukherjee et al.	(90) (91)
				↑ calcium and phosphate content in femur, lumbar vertebra, thoracic vertebra, thoracic rib (method of Adeniyi et al. (1993) and Lowry and Lopez (1946))	Mukherjee et al.	(91) (92)
				• ↑ tensile strength of the femur (method of Shapiro and Heaney (2003) • ↑ serum estradiol levels (ELISA) • serum PTH levels is not affected (ELISA)	Mukherjee et al.	(92)
Oil extract of *Allium sativum L.*	Rat model of OP (Female Wistar, ovariectomy)	Gavage, 100 mg/kg body wt/day, single evening dose for 30 days	Increase in bone strength and inhibition of bone resorption	• ↑ tensile strength of the femurs (method of Shapiro and Heaney (2003)	Mukherjee et al.	(93)

(Continued)

TABLE 3 Continued

Molecule tested	Experimental *in vivo* model description	Mode of administration, dose and duration	Main effect	Specific outcomes	Authors	Ref
from raw cloves				• ↓ serum TRAP activity (commercial kit)		
***Allium cepa L.* powder**	Rat model of OP (Female, 14-week-old, Wistar rats) treated or not with 1 mg/kg/day alendronate	Dietary administration, diet containing 3%, 7% and 14% (wt/wt) *Allium cepa L.* powder, for 6 weeks	Prevention of Ovx-induced bone loss and deterioration of biomechanical properties (efficacy was slightly inferior to that of alendronate)	• ↓ serum calcium (measured with an automatic chemistry analyzer) • ↑ serum OCN (ELISA) • ↑ BV/TV, Tb.N, ↓ Tb.Sp (histomorphometry on histological specimen) • ↓ osteoclasts (TRAP staining on histological specimen) • ↑ loading force to maximal load and tissue fracture, ↑ stiffness (three-point bending test)	Huang et al.	(94)
Diallyl disulfide (DADS) *	A mouse calvarial osteolysis model (Female, 6-wk-old, C57BL/6 mice, LPS treatment 5 mg/kg)	Subcutaneous injections, 20-40 mg/kg DADS, every alternate day for 14 days	Inhibition of LPS-induced osteolysis	• ↓ bone erosion as compared to LPS, ↑ BV/TV, ↓ porosity (microCT) • ↓ osteoclasts (histologic and histomorphometric analysis TRAP staining)	Yang et al.	(86)
Allyl sulfide (AS) *	Age-associated OP mouse model (Female, 20-months-old (aged), C57BL/6 J mice)	Oral gavage, 200 mg/kg, 3-months	Restored osteogenesis and bone density	• ↑ plasma levels of P1NP and CTX-I • ↑ bone density in the femur's metaphyseal area (X-ray *in vivo* imaging)	Behera et al.	(83)
Allicin *	Mice model of lead-induced bone loss (Male, 3-weeks-old, C57BL/6 J mice, 0.2% lead acetate in drinking water ad libitum for 12 weeks)	Intraperitoneally injection, 10 mg/kg, in the last 4 weeks	Prevention lead-induced bone loss	• ↑ BMD, BVF, Tb.N, Tb.Th, ↓ Tb.Sp (microCT) • ↑ CAT, SOD, reduced GSH; ↑ MDA on femur homogenates (commercial kits) • ↓ TRAP, CTSK, NFATc1, MMP-9 mRNA in femur (RT-PCR) • ↑ SIRT1 and ↓ of acetylated FOXO1 on femur homogenates (western blot)	Li et al.	(95)
Allicin *	Mice model of aging rats (Male, 13 months-old, F344 rats)	Intragastric administration, 4-8-16 mg/kg, once daily for 8 months	Reverse aging-associated bone loss and frailty	• ↑ femoral, spinal, tibial BMD (DEXA) • ↑ elastic load and maximum load in femur - ↑ bone strength (Three-Point Bending Test) • ↑ serum P1NP, ↑ serum CTX-I (ELISA)	Liu et al.	(96)

Most *in vivo* studies were conducted by using water or ethanol extracts of *Allium* edible plants (11 studies; *Allium macrostemon*, *Allium hookeri*, *Allium fistulosum*, *Allium sativum L.*, *Allium cepa L.*). A few studies used *Allium*-derived OSCs (4 studies; diallyl sulfide, allyl sulfide, allicin). Most studies were performed in normal control mice showing improved bone formation and inhibited bone resorption; and in osteoporosis mice showing prevention of bone loss. Notably, only the effects of purified OSCs (labeled with * in the table) can be attributable entirely to OSCs. Markers of bone formation in serum: procollagen 1 intact N-terminal propeptide (P1NP); osteocalcin (OCN); collagen I (Col I), alkaline phosphatase (ALP), parathormone (PTH). Markers of bone resorption in serum: serum type I collagen breakdown product (CTX-I). Markers of bone resorption in urine: urinary excretion of tritium. Bone microstructural parameters analyzed by microCT analysis: BMD (bone mineral density), bone volume fraction (BVF), spine BMD (s-SMD), tibia BMD (t-BMD), BMC (bone mineral content), bone volume (BV), bone volume/total volume (BV/TV), bone surface/bone volume (BS/BV), trabecular thickness (Tb.Th), trabecular number (Tb.N), trabecular space (Tb.Sp), bone volume fraction (BVF). Bone mineral density analyzed by dual-energy X-ray absorptiometry (DEXA). Markers of bone formation in histological specimen: ALP, Col I. Osteoid tissue detection by masson-goldner trichrome assay. Markers of osteoclasts/bone resorption in histological specimen: tartrate-resistant acid phosphatase (TRAP), nuclear factor of activated T-cells cytoplasmic 1 (NFATc1), cathepsin K (CTSK). Markers of redox stress response: catalase (CAT), superoxide dismutase (SOD), reduced glutathione (GSH), malondialdehyde (MDA). Measurements of bone strength: method of Shapiro and Heaney (2003); three-Point Bending Test. Other abbreviations: insulin-like growth factor 1 (IGF-1), bone morphogenetic protein 2 (BMP-2), lipopolysaccharide (LPS), sirtuin (SIRT); forkhead box O (FOXO). ↑ means up-regulation; ↓ means down-regulation.

TABLE 4 *Brassicaceae*-derived OSCs: effects on *in vitro* models of osteoclastogenesis and osteoblastogenesis.

Molecule (organosulfur compouds)	Experimental *in vitro* model	Concentration	Main effect	Specific outcomes	Authors	Ref
Sulforaphane *	MLO-Y4, an osteocyte – cell line	3-10-15-30-100 µM	Inhibits cells proliferation; induces apoptosis; and inhibits osteoclastogenesis	• ↓ viability and metabolic activity (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide-like assay (EZ4U) • ↑ in the activities of Caspase 3/7 and 8 (assay kit) • ↑ Fas mRNA expression (RT-PCR) • ↓ RANKL mRNA expression (RT-PCR)	Thaler et al.	(97)
Glucoraphanin *	*In vitro* culture of human mesenchymal stromal cells from tibial plateau	3.3-10-33-100 µM	Induction of osteogenesis	• ↑ mineralization (alizarin red staining) • ↑ BSP, CBS, SMAD-1 mRNA (RT-PCR) • ↓ ALP, WISP-1 mRNA (RT-PCR)	Gambari et al.	(98)
***Brassica rapa* L. root ethanol extract**	MG-63 cells line	1-5-10-25-50 µg/ml	Increased osteogenesis	• ↑ viability (Wst-8 assay) • ↑ ALP activity (pNPP measurements) • ↑ collagen (Sirius Red) • ↑ mineralization (alizarin red staining)	Jeong et al.	(54)
Sulforaphane *	MC3T3-E1	3-10-15-20-30-100 µM SFN	Promotion osteoblast differentiation and induction of apoptosis	• ↓ cells proliferation (3-(EZ4U) • ↑ in the activities of Caspase 3/7 and 8 (assay kit) • ↑ Fas mRNA expression (RT-PCR) • ↑ mineralization (alizarin red staining) • ↑ RUNX-2 mRNA expression (RT-PCR)	Thaler et al.	(97)
Sulforaphane *	BMMSCs from long bones of 6-week-old C57BL/6 mice	3 µM	Promotes osteoblast differentiation	• ↑ mineralization (alizarin red staining) • ↑ RUNX-2 mRNA expression (RT-PCR)	Thaler et al.	(97)
Hot water extract of *Brassica oleracea*	RAW 264.7 cell line	200 g/mL	Inhibition of osteoclast formation	↓ osteoclasts in femur, when in combination with P. ginseng extract (TRAP staining)	Kang et al.	(99)
Sulforaphane *	RAW 264.7 cell line	3-10-15-30-100 µM	Reduces proliferation and induces apoptosis	• ↓ viability and metabolic activity (EZ4U) • No alteration in Acp5, Clcr, and CTSK mRNA expression (RT-PCR) • ↑ Tet1 and Fas-Caspase 8-Caspase 3/7 pathway (western blot, assay kit)	Thaler et al.	(97)
Sulforaphane *	RAW 264.7 cell line	1-2-5-10 µM	Inhibition of osteoclastogenesis	• ↓osteoclasts (TRAP staining) • ↑ NRF2 protein accumulation (western blot); ↑ HO1, NQO1, GCLC and GCLM mRNA (RT-PCR) • ↓ ROS (2′,7′-Dichlorofluorescin diacetate) • ↓ NFATc1, C-FOS, TNFα, TRAP, CTSK, MMP-9, DC-STAMP mRNA (RT-PCR)	Xue et al.	(100)
Sulforaphane *	RAW 264.7 cell line	0.01-0.1-0.5-1 µM	1. Inhibits osteoclastogenesis 2. Inhibits osteoclasts cells-fusion	• induced cytotoxicity at > 5 µM (CCK-8 assay) • ↓ osteoclasts (TRAP assay) • ↓NFATc1, TRAP, CTSK mRNA (RT-PCR) • ↓ OSCAR, DC-STAMP, OC-STAMP mRNA (RT-PCR) • ↑ phosphorylation of STAT1 (Tyr701) (western blot)	Takagi et al.	(101)
Sulforaphane *	RAW 264.7 cell line	0.01-0.1-1-10 µM	Inhibition of osteoclastogenesis	• ↓ osteoclasts • ↓NF-kappaB activation	Kim et al.	(102)
Sulforaphane *	RAW 264.7 cell line	0.5, 1, 2.5, 5, 10, 20 µM	Decreased viability and osteoclastogenesis	• Marked cytotoxicity at concentration > 5 µM, low cytotoxicity 1-2.5 µM (CCK-8 assay) • ↓osteoclasts (TRAP staining)	Luo et al.	(103)

(Continued)

TABLE 4 Continued

Molecule (organosulfur compouds)	Experimental *in vitro* model	Concentration	Main effect	Specific outcomes	Authors	Ref
				• ↓ CTSK, MMP-9 mRNA and protein (RT-PCR) • ↓ in autophagosomes and LC3-II, Beclin1, and Atg5–Atg12 mRNA and protein; ↓ of JNK phosphorylation (RT-PCR, western blot) • ↓size of F-actin rings		
Sulforaphane *	Primary mouse osteoclasts from tibial and femoral bone marrow of 8-week-old C57BL/6 mice	3 μM	Inhibition of osteoclasts resorption	↓ resorption activity	Thaler et al.	(97)
Sulforaphane *	Primary osteoclast precursors isolated from BM of tibias and femurs of 8–12 weeks old male C57BL/6 mice	1-5 μM	Inhibition of osteoclastogenesis	↓ osteoclasts (TRAP staining)	Xue et al.	(100)
Sulforaphane *	BM cells obtained from the femur and tibia of 7–10-week-old ddY male mice	0.01-0.1-0.5-1 μM	Inhibition of osteoclastogenesis	• induced cytotoxicity at > 5 μM (CCK-8 assay) • ↓ osteoclasts (TRAP staining) • ↓ NFATc1, TRAP, CTSK mRNA expression (RT-PCR)	Takagi et al.	(101)
Sulforaphane *	BM cells isolated from femora and tibiae of 4- 6-week-old C57BL/6 mice	0.01-0.1-1-10 μM	Inhibition of osteoclastogenesis	• ↓ osteoclasts • Early inhibition of osteoclastogenesis • No effects on osteoclasts resorption • No effects on RANK or c-fms mRNA	Kim et al.	(102)
Sulforaphane *	BMMs from 5-week-old C57BL/6 female mice	1, 2.5, 5 μM	Decreased viability and inhibition of osteoclastogenesis	• Moderate cytotoxicity at concentration >2.5 μM (CCK-8 assay) • ↓ osteoclasts (TRAP staining)	Luo et al.	(103)
Sulforaphane *	Human monocytes isolated from peripheral blood of healthy volunteers	0.2-1-5 μM	Inhibition of osteoclastogenesis	• ↓ osteoclasts (TRAP staining) • ↑NRF2 accumulation (immunocytochemistry) • ↑ NQO1 and PRDX1 mRNA expression (RT-PCR)	Gambari et al.	(104)

Most in vitro studies were conducted using purified OSCs (6 studies, 15 in vitro models; sulforaphane, glucoraphanin); while only a few used water or ethanol extracts from Brassicaceae edible plants (2 studies, 2 in vitro models; Brassica rapa, Brassica oleracea). Most studies showed increased osteogenesis and decreased osteoclastogenesis. Notably, only the effects of purified OSCs (labeled with * in the table) can be attributable to OSCs. The concentrations tested ranged from 0.01 to 100 μg/ml. Murine in vitro models of osteoclastogenesis: osteoclasts derived from bone marrow of femora and tibiae of mice, RAW 264.7 cell line. Human in vitro models of osteoclastogenesis: human monocytes isolated from peripheral blood of healthy volunteers. Murine in vitro models of osteoblastogenesis: MC3T3-E1 (Mouse C57BL/6 calvaria cells line); murine bone marrow (BM) cells; bone marrow-derived mesenchymal stem cells (BMMSCs), bone marrow macrophages (BMMs). Human in vitro models of osteoblastogenesis: MC3T3-E1, MSCs isolated from human tibial plateau. Osteocyte – cell line: MLO-Y4. Functional assays for osteoclastogenesis: tartrate-resistant acid phosphatase positive (TRAP staining); pit assay. Functional assays for osteoblastogenesis: Alizarin red staining (marker of mineralization), Sirius red assay (marker of collagen I), p-nitrophenyl phosphate (pNPP) quantification. Proliferation/viability assays: cell counting kit-8 (CCK-8) cell viability assay, water-soluble tetrazolium-8 (WST-8) assay, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide-like assay (EZ4U). Markers of osteoclasts: nuclear factor of activated T-cells cytoplasmic 1 (NFATc1), cathepsin K (CTSK), receptor activator of NF-KB (RANK), osteoclast stimulatory transmembrane protein (OC-STAMP), tartrate-resistant acid phosphatase (TRAP), receptor activator of nuclear factor-κB ligand (RANKL), dendritic cell specific transmembrane protein (DC-STAMP), reactive oxygen species (ROS), c-fos, nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), matrix metallopeptidase 9 (MMP-9), osteoclasts-specific activating receptor (OSCAR), acid phosphatase 5, tartrate resistant (ACP5), calcitonin receptor-like receptor (Clcr), colony-stimulating factor-1 receptor (c-fsm), c-fos. Markers of osteoblastogenesis: cystathionine-β-synthase (CBS), bone sialoprotein (BSP), SMAD family member 1 (SMAD-1), alkaline phosphatase (ALP), WNT1-inducible-signaling pathway protein 1 (WISP-1), osteocalcin (OCN), runt-related transcription factor 2 (RUNX-2). Markers of cell viability – apoptosis: Fas, Caspase 3/7 and 8, nuclear factor erythroid-derived 2-related factor 2 (NRF2), heme oxygenase-1 (HO1), NAD(P)H: quinone oxidoreductase 1 (NQO1), peroxiredoxin-1 (PRDX-1), glutamate cysteine ligase catalytic subunit (GCLC), glutamate-cysteine ligase modifier subunit (GCLM), peroxiredoxin 1 (PRDX-1), microtubule-associated protein 1A/1B-light chain 3 (LC3-II), beclin1, autophagy related 5 (ATG5), Jun N-terminal kinases (JNK), autophagy related 12 (Atg12). ↑ means up-regulation; ↓ means down-regulation

(97); at the molecular level, SFN induced up-regulation of RUNX-2 in mouse MSCs (97). Gambari et al. showed increased mineralization and BSP, CBS and SMAD-1 mRNA up-regulation by GRA administration in primary human MSCs (98). Finally, with regards to osteocyte regulation, Thaler et al. showed that SFN inhibited proliferation in murine osteocyte-like cell line (MLO-Y4) (97).

Purified OSCs have also been tested in *in vivo* models of bone loss or osteolysis, showing beneficial effects on preserving bone mass. Oral administration of allyl sulfide in an age-associated osteoporosis mouse model resulted in increased bone density at X-ray analysis and increased serum levels of procollagen 1 intact N-terminal propeptide (P1NP; a marker of bone formation) (83). Similarly, intragastric administration of allicin increased BMD, as detected by dual energy X-ray

TABLE 5 *Brassicaceae*-derived OSCs: effects on *in vivo* models of bone loss.

Molecule tested	Experimental *in vivo* model description	Mode of administration, dose and duration	Main effect	Specific features	Authors	Ref
Sulforaphane	C57BL/6 mice, Mouse calvarial models treated with LPS (10 mg/kg body weight injected in calvaria)	Intraperitoneal injection, 10 mg/kg body weight, the day before LPS treatment for 6 days	Protection against LPS-induced calvarial bone erosion by inhibition of osteoclastogenesis	• ↑ BV/TV, Tb.N, ↓Tb.Sp (microCT) • ↓ osteoclasts (TRAP staining in histological samples) • ↓ CTSK (immunohistochemical and immunofluorescence analysis)	Luo et al.	(103)
Sulforaphane	*Ex vivo* culture of calvariae explants of 2–3-day-old and 7-week-old, C57BL/6 mice	3 µM	Promotes osteogenesis inhibits osteoclastogenesis	• ↑ ECM mineralization (alizarin red staining on calvaria tissue) • ↓ RANKL (RT-PCR on calvariae lysates)	Thaler et al.	(97)
Sulforaphane	Mice model of OP (Female, 8-week-old, C57BL/6 mice, ovariectomy)	Intraperitoneal injection, 7.5 mM DL-SFN, every other day for 5 weeks	Prevention of bone loss	• ↑ BV/TV, Tb.N ↓Tb.Sp, no effect on Tb.Th or Co.Th in tibiae (micro CT)	Thaler et al.	(97)
SFX- 01® (a stable form of Sulforaphane)	Osteoarthritis model (Male, 26-week-old, STR/Ortmice)	Oral administration, 100 mg/kg, daily for 3 months	Improvements in cortical bone mass	• ↑ TV, BV and BV/TV of tibial epiphyseal trabecular bone and metaphyseal trabecular bone (micro CT) • ↑serum P1NP (ELISA) • ↓serum CTX-I (ELISA)	Javaheri et al.	(105)
***Brassica rapa L.* root ethanol extract**	Female, 3-week-old, Sprague-Dawley rats	Oral administration, 500 mg/kg/day, single daily dose for 6 weeks	Increased bone formation	• ↑ BMD, BV, BV/TV, Tb.N, Tb.Th., ↓Tb.Sp. (microCT) • ↑ serum OCN (immunoassay)	Jeong et al.	(54)
***Lepidum sativum* seed extract**	Rat model of OP (Female Wistar rats, ovariectomy)	Oral gavage 50 and 100 mg/kg	Prevention of bone loss and bone strengthening activity	• ↑ femur weight (weights were calculated as wet femur weight/body weight) • ↑ femur compression strength (hardness tester (Erweka GmbH, Heusen-stamm, Germany) • ↑ ALP, OCN serum levels; ↓ TRAP, CTX-I serum levels (ELISA) • ↓ RANKL, ↑ OPG mRNA (RT-PCR)	Abdallah et al.	(59)
***Lepidum sativum* seed**	Glucocorticoid-induced OP (GIO) model (Female Wistar rat, subcutaneous injection of methylprednisolone 3.5 mg/kg per day for 4 weeks)	Oral gavage, 6 g of LS seeds in diet daily	Prevention of GIO-dependent bone loss	• ↑ percentage of trabecular bone *vs* GIO (histopathological examination and Image J quantification) • ↓ serum TRAP *vs* GIO (commercial kit) • ↑ serum b-ALP (immunoassay), phosforous and calcium (automated analyser) *vs* GIO	Elshal et al.	(106)
***Lepidum* sativum seed**	Fracture-induced healing model (New Zealand White rabbits, induced fractures in the midshaft of the left femur)	Oral gavage, 6 g of *Lepidum sativum* seeds in their food daily after surgery	Increased healing of fractures	Increased callus formation in fractures (x-rays and quantification)	Juma et al.	(83)
Methanolic and aqueous extract of *Lepidium sativum* seeds	Fracture healing model (Charles foster rats, hand held three-point bending technique)	Oral administration, methanolic extracts 400 mg/kg or aqueous extracts 550 mg/kg, from the day of fracture induction for 2 months	Increased healing of fractures	• Larger callus formation (x-rays and quantification) • ↑ calcium, phosphorus,	Dixit Jr Iii et al.	(28)

(Continued)

TABLE 5 Continued

Molecule tested	Experimental *in vivo* model description	Mode of administration, dose and duration	Main effect	Specific features	Authors	Ref
				and ALP serum levels (commercial kits)		
***Lepidium sativum* seeds**	Glucocorticoid-induced OP (Adult male guinea pigs, methyl prednisolone 3.5 mg/kg per day for 4 weeks subcutaneously)	Oral administration trough a gastric tube, 300 mg/kg, for 4 weeks	Prevention of bone loss in femur	• Prevention of caspase-3 activation (caspase-3 immunostaining) • Prevention of decrease of OPN (immunohystochemistry) • Prevention of decrease in osteoblast and Co.th. in femur (histomorphometric analysis) • Prevention of increase of osteoclasts in femur (histomorphometric analysis)	EL-Haroun et al.	(107)
Ethanol extracts of Maca root (*Lepidium meyenii Walp.*)	Rat model of OP (Female, 90-day-old, Sprague-Dawley rats, ovariectomy)	Oral gavage, 0.096 and 0.24 g/kg, for 28 weeks	Prevention of estrogen deficient bone loss	• ↑ calcium content of femur (Atomic Absorption Spectrophotometer) • ↑ BMD and trabecular bone of the lumbar vertebrae (DEXA) • ↑ serum OCN (radioimmunoassay commercial kit)	Zhang et al.	(108)
Hot water extract of *Brassica oleracea* (Bo)	Mice model of OP (Female, 7-week-old, C57BL/6 mice, ovariectomy)	Oral administration, 500 mg/kg, daily for 10 weeks	Inhibits OVX-induced bone loss	• ↑ BMD when in combination with Panax ginseng (DEXA) • ↓ osteoclast number when in combination with Panax ginseng (immunohistochemistry, TRAP staining)	Kang et al.	(99)

Most *in vivo* studies were conducted by using water or ethanol extracts of *Brassica* edible plants (8 studies; *Brassica rapa, Lepidum sativum, Lepidum meyenii Walp, Brassica oleracea*). A minority of studies used *Brassicaceae*-purified OSCs (3 studies; 4 models; SFN, SFX-01). Most studies were performed in osteoporosis mice showing prevention of bone loss. Notably, only the effects of purified OSCs (labeled with * in the table) can be attributable entirely to OSCs. The route of administration was mainly by oral administration. Markers of bone formation in serum: procollagen 1 intact N-terminal propeptide (P1NP); osteocalcin (OCN). Markers of bone resorption in serum: serum type I collagen breakdown product (CTX-I), tartrate-resistant acid phosphatase (TRAP), osteoprotegerin (OPG), cortical thickness (Co.Th). Bone microstructural parameters analyzed by microCT analysis: BMD (bone mineral density), bone volume (BV), bone volume/total volume (BV/TV), trabecular thickness (Tb.Th), trabecular number (Tb.N), trabecular space (Tb.Sp.). Bone mineral density analyzed by Dual-energy X-ray absorptiometry (DEXA). Markers of bone formation in histological specimen: alkaline phosphatase (ALP), osteopontin (OPN). Markers of osteoclasts / bone resorption in histological specimen: tartrate-resistant acid phosphatase (TRAP), cathepsin K (CTSK). Measurements of bone strength: Erweka GmbH, Heusen-stamm Germany. Extracellular matrix (ECM). Markers of osteoclast in histological specimen: receptor activation of nuclear factor-kB ligand (RANKL). ↑ means up-regulation; ↓ means down-regulation.

absorptiometry, and bone strength, as measured by three-point bending assay, in a model of aging osteoporotic rats (96). Intraperitoneal administration of allicin prevented the bone loss in a mice model of lead-induced bone loss (osteoporosis induced by a toxic heavy metal), as measured by increased BMD, trabecular number (Tb.N), trabecular thickness (Tb.Th) and decreased trabecular space (Tb.Sp), quantified using micro-CT analysis (95). Finally, SFN showed to be protective against bone loss in different *in vivo* models. Intraperitoneal injection of SFN in lipopolysaccharide (LPS)-induced erosion of the mice calvaria bone induced increased trabecular bone volume (BV/TV), increased Tb.N and decreased Tb.Sp, as measured by micro-CT analysis (103); moreover, intraperitoneal injection of SFN in a mice model of ovariectomy-induced bone loss stimulated trabecular bone formation, increased Tb.N and decreased Tb.Sp (97); finally, the oral administration of SFN-01 (a stabilized form of SFN) in a mice model of osteoarthritis, resulted in increased trabecular bone volume and serum P1NP (105).

Regulation of osteoclastogenesis and bone resorption

Osteoclasts are bone-resorbing cells which arise from immature monocytes and mature tissue macrophages (124). Osteoclasts differentiation stems from the signaling triggered by two critical cytokines produced by MSCs, osteoblasts and osteocytes: macrophage colony-stimulating factor (M-CSF) and RANKL binding, respectively, to the receptors colony-

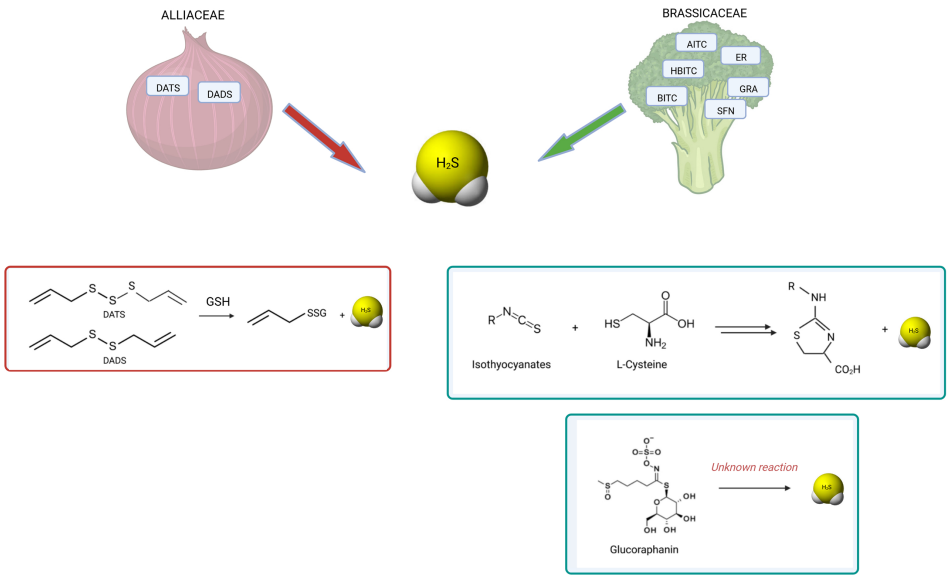

FIGURE 3
H_2S release by OSCs derived from *Alliaceae* and *Brassicaceae*. The known reactions occurring for H_2S release by polysulfides and isothiocyanates are shown. Among garlic-derived polysulfides, diallyl disulfide (DADS) and diallyl trisulfide (DATS) have been shown to release H_2S by reaction with glutathione (GSH) by polarographic H_2S sensor (154) (147) (148). Among glucosinolates, GRA has been found to release H_2S by amperometric approach (149). Similarly, several isothiocyanates showed H_2S-releasing activity: allyl isothiocyanate (AITC), 4-hydroxybenzyl isothiocyanate (HBITC), benzyl isothiocyanate (BITC), erucin (ER), sulforaphane (SFN) (149) (150). While the mechanism of release is unknown for glucosinolates, the mechanism of release by isothiocyanates is dependent on L-cysteine reaction (155). Moreover, different OSCs have different kinetics of H_2S release.

stimulating factor-1 receptor (c-fms) and receptor activator of nuclear factor κ B (RANK) (125, 126). RANKL signaling activation induces various intracellular signal transduction cascades such as tumor necrosis factor receptor-associated factor 6 (TRAF-6), NADPH oxidase 1 (NOX-1), RAC family small GTPase 1 (RAC1), nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), and nuclear factor-activated T cells c1 (NFATc1), c-fos (127–129). Other

TABLE 6 Clinical studies on musculoskeletal effects of OSCs-rich food and extracts.

Molecule tested	Patients data	Mode of administration, concentration, treatments	Main effect	Specific features	Authors	Ref
Onion	Perimenopausal and postmenopausal non-Hispanic white women, 50 years and older	Onion consumption ≥ once a day; 3-5 a week; 2 a month to 2 a week, 1 a month or less	Prevention of bone loss	↑ BMD by increased consumption	Matheson et al.	(179)
Onion juice	Healthy subjects, male and female, 40-80 years	100 mL of onion juice or placebo for 8 weeks	Decreased bone anabolic markers	• ↓ALP serum level (commercial kit)	Law et al.	(85)
Onion juice	Postmenopausal women	100 mL of onion juice or placebo for 8 weeks	Mild changes in BMD	• ↓ALP serum levels (commercial kit) • Mildly improved BMD (DEXA of the lumbar, right and left hip)	Law et al.	(85)
***Allium* vegetables (onion, leek, and garlic)**	Women, ≥70 years	Habitual intakes of *Allium* intake	Inversely associated with all fractures	Inversely associated with all fractures	Blekkenhorst et al.	(18)
Cruciferous (cabbage, brussel sprouts, cauliflower, and broccoli)	Women aged >70 years	Cruciferous vegetables intake	Inversely associated with all fractures	Inversely associated with all fractures	Blekkenhorst et al.	(18)
Raw garlic consumption	28958 patients (males and females)	Habitual intakes of raw garlic	Positive correlation with handgrip strength		Gu et al.	(139)

Analysis of bone mineral density (BMD) by Dual-energy X-ray absorptiometry (DEXA). Measurement of alkaline phosphatase (ALP). ↑ means up-regulation; ↓ means down-regulation.

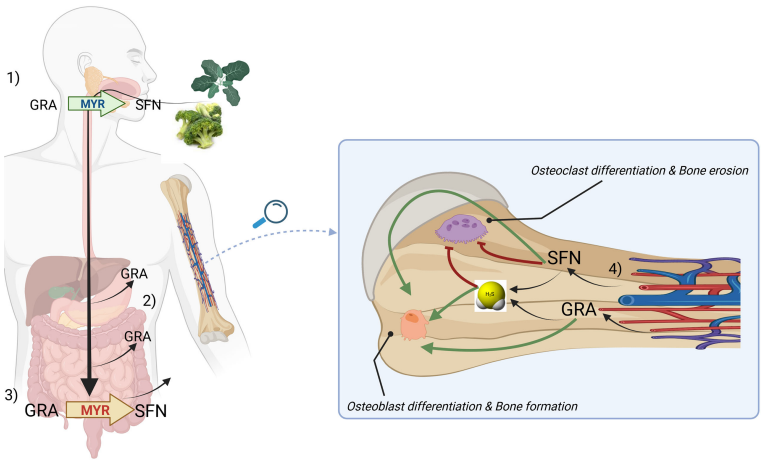

FIGURE 4
A general model describing the routes of absorption of GRA and SFN and a proposed mechanism of action on bone cells based on H_2S-release. Briefly, upon chewing of plants belonging to *Brassica* genus, myrosinase (MYR, green) is released and can convert glucoraphanin (GRA) to sulforaphane (SFN) (1). GRA can be adsorbed in the stomach or in the small intestine (2). Microbacterial thioglucosidases (MYR, red) converts GRA to SFN which is further adsorbed in large quantities (3). SFN and GRA are released by circulation in bone tissue where can release H_2S and exert anabolic and anticatabolic properties on bone cells (4). The mechanism by which H_2S can be directly released from GRA has not been clarified yet.

receptors involved in osteoclastogenesis are calcitonin receptor (CTR), ITAM bearing Fc receptor standard g chain (FcRγ), osteoclasts-specific activating receptor (OSCAR) (126, 130); key signaling is mediated by mitogen-activated protein kinases (MAPK), and includes extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38 activation. Moreover, critical to osteoclast differentiation and function are: intracellular reactive oxygen species (ROS) generation, which act as key signaling molecules (82, 88, 94); osteoclast fusion mediated among other factors, by the fusogenic molecules osteoclasts-stimulatory transmembrane protein (OC-STAMP) and dendritic cell-specific transmembrane protein (DC-STAMP) (126, 131, 132); and expression of specific enzymes such as tartrate-resistant acid phosphatase (TRAP), cathepsin K (CTSK) (126, 130), tartrate-resistant acid phosphatase 5b (TRAP5b) (83) and matrix metallopeptidase 9 (MMP-9).

Extracts from both *Allium* and *Brassica* species were shown to attenuate osteoclast differentiation *in vitro* in the murine macrophage cell line, RAW 264.7. In particular, extracts of onion (85), freeze dried onion juice (85), solution of onion crude powder (81) inhibited osteoclastogenesis, as measured by TRAP staining *in vitro*. A similar effect was achieved by an extract of *Brassica oleracea* but only in combination with extract from *Panax ginseng* (99). Using human THP1 monocytes, Bose et al. showed that ginger and garlic extracts reduce the frequency and the size of resorption pits carved by osteoclasts (82); inhibition of osteoclast number was found also by onion and commercial onion extracts in rat and rabbit osteoclasts (81). Notably, Wetli et al. demonstrated that onion extract reduced rat osteoclast differentiation and were able to isolate a specific sulfoxide component of onion powder, γ-glutamyl-*trans*-*S*-1-propenyl-L-cysteine sulfoxide (GPCS), which the authors found to be the key responsible of this biological activity (41).

In vivo administration of extracts rich in OSCs decreased osteoclastogenesis and bone erosion in rodent model of osteoporosis; Huang et al. showed that ovariectomized rats fed with different concentrations of onion extracts (up to 14% wt/wt in the diet powder) were partly protected against loss of bone mass and bone material properties (94); moreover, histomorphometry revealed that treatment with onion extracts was associated with a lower number of osteoclasts *in vivo* (94). Similar findings were reported by Kang et al. using ovariectomized mice fed with a combination of extracts obtained from *Panax ginseng* and *Brassica oleracea* (99). Furthermore, Abdallah HM et al. reported that ovariectomized rats treated with extracts of *Lepidium sativum* were partly protected against osteoporosis and showed a sharply decreased RANKL/osteoprotegerin (OPG) ratio in femur bones (59).

Studies that used purified OSCs molecules further supported efficacy and specificity. Yang et al. demonstrated a dose-dependent inhibition of osteoclast differentiation and a decreased bone resorption by mature osteoclasts upon treatment with DADS (86). Monocytes proliferation and viability was inhibited by SFN (97).

Luo et al. (103) and Xue et al. (100) showed that SFN inhibits osteoclast differentiation in RAW 264.7 murine macrophagic cell line; Takagi et al. (101) and Kim et al. (102) showed similar findings in murine BM cells and so did Gambari et al. (104) in a model of osteoclast derived from human monocytes. Moreover, Chen et al. reported the inhibition of osteoclast differentiation by alliin in RAW 264.7 *via* scavenging of ROS signaling (87).

Mechanisms of regulation of osteoclastic differentiation by OSCs involved different molecular targets. Li et al. reported that the anti-osteoclastogenic activity of allicin in mice is associated to the activation of the SIRT1/FOXO1 pathway and ROS scavenging (95). Similarly, one key mechanism of action of SFN is the activation of the master regulator of the antioxidant defense system, nuclear factor erythroid-derived 2-related factor 2 (NRF2), and its downstream target antioxidant and detoxifying enzymes (133), which is known to actively inhibit mouse osteoclasts differentiation *in vitro* (104, 134). SFN modifies sulfhydryl groups in kelch-like erythroid-cell-derived protein with CNC homology (ECH)-associated protein (KEAP-1), causing KEAP-1 dislocation, NRF2 stabilization and nuclear translocation (135); moreover, SFN regulates NRF2 expression *via* epigenetic mechanisms (136). Coherently, SFN was shown to increase NRF2 protein accumulation in RAW 264.7 murine cell line, to increase the expression of some NRF2-mediated antioxidant genes (heme oxygenase-1, HO1; NAD(P)H: quinone oxidoreductase 1, NQO1; glutamate cysteine ligase catalytic subunit, GCLC; ligase modifier subunit, GCLM) and decrease intracellular ROS production, and the overall number of osteoclasts as shown by Xue et al. (100). Similarly, SFN was shown to inhibit the osteoclast differentiation of human monocytes while increasing NRF2 nuclear translocation and protein expression of NRF2-mediated antioxidant genes (NQO1; Peroxiredoxin 1, PRDX-1), as published by Gambari et al. (104). Finally, SFN induces Caspase 8 and 3/7, thus inducing apoptosis in a RAW 264.7 murine cell line as shown by Thaler et al. (97).

Moreover, downregulation of the key transcription factor NFATc1 is implicated in several studies showing inhibition of osteoclast development: Yang et al. reported a dose-dependent down-regulation of NFATc1 in a RAW 264.7 murine cell line after DADS treatment (86); Xue et al. (100) and Takagi et al. (101), respectively, reported similar findings in RAW 264.7 murine cell line and in murine BM cells after SFN treatment; Behera et al. in murine BM cells after allyl sulfide treatment (83). The inhibition of other key transcription factor c-Fos and Nf-kB was shown by Yang et al. in a RAW 264.7 murine cell line after DADS treatment (86).

Several other proteins implicated in the adhesion and proteolytic extracellular matrix degradation, such as TRAP, CTSK, CTR or MMPs were shown to be affected by OSCs, specifically by allicin, DADS, SFN, allyl sulfide (83, 95, 100, 137), in RAW264.7 cells and murine BM and are detailed in Tables 2, 4.

OSCs can modulate the expression of osteoclasts-specific activating receptors, necessary for the co-stimulatory signaling with immunoreceptors and prevented osteoclast fusion by inhibiting fusogenic molecules. Takagi et al. showed in RAW 264.7 murine cell line that OSCAR is inhibited by SFN (101). DC-STAMP was found inhibited in RAW 264.7 murine cell line after SFN treatment as shown by Takagi et al. (101) and by Xue et al. (100) and after DADS treatment as shown by Yang et al. (86). OC-STAMP was found inhibited in RAW 264.7 murine cell line after SFN treatment as shown by Takagi et al. (101).

Finally, OSCs compounds were shown to inhibit osteoclast differentiation *via* a paracrine mechanism, acting on osteoclasts-supporting cells. Thaler et al. showed that RANKL was inhibited by SFN in a murine osteocytes cell line (MLO-Y4) (97). Behera et al. showed that RANKL was inhibited while OPG was increased in supernatants of murine MSCs cells culture treated with allyl sulfide (83); and that treatment with this conditioned medium inhibited the expression of RANK and osteoclast differentiation of murine bone marrow (BM) cells (83).

Only a few *in vivo* studies used purified OSCs to investigate bone metabolism. In a mice model of lead-induced bone loss, intraperitoneal injection of allicin alleviates bone loss by preventing oxidative stress and osteoclastogenesis by modulating SIRT1/FOXO1 pathway (95). SFN treatment in a mouse calvaria model treated with LPS decreased the number of osteoclasts (103). Treatment of *Lepidium sativum* in a rat model of ovariectomy-induced osteoporosis improved mechanical properties of femurs while decreasing TRAP, serum type I collagen breakdown product (CTX-I), RANKL (59) and the number of osteoclasts (107).

H_2S release from OSCs as a potential mechanism of bioactivity in bone

H_2S is a pleiotropic molecule which provides numerous health benefits by improving hypertension and cardiometabolic disorders (138) (139), relieving pain (140, 141), and increasing insulin sensitivity (142); protecting against neurological diseases including Alzheimer disease (143). Moreover, H_2S is critically involved in the extension of lifespan provided by caloric restriction (144, 145). Supraphysiological levels of H_2S may be generated in certain pathological conditions and lead to toxicity, inducing inflammation or tissue damage (146).

The intriguing overlap between biological effects attributed to some *Allium* and *Brassica* species and those exhibited by the gasotransmitter H_2S prompted several researchers to verify the H_2S releasing capacity of those molecules. Recently, the ability of releasing H_2S was found as a distinctive feature of several OSCs, and a plausible mechanism for their biological effects across different organs and tissues was described. The biological relevance of H_2S release by OSCs was first demonstrated by

Benavides et al. in the context of a study on the vasoactivity of garlic. The authors showed that garlic polysulfides DATS and DADS, the downstream metabolites of alliin, released H_2S in red blood cells; importantly, pre-treating the cells with the thiol-blocking reagent iodoacetamide inhibited the release of H_2S, thereby demonstrating that the mechanism by which polysulfides release H_2S is dependent on intracellular thiols, such as glutathione (GSH) (147). Chemically, this reaction involves a nucleophilic substitution from thiol at the α carbon of the H_2S-donor moiety and a subsequent release of H_2S (148). This mechanism is biologically relevant as the relaxation induced by both garlic extract and DADS on isolated rat aortic rings strongly correlated to the amount of H_2S released. In the wake of this work, Citi et al. first revealed that a similar mechanism accounts for the ability of several *Brassicaceae*-derived ITCs to release pharmacologically relevant concentrations of H_2S in an L-cysteine dependent manner (149): allyl isothiocyanate (AITC), 4-hydroxybenzyl isothiocyanate (HBITC), benzyl isothiocyanate (BITC), erucin (ER), SFN (149, 150). The same group reported that H_2S-release is associated with the *in vivo* anti-hypertensive, hypoglycemic, pain-relieving, and anti-inflammatory effects of OSCs derived from the *Brassicacea Eruca Sativa* (138, 151–153). Interestingly, Lucarini et al. first demonstrated that GRA, a GLS, can release H_2S in aqueous solution independent of myrosinase, but the chemical mechanism underlying this phenomenon is still unclear (150). Whether other *Alliaceae* or *Brassicaceae*-derived OSCs releases H_2S is still unknown.

Figure 3 summarizes the known reactions leading to H_2S release from polysulfides, GLS or ITCs.

This mechanism holds important implications for bone. Recent findings by our group and others demonstrated that H_2S plays an important role in the regulation of bone cell differentiation and function. *In vitro*, H_2S-donors promote osteogenic differentiation and stimulate mineralization by increasing calcium intake (156) and the expression of genes directly involved in the biosynthesis of hydroxyapatite, such BSP (157). Furthermore, the expression of the enzymes CBS and CSE, which are responsible for endogenous H_2S production, steadily increased during osteogenic differentiation and correlated to mineral apposition (119). Moreover, H_2S-donors inhibit osteoclast maturation and resorption by activating the antioxidant response elicited by the NRF2 transcription factor (104, 158). Further attesting to the relevance of H_2S in bone homeostasis, evidence from several *in vivo* preclinical models showed that the depletion of H_2S levels is associated with loss of bone mass; similar findings were reported in ovariectomized mice (157), in H_2S-deficient CBS+/− mice (156), in glucocorticoids-induced osteoporosis (159). Interestingly, when animals were treated with pharmacological H_2S-donors to normalize the plasma level of H_2S, bone loss was prevented or reversed (156, 157). The ability of H_2S to stimulate bone formation appears to be maintained across various conditions, even unrelated to systemic or genetic disfunctions: for example, the exogenous administration of H_2S by means of the pharmacological donor GYY4137 was effective to attenuate the bone loss induced by modelled microgravity (160) and to promote osteogenesis in a model of distraction osteogenesis (161).

Overall, these data demonstrate that H_2S regulates osteogenesis and bone formation in both healthy and pathological conditions.

Therefore, H_2S release by OSCs could account, at least in part, for their biological properties. However, up to date no clinical or preclinical *in vivo* studies have investigated the effect of OSCs by correlating their bioactivity to the H_2S levels.

The GRA/SFN system: A case-model for OSCs bioactivity based on H_2S release

GRA is a glucosinolate abundant in aerial portions, developing florets (flower buds), sprouts, seeds and mature plants of cabbage, broccoli, cauliflower, kale and Brussels sprouts (77). GRA conversion to SFN, an ITC, requires the enzyme myrosinase, an intracellular thioglucosidase, which catalyzes its hydrolysis to an unstable aglucone that spontaneously rearranges to give rise to a range of products, including SFN. SFN is the progenitor of a family of compounds widely studied in the literature mostly due to their antioxidant and anticancer properties. In mammalians, GRA conversion to SFN is primarily mediated by bacterial microflora of the gastrointestinal tract; while a small proportion is generated in the mouth by plant myrosinase when released by plants after chewing. Our current knowledge on the bioavailability and the rate of conversion of GSL into ITCs are largely based on studies on the GRA/SFN system.

Although most of GRA introduced with diet undergoes hydrolysis in the gut by microbial thioglucosidases, a fraction of GRA (around 10-15%) is absorbed directly in the stomach and in the small intestine, before the catabolic breakdown to SFN is triggered by gut microbiota (77, 162).

Gastric acidity appears to attenuate GSL bioavailability (163). However, GRA is not destroyed by digestive enzymes during passage through the digestive tract and is able to reach the rat cecum intact, when is hydrolyzed to SFN which is able to cross the cecal enterocyte for systemic absorption and enterohepatic circulation (164, 165). Conversion of GRA to bioactive SFN by the rat cecal microbiota requires four or more days after broccoli consumption and is reversible (166); however, recent randomized clinical trials have ascertained that upon ingestion of GRA-enriched soups, increased SFN levels were detectable as early as 30' in plasma and 1h in the urine of patients (162). Attesting the tissue systemic absorption of SFN and ITCs in general, they have been detected in both plasma and synovial fluid of osteoarthritis patients undergoing consumption of GLS-rich diets for 2 weeks

(167). On the other hand, the direct delivery of SFN from foods is possible and was demonstrated in recent clinical studies (168, 169) where SFN was shown to be readily bioavailable (170); however, SFN is unstable, requires storage at freezing temperature, and SFN-enriched extracts are difficult to prepare and very expensive (163).

Although most of the research on the biological effects of SFN is focused on cancer because of its effect on cell cycle and apoptosis (171–173), it also regulates bone cells: *in vitro*, SFN inhibits monocyte cell proliferation and osteoclast differentiation in multiple ways, detailed above (100–104), while increases mineralization in mouse MSCs and in an *ex vivo* culture of calvariae explants (97). Notably, in one *in vivo* study the administration of SFN for 5 weeks to normal and ovariectomized mice lead to an approximate 20% increase in bone mass (97), shifting the balance of bone homeostasis and favoring bone acquisition and/or mitigation of bone resorption.

Of note, our group recently demonstrated that GRA obtained from Tuscan black kale promotes osteogenesis in human MSCs, independent of SFN, and this effect is associated to the release of H_2S and an increased H_2S uptake inside the cells (98). This is relatively unexpected as GLS have been considered for many years a relatively inert precursor of reactive derivatives ITCs. Although the chemistry underlying this phenomenon is still unclear and will require further investigation, this finding suggests that GLS may exert inherent biological activity based on their capacity to release H_2S.

As the hydrolytic product of GRA, SFN, had been already shown to inhibit the activity of osteoclast in bone, it can be suggested that the 'GRA-SFN system' exerts a beneficial effect on bone both at level of GLS and of its cognate ITC. The routes of absorption of GRA and SFN as well as the proposed mode of action on bone cells is summarized in Figure 4.

Clinical studies

OSCs and chronic diseases

Despite this review focuses primarily on the skeletal effects of OSCs, much of the clinical research on the health benefits of OSCs is aimed at metabolic or cardiovascular disease and cancer.

Vegetables or extracts rich in OSCs improved dyslipidemia, insulin resistance, hypertension and cardiovascular risk linked to atherosclerotic plaques in human studies.

Among interventional, randomized clinical trials, Jeon et al. evidenced that ethanol extracts from *Brassica rapa*, administrated as a part of the diet of overweight human for 10 weeks, induce a significant increase in the HLDL-cholesterol concentration and a significant reduction in the total cholesterol/HDL-cholesterol ratio, free fatty acid, and adipsin levels (174). A randomized double-blind trial, performed by Bahadoran et. al., investigated the effects of broccoli sprouts powder containing high concentration of SFN for four weeks in type 2 diabetic patients and showed that broccoli sprouts improve insulin resistance by decreasing serum insulin concentration and 'homeostatic model assessment for insulin resistance' (HOMA-IR) score (175).

In a prospective cohort study on Australian women aged 70 years and older, without clinical atherosclerotic vascular disease (ASVD) or diabetes mellitus at baseline, Blekkenhorst et al. investigated the occurrence of ASVD-related deaths during 15 years of follow-up and correlated it with several dietary intake, through a multivariable-adjusted model. Among the nutrients tested, intakes of cruciferous and *Allium* vegetables were inversely associated with ASVD mortality supporting the evidence that the effect of increased intake of cruciferous and *Allium* vegetables lowered cardiovascular disease risk (176).

In cancer, treatment with OSCs-rich food showed promising results as chemopreventive.

A placebo double-blind randomized controlled trial on men scheduled for prostate biopsy and treated with broccoli sprout extract (BSE) supplementation (providing SFN and myrosinase) for 4.4 wk, performed by Zhang et. al., showed that BSE supplementation correlated with changes in gene expression but not with other prostate cancer immunohistochemistry biomarkers (173). In a double-blind placebo randomized clinical trial in patients with colorectal adenomas-precancerous lesions of the large bowel treated with aged garlic extract (AGE), Tanaka et al. demonstrated that AGE significantly reduced the size and number of colon adenomas in patients after 12 month (25). Several epidemiological studies showed that SFN consumption has been reported to be associated with a lower risk of cancer development (breast, lung, stomach, esophagus, mammary glands, gastric, colorectal, prostate, skin, head and neck, and liver) (172). In a large cohort study Millen et al. correlated the presence of adenoma with food intake of several fruit and vegetables, as assessed by a food-frequency questionnaire, and showed that onions and garlic were significantly related to lower risk of adenoma (177). Notably, a randomized double-blinded intervention study, performed by Traka et. al., showed that consuming GRA-rich broccoli for 12 months reduced the risk of prostate cancer progression (178). In particular, patients administrated with a weekly portion of soup made from a standard broccoli or 2 experimental broccoli genotypes with enhanced concentrations of GRA, showed dose-dependent attenuated activation of gene expression associated to oncogenic pathways in transperineal biopsies; and an inverse association between consumption of cruciferous vegetables and cancer progression was observed (178).

Overall, these studies highlighted the significant role of diet administration of OSCs in several chronic diseases and substantiate the relevance of creating specific dietary regimen for their prevention.

OSCs in the prevention of bone loss and skeletal frailty

A few clinical trials or population-based studies have revealed positive relationships between the consumption of vegetables, bone density, muscle strength and fractures in women/men, as summarized in Table 6.

Matheson et al. used a food frequency questionnaire added to the Nutritional Health and Nutrition Examination Survey (2003–2004) to examine the correlation between habitual consumption of onion over the past 12 months to BMD (N unweighted =507; N weighed =35.7 million). They found that higher consumption of onion increased the BMD by 5% (179). Law et al. administrated onion juice to healthy men and women and post-menopausal women for 8 weeks and investigated the association with bone BMD; the results found that the BMD of 3 postmenopausal women was mildly improved at the end of the treatment (85).

In an intriguing study, Blekkenhorst et al. used a food frequency questionnaire to examine the associations of vegetable and fruit intakes, separately, and specific types of vegetables and fruits with fracture-related hospitalizations in a prospective cohort of elderly women (mean age ≥ 70; n=1468); the authors found that the consumption of vegetable, but not fruit, is associated to a lower incidence of fracture; of note, the habitual consumption of cruciferous vegetables and *Allium* vegetables was significantly inversely associated with all fractures (18); importantly, these results were adjusted for energy intake and physical activity.

In musculoskeletal ageing, sarcopenia and declining physical activity are often associated with osteoporosis as the clinical hallmarks of frailty (180).

Interestingly, a prospective cohort study performed on elderly women (mean age ≥ 70; n=1429) investigated the correlation between vegetable consumption and incident falls-related hospitalization over a time-period of 14 years. The authors found that hospitalizations were lower in participants consuming more vegetables, but the consumption of cruciferous vegetables was most strongly associated with lower falls-related hospitalization (181) and was associated with increased muscle strength.

Finally, cross-sectional study, by Gu et. al., demonstrated a positive correlation between raw garlic consumption, assessed using a food frequency questionnaire, and handgrip strength in both males and females (182). The results were adjusted for age, body mass index, smoking status, alcohol-consumption status, education levels, employment status, household income, family history of diseases (cardiovascular disease, hypertension, hyperlipidemia, and diabetes), metabolic syndromes, physical activity, total energy intake, dietary pattern, onion intake. Although this study did not directly assess indexes of bone quantity, it supports an overall protective effect of OSCs-rich vegetables on the musculoskeletal system (181).

Perspectives and challenges

The present literature revision stems from the increasing appreciation of the link between dietary habits, and particularly the use of phytochemicals, and bone health. We show that a growing body of evidence supports a beneficial effect of dietary OSCs on skeletal health. Of note, although a few population-based studies offer interesting clues on the clinical relevance of OSCs-rich vegetables for the prevention of bone fragility (18, 85, 179, 183), no clinical studies have been performed yet to specifically address the potential protective role of OSCs against osteoporosis or bone fractures; this goal would require a study design including a controlled intake of OSCs-rich nutrients for long time-periods and/or the evaluation of purified OSCs molecules.

The ability of OSCs to work a as dietary source of the bioactive molecule H_2S provide interesting future perspectives. OSCs-rich vegetables appear as the ideal candidate for clinical investigations on whether nutrients rich in sulfur can affect the pool of circulating reactive sulfur species (RSS), which include H_2S; this may have a broad implication for the prevention of those pathologies, sometimes referred to as 'H_2S-poor diseases', where the onset of the disease was associated to a lower systemic concentration of RSS compared to healthy controls. Increasing systemic RSS levels may also have important implication for bone-wasting diseases such as osteoporosis: indeed, animal studies have established that the bone loss associated to estrogen deficiency or to corticosteroid therapy is associated to a low systemic level of H_2S (157, 159). However, these preclinical data still await confirmation in observational clinical studies in humans. To obtain reliable data on this topic, it will be critical to include in the study design a robust analytical methodology to quantitatively measure the different sulfur species in human serum or plasma since they may hold different importance in different pathologies (184, 185) and the high reactivity of these gaseous molecules implies a complex chemistry (186).

Further investigations may be addressed to the evaluation of the effect of these compounds on the gut-bone axis. OSCs show a considerable ability to modulate the gut microbiome and its secondary metabolites (187–190) and to mitigate the gut-based inflammatory response; given the paramount importance of metabolites and cytokines originated from the gut on the regulation on bone metabolism (191), it is conceivable that dietary OSCs may modulate the bone-bioactive components of the microbiota.

In the end, it is apparent that members of the OSCs family of phytochemicals affect bone homeostasis in several ways and may provide new insights into the potential bone health benefits of plant-derived food and leading to a more effective prevention of osteoporosis by non-pharmacological tools.

This review may be useful to fuel clinical trials that may use a robust set of outcome measurements, aiming at assessing both bone quantity and bone quality before and after specific nutrition protocols; correlation between nutrients intake, H_2S blood levels and bone status would help to define preventive/clinical dietary protocols for patients with an increased risk of bone fragility.

Author contributions

FG and LG contributed to the conception and design of the review. FG ad LG wrote the first draft of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

References

1. Clynes MA, Harvey NC, Curtis EM, Fuggle NR, Dennison EM, Cooper C. The epidemiology of osteoporosis. *Br Med Bull* (2020) 133(1):105–17. doi: 10.1093/bmb/ldaa005

2. Hernlund E, Svedbom A, Ivergard M, Compston J, Cooper C, Stenmark J, et al. Osteoporosis in the European union: medical management, epidemiology and economic burden. a report prepared in collaboration with the international osteoporosis foundation (IOF) and the European federation of pharmaceutical industry associations (EFPIA). *Arch Osteoporos.* (2013) 8:136. doi: 10.1007/s11657-013-0136-1

3. Kanis JA, Norton N, Harvey NC, Jacobson T, Johansson H, Lorentzon M, et al. SCOPE 2021: a new scorecard for osteoporosis in Europe. *Arch Osteoporos.* (2021) 16(1):82. doi: 10.1007/s11657-020-00871-9

4. Zaidi M. Skeletal remodeling in health and disease. *Nat Med* (2007) 13 (7):791–801. doi: 10.1038/nm1593

5. Meunier P, Aaron J, Edouard C, Vignon G. Osteoporosis and the replacement of cell populations of the marrow by adipose tissue. a quantitative study of 84 iliac bone biopsies. *Clin Orthop Relat Res* (1971) 80:147–54. doi: 10.1097/00003086-197110000-00021

6. Verma S, Rajaratnam JH, Denton J, Hoyland JA, Byers RJ. Adipocytic proportion of bone marrow is inversely related to bone formation in osteoporosis. *J Clin Pathol* (2002) 55(9):693–8. doi: 10.1136/jcp.55.9.693

7. Infante A, Rodriguez CI. Osteogenesis and aging: lessons from mesenchymal stem cells. *Stem Cell Res Ther* (2018) 9(1):244. doi: 10.1186/s13287-018-0995-x

8. Papaioannou A, Kennedy CC, Dolovich L, Lau E, Adachi JD. Patient adherence to osteoporosis medications: problems, consequences and management strategies. *Drugs Aging.* (2007) 24(1):37–55. doi: 10.2165/00002512-200724010-00003

9. Siris ES, Harris ST, Rosen CJ, Barr CE, Arvesen JN, Abbott TA, et al. Adherence to bisphosphonate therapy and fracture rates in osteoporotic women: relationship to vertebral and nonvertebral fractures from 2 US claims databases. *Mayo Clin Proc* (2006) 81(8):1013–22. doi: 10.4065/81.8.1013

10. Willems HME, van den Heuvel E, Schoemaker RJW, Klein-Nulend J, Bakker AD. Diet and exercise: a match made in bone. *Curr Osteoporos Rep* (2017) 15 (6):555–63. doi: 10.1007/s11914-017-0406-8

11. Karpouzos A, Diamantis E, Farmaki P, Savvanis S, Troupis T. Nutritional aspects of bone health and fracture healing. *J Osteoporos.* (2017) 2017:4218472. doi: 10.1155/2017/4218472

12. Munoz-Garach A, Garcia-Fontana B, Munoz-Torres M. Nutrients and dietary patterns related to osteoporosis. *Nutrients* (2020) 12(7):1986 . doi: 10.3390/nu12071986

13. Rizzoli R. Dairy products and bone health. *Aging Clin Exp Res* (2022) 34 (1):9–24. doi: 10.1007/s40520-021-01970-4

14. Rizzoli R, Biver E, Brennan-Speranza TC. Nutritional intake and bone health. *Lancet Diabetes Endocrinol* (2021) 9(9):606–21. doi: 10.1016/S2213-8587(21)00119-4

15. Warensjo Lemming E, Byberg L. Is a healthy diet also suitable for the prevention of fragility fractures? *Nutrients* (2020) 12(9):2642. doi: 10.3390/nu12092642

16. Benetou V, Orfanos P, Feskanich D, Michaelsson K, Pettersson-Kymmer U, Byberg L, et al. Mediterranean Diet and hip fracture incidence among older adults: the CHANCES project. *Osteoporos Int* (2018) 29(7):1591–9. doi: 10.1007/s00198-018-4517-6

17. Benetou V, Orfanos P, Pettersson-Kymmer U, Bergstrom U, Svensson O, Johansson I, et al. Mediterranean Diet and incidence of hip fractures in a European cohort. *Osteoporos Int* (2013) 24(5):1587–98. doi: 10.1007/s00198-012-2187-3

18. Blekkenhorst LC, Hodgson JM, Lewis JR, Devine A, Woodman RJ, Lim WH, et al. Vegetable and Fruit Intake and Fracture-Related Hospitalisations: A Prospective Study of Older Women. (2017) 9(5):11. doi: 10.3390/nu9050511

19. Byberg L, Bellavia A, Orsini N, Wolk A, Michaelsson K. Fruit and vegetable intake and risk of hip fracture: a cohort study of Swedish men and women. *J Bone Miner Res* (2015) 30(6):976–84. doi: 10.1002/jbmr.2384

20. Tucker KL, Chen H, Hannan MT, Cupples LA, Wilson PW, Felson D, et al. Bone mineral density and dietary patterns in older adults: the framingham osteoporosis study. *Am J Clin Nutr* (2002) 76(1):245–52. doi: 10.1093/ajcn/76.1.245

21. de Haas SCM, de Jonge EAL, Voortman T, Graaff JS, Franco OH, Ikram MA, et al. Dietary patterns and changes in frailty status: the Rotterdam study. *Eur J Nutr* (2018) 57(7):2365–75. doi: 10.1007/s00394-017-1509-9

22. de Jonge EAL, Rivadeneira F, Erler NS, Hofman A, Uitterlinden AG, Franco OH, et al. Dietary patterns in an elderly population and their relation with bone mineral density: the Rotterdam study. *Eur J Nutr* (2018) 57(1):61–73. doi: 10.1007/s00394-016-1297-7

23. Somani SJ, Modi KP, Majumdar AS, Sadarani BN. Phytochemicals and their potential usefulness in inflammatory bowel disease. *Phytother Res* (2015) 29 (3):339–50. doi: 10.1002/ptr.5271

24. Mathew BC, Prasad NV, Prabodh R. Cholesterol-lowering effect of organosulphur compounds from garlic: a possible mechanism of action. *(KUMJ)* (2004) 2(2):100–2.

25. Tanaka S, Haruma K, Yoshihara M, Kajiyama G, Kira K, Amagase H, et al. Aged garlic extract has potential suppressive effect on colorectal adenomas in humans. *J Nutr* (2006) 136(3 Suppl):821S–6S. doi: 10.1093/jn/136.3.821S

26. Panjwani AA, Liu H, Fahey JW. Crucifers and related vegetables and supplements for neurologic disorders: what is the evidence? *Curr Opin Clin Nutr Metab Care* (2018) 21(6):451–7. doi: 10.1097/MCO.0000000000000511

27. Del Chierico F, Vernocchi P, Dallapiccola B, Putignani L. Mediterranean Diet and health: food effects on gut microbiota and disease control. *Int J Mol Sci* (2014) 15(7):11678–99. doi: 10.3390/ijms150711678

28. Dixit VJr Iii, Kumar I, Palandurkar K, Giri R, Giri K. Lepidium sativum: Bone healer in traditional medicine, an experimental validation study in rats. *J Family Med Prim Care* (2020) 9(2):812–8. doi: 10.4103/jfmpc.jfmpc_761_19

29. Ramirez D, Abellan-Victorio A, Beretta V, Camargo A, Moreno DA. Functional ingredients from brassicaceae species: Overview and perspectives. *Int J Mol Sci* (2020) 21(6):1998. doi: 10.3390/ijms21061998

30. Iberl B, Winkler G, Muller B, Knobloch K. Quantitative determination of allicin and alliin from garlic by HPLC*. *Planta Med* (1990) 56(3):320–6. doi: 10.1055/s-2006-960969

31. Bose S, Laha B, Banerjee S. Quantification of allicin by high performance liquid chromatography-ultraviolet analysis with effect of post-ultrasonic sound and microwave radiation on fresh garlic cloves. *Pharmacogn Mag.* (2014) 10(Suppl 2): S288–93. doi: 10.4103/0973-1296.133279

32. Iberl B, Winkler G, Knobloch K. Products of allicin transformation: Ajoenes and dithiins, characterization and their determination by HPLC*. *Planta Med* (1990) 56(2):202–11. doi: 10.1055/s-2006-960926

33. Egen-Schwind C, Eckard R, Jekat FW, Winterhoff H. Pharmacokinetics of vinyldithiins, transformation products of allicin. *Planta Med* (1992) 58(1):8–13. doi: 10.1055/s-2006-961379

34. Lawson LD, Wang ZJ, Hughes BG. Identification and HPLC quantitation of the sulfides and dialk(en)yl thiosulfinates in commercial garlic products. *Planta Med* (1991) 57(4):363–70. doi: 10.1055/s-2006-960119

35. Rabinkov A, Miron T, Mirelman D, Wilchek M, Glozman S, Yavin E, et al. S-allylmercaptoglutathione: the reaction product of allicin with glutathione possesses SH-modifying and antioxidant properties. *Biochim Biophys Acta* (2000) 1499(1-2):144–53. doi: 10.1016/S0167-4889(00)00119-1

36. Bastaki SMA, Ojha S, Kalasz H, Adeghate E. Chemical constituents and medicinal properties of allium species. *Mol Cell Biochem* (2021) 476(12):4301–21. doi: 10.1007/s11010-021-04213-2

37. Iciek M, Kwiecien I, Wlodek L. Biological properties of garlic and garlic-derived organosulfur compounds. *Environ Mol Mutagen* (2009) 50(3):247–65. doi: 10.1002/em.20474

38. Rauf A, Abu-Izneid T, Thiruvengadam M, Imran M, Olatunde A, Shariati MA, et al. Garlic (Allium sativum l.): Its chemistry, nutritional composition, toxicity and anticancer properties. *Curr Top Med Chem* (2021) 22(11):957–72. doi: 10.2174/1568026621666211105094939

39. Cascajosa-Lira A, Prieto Ortega AI, Guzman-Guillen R, Catunescu GM, de la Torre JM, Guillamon E, et al. Simultaneous determination of allium compounds (Propyl propane thiosulfonate and thiosulfinate) in animal feed using UPLC-MS/MS. *Food Chem Toxicol* (2021) 157:112619. doi: 10.1016/j.fct.2021.112619

40. Kim S, Lee S, Shin D, Yoo M. Change in organosulfur compounds in onion (Allium cepa l.) during heat treatment. *Food Sci Biotechnol* (2016) 25(1):115–9. doi: 10.1007/s10068-016-0017-7

41. Wetli HA, Brenneisen R, Tschudi I, Langos M, Bigler P, Sprang T, et al. A gamma-glutamyl peptide isolated from onion (Allium cepa l.) by bioassay-guided fractionation inhibits resorption activity of osteoclasts. *J Agric Food Chem* (2005) 53(9):3408–14. doi: 10.1021/jf040457i

42. Nohara T, Fujiwara Y, Kudo R, Yamaguchi K, Ikeda T, Murakami K, et al. Isolation and characterization of new onionins A2 and A3 from allium cepa, and of onionins A1, A2, and A3 from allium fistulosum. *Chem Pharm Bull (Tokyo).* (2014) 62(11):1141–5. doi: 10.1248/cpb.c14-00461

43. Ueda Y, Tsubuku T, Miyajima R. Composition of sulfur-containing components in onion and their flavor characters. *Biosci Biotechnol Biochem* (1994) 58(1):108–10. doi: 10.1271/bbb.58.108

44. Wang H, Li J, Wang Z, Zhang X, Ni Y. Modified method for rapid quantitation of s-alk(en)yl-L-cysteine sulfoxide in yellow onions (Allium cepa l.). *J Agric Food Chem* (2007) 55(14):5429–35. doi: 10.1021/jf070298d

45. Fernandes S, Gois A, Mendes F, Perestrelo R, Medina S, Camara JS. Typicality assessment of onions (Allium cepa) from different geographical regions based on the volatile signature and chemometric tools. *Foods* (2020) 9 (3):375. doi: 10.3390/foods9030375

46. Tigu AB, Moldovan CS, Toma VA, Farcas AD, Mot AC, Jurj A, et al. Phytochemical analysis and *In vitro* effects of allium fistulosum l. and allium sativum l. extracts on human normal and tumor cell lines: A comparative study. *Molecules* (2021) 26(3):574. doi: 10.3390/molecules26030574

47. Yang HJ, Kim MJ, Qiu JY, Zhang T, Wu X, Jang DJ, et al. Rice porridge containing welsh onion root water extract alleviates osteoarthritis-related pain behaviors, glucose levels, and bone metabolism in osteoarthritis-induced ovariectomized rats. *Nutrients* (2019) 11(7):1503. doi: 10.3390/nu11071503

48. Park H, Jeong J, Hyun H, Kim J, Kim H, Oh HI, et al. Effects of a hot-water extract of allium hookeri roots on bone formation in human osteoblast-like MG-63 cells *In vitro* and in rats in vivo. *Planta Med* (2016) 82(16):1410–5. doi: 10.1055/s-0042-108733

49. Kim S, Kim DB, Lee S, Park J, Shin D, Yoo M. Profiling of organosulphur compounds using HPLC-PDA and GC/MS system and antioxidant activities in hooker chive (Allium hookeri). *Nat Prod Res* (2016) 30(24):2798–804. doi: 10.1080/14786419.2016.1164700

50. Jang JY, Lee MJ, You BR, Jin JS, Lee SH, Yun YR, et al. Allium hookeri root extract exerts anti-inflammatory effects by nuclear factor-kappaB down-regulation in lipopolysaccharide-induced RAW264.7 cells. *BMC Complement Altern Med* (2017) 17(1):126. doi: 10.1186/s12906-017-1633-3

51. Kim HJ, Lee SH, Lee SH, Lee J, Kim H, Chang GT, et al. Longitudinal bone growth stimulating effect of allium macrostemon in adolescent female rats. *Molecules* (2020) 25(22):5449. doi: 10.3390/molecules25225449

52. Bernaert N, Goetghebeur L, De Clercq H, De Loose M, Daeseleire E, Van Pamel E, et al. Influence of cultivar and harvest time on the amounts of isoalliin and methiin in leek (Allium ampeloprasum var. porrum). *J Agric Food Chem* (2012) 60 (44):10910–9. doi: 10.1021/jf302132a

53. Kubec R, Dadakova E. Chromatographic methods for determination of s-substituted cysteine derivatives–a comparative study. *J Chromatogr A.* (2009) 1216 (41):6957–63. doi: 10.1016/j.chroma.2009.08.032

54. Jeong J, Park H, Hyun H, Kim J, Kim H, Oh HI, et al. Effects of glucosinolates from turnip (Brassica rapa l.) root on bone formation by human osteoblast-like MG-63 cells and in normal young rats. *Phytother Res* (2015) 29 (6):902–9. doi: 10.1002/ptr.5331

55. Barbieri GPR, Maggio A, De Pascale S, Fogliano V. Glucosinolates profile of brassica rapa l. subsp. sylvestris l. janch. var. esculenta hort. *Food Chem* (2008) 107 (4):1687–91. doi: 10.1016/j.foodchem.2007.09.054

56. Cramer JM, Teran-Garcia M, Jeffery EH. Enhancing sulforaphane absorption and excretion in healthy men through the combined consumption of fresh broccoli sprouts and a glucoraphanin-rich powder. *Br J Nutr* (2012) 107 (9):1333–8. doi: 10.1017/S0007114511004429

57. Liang H, Li C, Yuan Q, Vriesekoop F. Separation and purification of sulforaphane from broccoli seeds by solid phase extraction and preparative high-performance liquid chromatography. *J Agric Food Chem* (2007) 55(20):8047–53. doi: 10.1021/jf0706833

58. Hashem FA, Motawea H, El-Shabrawy AE, Shaker K, El-Sherbini S. Myrosinase hydrolysates of brassica oleraceae l. var. italica reduce the risk of colon cancer. *Phytother Res* (2012) 26(5):743–7. doi: 10.1002/ptr.3591

59. Abdallah HM, Farag MA, Algandaby MM, Nasrullah MZ, Abdel-Naim AB, Eid BG, et al. Osteoprotective activity and metabolite fingerprint *via* UPLC/MS and GC/MS of lepidium sativum in ovariectomized rats. *Nutrients* (2020) 12(7):2075. doi: 10.3390/nu12072075

60. Robin AH, Yi GE, Laila R, Yang K, Park JI, Kim HR, et al. Expression profiling of glucosinolate biosynthetic genes in brassica oleracea l. var. capitata inbred lines reveals their association with glucosinolate content. *Molecules* (2016) 21(6):787. doi: 10.3390/molecules21060787

61. Soengas P, Velasco P, Fernandez JC, Cartea ME. New vegetable brassica foods: A promising source of bioactive compounds. *Foods* (2021) 10(12):2911. doi: 10.3390/foods10122911

62. Bell L, Oruna-Concha MJ, Wagstaff C. Identification and quantification of glucosinolate and flavonol compounds in rocket salad (Eruca sativa, eruca vesicaria and diplotaxis tenuifolia) by LC-MS: highlighting the potential for improving nutritional value of rocket crops. *Food Chem* (2015) 172:852–61. doi: 10.1016/j.foodchem.2014.09.116

63. Gerendas J, Breuning S, Stahl T, Mersch-Sundermann V, Muhling KH. Isothiocyanate concentration in kohlrabi (Brassica oleracea l. var. gongylodes) plants as influenced by sulfur and nitrogen supply. *J Agric Food Chem* (2008) 56 (18):8334–42. doi: 10.1021/jf800399x

64. Marcinkowska M, Frank S, Steinhaus M, Jelen HH. Key odorants of raw and cooked green kohlrabi (Brassica oleracea var. gongylodes l.). *J Agric Food Chem* (2021) 69(41):12270–7. doi: 10.1021/acs.jafc.1c04339

65. Manivannan A, Kim JH, Kim DS, Lee ES, Lee HE. Deciphering the nutraceutical potential of raphanus sativus-a comprehensive overview. *Nutrients* (2019) 11(2):402. doi: 10.3390/nu11020402

66. Giorgetti L, Giorgi G, Cherubini E, Gervasi PG, Della Croce CM, Longo V, et al. Screening and identification of major phytochemical compounds in seeds, sprouts and leaves of Tuscan black kale brassica oleracea (L.) ssp acephala (DC) var. sabellica l. *Nat Prod Res* (2018) 32(14):1617–26. doi: 10.1080/14786419.2017.1392953

67. Liu Z, Hirani AH, McVetty PB, Daayf F, Quiros CF, Li G. Reducing progoitrin and enriching glucoraphanin in brassica napus seeds through silencing of the GSL-ALK gene family. *Plant Mol Biol* (2012) 79(1-2):179–89. doi: 10.1007/s11103-012-9905-2

68. Ku KM, Kim MJ, Jeffery EH, Kang YH, Juvik JA. Profiles of glucosinolates, their hydrolysis products, and quinone reductase inducing activity from 39 arugula (Eruca sativa mill.) accessions. *J Agric Food Chem* (2016) 64(34):6524–32. doi: 10.1021/acs.jafc.6b02750

69. Rektorisova M, Hrbek V, Jiru M, Ovesna J, Hajslova J. Variability in s-Alk(en)yl-L-Cysteine sulfoxides in garlic within a seven-month period determined by a liquid chromatography - tandem mass spectrometry method. *Plant Foods Hum Nutr* (2020) 75(3):376–82. doi: 10.1007/s11130-020-00817-z

70. Rose P, Whiteman M, Moore PK, Zhu YZ. Bioactive s-alk(en)yl cysteine sulfoxide metabolites in the genus allium: the chemistry of potential therapeutic agents. *Nat Prod Rep* (2005) 22(3):351–68. doi: 10.1039/b417639c

71. Kodera Y, Kurita M, Nakamoto M, Matsutomo T. Chemistry of aged garlic: Diversity of constituents in aged garlic extract and their production mechanisms *via* the combination of chemical and enzymatic reactions. *Exp Ther Med* (2020) 19 (2):1574–84. doi: 10.3892/etm.2019.8393

72. Yoshimoto N, Saito K. S-alk(en)ylcysteine sulfoxides in the genus allium: proposed biosynthesis, chemical conversion, and bioactivities. *J Exp Bot* (2019) 70 (16):4123–37. doi: 10.1093/jxb/erz243

73. Block E. Recent results in the organosulfur and organoselenium chemistry of genus allium and brassica plants. relevance for cancer prevention. *Adv Exp Med Biol* (1996) 401:155–69. doi: 10.1007/978-1-4613-0399-2_13

74. Calvey EM, White KD, Matusik JE, Sha D, Block E. Allium chemistry: identification of organosulfur compounds in ramp (Allium tricoccum) homogenates. *Phytochemistry* (1998) 49(2):359–64. doi: 10.1016/S0031-9422(98)00191-5

75. Prieto MA, Lopez CJ, Simal-Gandara J. Glucosinolates: Molecular structure, breakdown, genetic, bioavailability, properties and healthy and adverse effects. *Adv Food Nutr Res* (2019) 90:305–50. doi: 10.1016/bs.afnr.2019.02.008

76. Shekarri Q, Dekker M. A physiological-based model for simulating the bioavailability and kinetics of sulforaphane from broccoli products. *Foods* (2021) 10(11):2761. doi: 10.3390/foods10112761

77. Angelino DJ E. Glucosinolate hydrolysis and bioavailability of resulting isothiocyanates: Focus on glucoraphanin. *J Funct Foods* (2014) 7:67–76. doi: 10.1016/j.jff.2013.09.029

78. Shapiro TA, Fahey JW, Wade KL, Stephenson KK, Talalay P. Human metabolism and excretion of cancer chemoprotective glucosinolates and isothiocyanates of cruciferous vegetables. *Cancer Epidemiol Biomarkers Prev* (1998) 7(12):1091–100.

79. Datta HK, Ng WF, Walker JA, Tuck SP, Varanasi SS. The cell biology of bone metabolism. *J Clin Pathol* (2008) 61(5):577–87. doi: 10.1136/jcp.2007.048868

80. Ryuk JA. Effect of allium fistulosum extracts on the stimulation of longitudinal bone growth in animal modeling diet-induced calcium and vitamin d deficiencies. *Appl Sci* (2021) 11(17):7786. doi: 10.3390/app11177786

81. Tang CH, Huang TH, Chang CS, Fu WM, Yang RS. Water solution of onion crude powder inhibits RANKL-induced osteoclastogenesis through ERK, p38 and NF-kappaB pathways. *Osteoporos Int* (2009) 20(1):93–103. doi: 10.1007/s00198-008-0630-2

82. Bose S, Banerjee D, Vu AA. Ginger and garlic extracts enhance osteogenesis in 3D printed calcium phosphate bone scaffolds with bimodal pore distribution. *ACS Appl Mater Interfaces* (2022) 4(11):12964–75. doi: 10.1021/acsami.1c19617

83. Behera J, Ison J, Rai H, Tyagi N. Allyl sulfide promotes osteoblast differentiation and bone density *via* reducing mitochondrial DNA release mediated Kdm6b/H3K27me3 epigenetic mechanism. *Biochem Biophys Res Commun* (2021) 543:87–94. doi: 10.1016/j.bbrc.2021.01.016

84. Monarrez-Cordero BE, Rodriguez-Gonzalez CA, Valencia-Gomez LE, Hernandez-Paz JF, Martel-Estrada SA, Camacho-Montes H, et al. The effect of allium cepa extract on the chitosan/PLGA scaffolds bioactivity. *J Appl Biomater Funct Mater* (2021) 19:2280800021989701. doi: 10.1177/2280800021989701

85. Law YY, Chiu HF, Lee HH, Shen YC, Venkatakrishnan K, Wang CK. Consumption of onion juice modulates oxidative stress and attenuates the risk of bone disorders in middle-aged and post-menopausal healthy subjects. *Food Funct* (2016) 7(2):902–12. doi: 10.1039/C5FO01251A

86. Yang J, Tang R, Yi J, Chen Y, Li X, Yu T, et al. Diallyl disulfide alleviates inflammatory osteolysis by suppressing osteoclastogenesis *via* NF-kappaB-NFATc1 signal pathway. *FASEB J* (2019) 33(6):7261–73. doi: 10.1096/fj.201802172R

87. Chen Y, Sun J, Dou C, Li N, Kang F, Wang Y, et al. Alliin attenuated RANKL-induced osteoclastogenesis by scavenging reactive oxygen species through inhibiting Nox1. *Int J Mol Sci* (2016) 17(9):1516. doi: 10.3390/ijms17091516

88. Weisstaub AR, Salinas MV, Correa MJ, Barchuk M, Berg G, Zuleta A. Effects of the intake of white wheat bread added with garlic and resistant starch: action on calcium bioavailability and metabolic parameters of growing wistar rats. *Food Funct* (2018) 9(11):5707–14. doi: 10.1039/C8FO01407H

89. El-Sayyad HI, Abou-El-Naga AM, Gadallah AA, Bakr IH. Protective effects of allium sativum against defects of hypercholesterolemia on pregnant rats and their offspring. *Int J Clin Exp Med* (2010) 3(2):152–63.

90. Mukherjee M, Das AS, Mitra S, Mitra C. Prevention of bone loss by oil extract of garlic (Allium sativum linn.) in an ovariectomized rat model of osteoporosis. *Phytother Res* (2004) 18(5):389–94. doi: 10.1002/ptr.1448

91. Mukherjee M, Das AS, Das D, Mukherjee S, Mitra S, Mitra C. Effects of garlic oil on postmenopausal osteoporosis using ovariectomized rats: comparison with the effects of lovastatin and 17beta-estradiol. *Phytother Res* (2006) 20(1):21–7. doi: 10.1002/ptr.1795

92. Mukherjee M, Das AS, Das D, Mukherjee S, Mitra S, Mitra C. Role of oil extract of garlic (Allium sativum linn.) on intestinal transference of calcium and its possible correlation with preservation of skeletal health in an ovariectomized rat model of osteoporosis. *Phytother Res* (2006) 20(5):408–15. doi: 10.1002/ptr.1888

93. Mukherjee M, Das AS, Das D, Mukherjee S, Mitra S, Mitra C. Role of peritoneal macrophages and lymphocytes in the development of hypogonadal osteoporosis in an ovariectomized rat model: possible phytoestrogenic efficacy of oil extract of garlic to preserve skeletal health. *Phytother Res* (2007) 21(11):1045–54. doi: 10.1002/ptr.2209

94. Huang TH, Muhlbauer RC, Tang CH, Chen HI, Chang GL, Huang YW, et al. Onion decreases the ovariectomy-induced osteopenia in young adult rats. *Bone* (2008) 42(6):1154–63. doi: 10.1016/j.bone.2008.01.032

95. Li D, Liang H, Li Y, Zhang J, Qiao L, Luo H. Allicin alleviates lead-induced bone loss by preventing oxidative stress and osteoclastogenesis *Via* SIRT1/FOXO1 pathway in mice. *Biol Trace Elem Res* (2021) 199(1):237–43. doi: 10.1007/s12011-020-02136-5

96. Liu Y, You M, Shen J, Xu Y, Li L, Wang D, et al. Allicin reversed the process of frailty in aging Male Fischer 344 rats with osteoporosis. *J Gerontol A Biol Sci Med Sci* (2020) 75(5):821–5. doi: 10.1093/gerona/glz205

97. Thaler R, Maurizi A, Roschger P, Sturmlechner I, Khani F, Spitzer S, et al. Anabolic and antiresorptive modulation of bone homeostasis by the epigenetic modulator sulforaphane, a naturally occurring isothiocyanate. *J Biol Chem* (2016) 291(13):6754–71. doi: 10.1074/jbc.M115.678235

98. Gambari L, Barone M, Amore E, Grigolo B, Filardo G, Iori R, et al. Glucoraphanin increases intracellular hydrogen sulfide (H2S) levels and stimulates osteogenic differentiation in human mesenchymal stromal cell. *Nutrients* (2022) 14(3):435. doi: 10.3390/nu14030435

99. Kang IS, Agidigbi TS, Kwon YM, Kim DG, Kim RI, In G, et al. Effect of Co-administration of panax ginseng and brassica oleracea on postmenopausal osteoporosis in ovariectomized mice. *Nutrients* (2020) 12(8):2415. doi: 10.3390/nu12082415

100. Xue P, Hu X, Powers J, Nay N, Chang E, Kwon J, et al. CDDO-me, sulforaphane and tBHQ attenuate the RANKL-induced osteoclast differentiation *via* activating the NRF2-mediated antioxidant response. *Biochem Biophys Res Commun* (2019) 511(3):637–43. doi: 10.1016/j.bbrc.2019.02.095

101. Takagi T, Inoue H, Takahashi N, Katsumata-Tsuboi R, Uehara M. Sulforaphane inhibits osteoclast differentiation by suppressing the cell-cell fusion molecules DC-STAMP and OC-STAMP. *Biochem Biophys Res Commun* (2017) 483(1):718–24. doi: 10.1016/j.bbrc.2016.12.075

102. Kim SJ, Kang SY, Shin HH, Choi HS. Sulforaphane inhibits osteoclastogenesis by inhibiting nuclear factor-kappaB. *Mol Cells* (2005) 20 (3):364–70.

103. Luo T, Fu X, Liu Y, Ji Y, Shang Z. Sulforaphane inhibits osteoclastogenesis via suppression of the autophagic pathway. *Molecules* (2021) 26(2):34. doi: 10.3390/molecules26020347

104. Gambari L, Lisignoli G, Cattini L, Manferdini C, Facchini A, Grassi F. Sodium hydrosulfide inhibits the differentiation of osteoclast progenitor cells *via* NRF2-dependent mechanism. *Pharmacol Res* (2014) 87:99–112. doi: 10.1016/j.phrs.2014.06.014

105. Javaheri B, Poulet B, Aljazzar A, de Souza R, Piles M, Hopkinson M, et al. Stable sulforaphane protects against gait anomalies and modifies bone microarchitecture in the spontaneous STR/Ort model of osteoarthritis. *Bone* (2017) 103:308–17. doi: 10.1016/j.bone.2017.07.028

106. Elshal MF, Almalki AL, Hussein HK, Khan JA. Synergistic antiosteoporotic effect of lepidium sativum and alendronate in glucocorticoid-induced osteoporosis in wistar rats. *Afr J Tradit Complement Altern Med* (2013) 10(5):267–73. doi: 10.4314/ajtcam.v10i5.8

107. El-Haroun H. Comparative study on the possible protective effect of lepidium sativum versus teriparatide in induced osteoporosis in adult Male Guinea pigs. *Egiptian J Hystol* (2020) 43(3):931–47. doi: 10.21608/ejh.2020.18855.1193

108. Zhang Y, Yu L, Ao M, Jin W. Effect of ethanol extract of lepidium meyenii walp. on osteoporosis in ovariectomized rat. *J Ethnopharmacol* (2006) 105(1-2):274–9. doi: 10.1016/j.jep.2005.12.013

109. Gorinstein S, Leontowicz H, Leontowicz M, Namiesnik J, Najman K, Drzewiecki J, et al. Comparison of the main bioactive compounds and antioxidant activities in garlic and white and red onions after treatment protocols. *J Agric Food Chem* (2008) 56(12):4418–26. doi: 10.1021/jf800038h

110. Kothari D, Lee WD, Niu KM, Kim SK. The genus allium as poultry feed additive: A review. *Anim (Basel).* (2019) 9(12):1032. doi: 10.3390/ani9121032

111. Sobolewska D, Michalska K, Podolak I, Grabowska K. Steroidal saponins from the genus allium. *Phytochem Rev* (2016) 15:1–35. doi: 10.1007/s11101-014-9381-1

112. Raiola A, Errico A, Petruk G, Monti DM, Barone A, Rigano MM. Bioactive compounds in brassicaceae vegetables with a role in the prevention of chronic diseases. *Molecules* (2017) 23(1):15. doi: 10.3390/molecules23010015

113. Darvin P, Joung YH, Yang YM. JAK2-STAT5B pathway and osteoblast differentiation. *JAKSTAT* (2013) 2(4):e24931. doi: 10.4161/jkst.24931

114. Lin GL, Hankenson KD. Integration of BMP, wnt, and notch signaling pathways in osteoblast differentiation. *J Cell Biochem* (2011) 112(12):3491–501. doi: 10.1002/jcb.23287

115. Kang S, Bennett CN, Gerin I, Rapp LA, Hankenson KD, Macdougald OA. Wnt signaling stimulates osteoblastogenesis of mesenchymal precursors by suppressing CCAAT/enhancer-binding protein alpha and peroxisome proliferator-activated receptor gamma. *J Biol Chem* (2007) 282(19):14515–24. doi: 10.1074/jbc.M700030200

116. Jeon MJ, Kim JA, Kwon SH, Kim SW, Park KS, Park SW, et al. Activation of peroxisome proliferator-activated receptor-gamma inhibits the Runx2-mediated transcription of osteocalcin in osteoblasts. *J Biol Chem* (2003) 278(26):23270–7. doi: 10.1074/jbc.M211610200

117. Wang M, Jin H, Tang D, Huang S, Zuscik MJ, Chen D. Smad1 plays an essential role in bone development and postnatal bone formation. *Osteoarthr Cartil* (2011) 19(6):751–62. doi: 10.1016/j.joca.2011.03.004

118. Meyers CA, Xu J, Asatrian G, Ding C, Shen J, Broderick K, et al. WISP-1 drives bone formation at the expense of fat formation in human perivascular stem cells. *Sci Rep* (2018) 8(1):15618. doi: 10.1038/s41598-018-34143-x

119. Gambari L, Lisignoli G, Gabusi E, Manferdini C, Paolella F, Piacentini A, et al. Distinctive expression pattern of cystathionine-beta-synthase and cystathionine-gamma-lyase identifies mesenchymal stromal cells transition to mineralizing osteoblasts. *J Cell Physiol* (2017) 232(12):3574–85. doi: 10.1002/jcp.25825

120. Robling AG, Bonewald LF. The osteocyte: New insights. *Annu Rev Physiol* (2020) 82:485–506. doi: 10.1146/annurev-physiol-021119-034332

121. Kitaura H, Marahleh A, Ohori F, Noguchi T, Shen WR, Qi J, et al. Osteocyte-related cytokines regulate osteoclast formation and bone resorption. *Int J Mol Sci* (2020) 21(14):5169. doi: 10.3390/ijms21145169

122. Pautke C, Schieker M, Tischer T, Kolk A, Neth P, Mutschler W, et al. Characterization of osteosarcoma cell lines MG-63, saos-2 and U-2 OS in comparison to human osteoblasts. *Anticancer Res* (2004) 24(6):3743–8.

123. Juma A. The effects of lepidium sativum seeds on fracture-induced healing in rabbits. *MedGenMed* (2007) 9(2):23. doi: 10.1016/j.jep.2005.12.013

124. Boyce BF. Advances in the regulation of osteoclasts and osteoclast functions. *J Dent Res* (2013) 92(10):860–7. doi: 10.1177/0022034513500306

125. Kim JM, Lin C, Stavre Z, Greenblatt MB, Shim JH. Osteoblast-osteoclast communication and bone homeostasis. *Cells* (2020) 9(9). doi: 10.3390/cells9092073

126. Gambari L, Grassi F, Roseti L, Grigolo B, Desando G. Learning from monocyte-macrophage fusion and multinucleation: Potential therapeutic targets for osteoporosis and rheumatoid arthritis. *Int J Mol Sci* (2020) 21(17):6001. doi: 10.3390/ijms21176001

127. Kim JH, Kim N. Regulation of NFATc1 in osteoclast differentiation. *J Bone Metab* (2014) 21(4):233–41. doi: 10.11005/jbm.2014.21.4.233

128. Lee NK, Choi YG, Baik JY, Han SY, Jeong DW, Bae YS, et al. A crucial role for reactive oxygen species in RANKL-induced osteoclast differentiation. *Blood* (2005) 106(3):852–9. doi: 10.1182/blood-2004-09-3662

129. Youn MY, Takada I, Imai Y, Yasuda H, Kato S. Transcriptionally active nuclei are selective in mature multinucleated osteoclasts. *Genes Cells* (2010) 15 (10):1025–35. doi: 10.1111/j.1365-2443.2010.01441.x

130. Costa AG, Cusano NE, Silva BC, Cremers S, Bilezikian JP. Cathepsin K: its skeletal actions and role as a therapeutic target in osteoporosis. *Nat Rev Rheumatol* (2011) 7(8):447–56. doi: 10.1038/nrrheum.2011.77

131. Miyamoto K, Ninomiya K, Sonoda KH, Miyauchi Y, Hoshi H, Iwasaki R, et al. MCP-1 expressed by osteoclasts stimulates osteoclastogenesis in an autocrine/paracrine manner. *Biochem Biophys Res Commun* (2009) 383(3):373–7. doi: 10.1016/j.bbrc.2009.04.020

132. Witwicka H, Hwang SY, Reyes-Gutierrez P, Jia H, Odgren PE, Donahue LR, et al. Studies of OC-STAMP in osteoclast fusion: A new knockout mouse model, rescue of cell fusion, and transmembrane topology. *PloS One* (2015) 10(6): e0128275. doi: 10.1371/journal.pone.0128275

133. Kensler TW, Wakabayashi N, Biswal S. Cell survival responses to environmental stresses *via* the Keap1-Nrf2-ARE pathway. *Annu Rev Pharmacol Toxicol* (2007) 47:89–116. doi: 10.1146/annurev.pharmtox.46.120604.141046

134. Kanzaki H, Shinohara F, Kajiya M, Kodama T. The Keap1/Nrf2 protein axis plays a role in osteoclast differentiation by regulating intracellular reactive oxygen species signaling. *J Biol Chem* (2013) 288(32):23009–20. doi: 10.1074/jbc.M113.478545

135. Dinkova-Kostova AT, Holtzclaw WD, Cole RN, Itoh K, Wakabayashi N, Katoh Y, et al. Direct evidence that sulfhydryl groups of Keap1 are the sensors regulating induction of phase 2 enzymes that protect against carcinogens and oxidants. *Proc Natl Acad Sci U S A.* (2002) 99(18):11908–13. doi: 10.1073/pnas.172398899

136. Bhattacharjee S, Dashwood RH. Epigenetic regulation of NRF2/KEAP1 by phytochemicals. *Antioxid (Basel).* (2020) 9(9):865. doi: 10.3390/antiox9090865

137. Yang H, Qin J, Wang X, Ei-Shora HM, Yu B. Production of plant-derived anticancer precursor glucoraphanin in chromosomally engineered escherichia coli. *Microbiol Res* (2020) 238:126484. doi: 10.1016/j.micres.2020.126484

138. Martelli A, Piragine E, Citi V, Testai L, Pagnotta E, Ugolini L, et al. Erucin exhibits vasorelaxing effects and antihypertensive activity by H2 s-releasing properties. *Br J Pharmacol* (2020) 177(4):824–35. doi: 10.1111/bph.14645

139. Yang G, Wu L, Jiang B, Yang W, Qi J, Cao K, et al. H2S as a physiologic vasorelaxant: hypertension in mice with deletion of cystathionine gamma-lyase. *Science* (2008) 322(5901):587–90. doi: 10.1126/science.1162667

140. Di Cesare Mannelli L, Lucarini E, Micheli L, Mosca I, Ambrosino P, Soldovieri MV, et al. Effects of natural and synthetic isothiocyanate-based H2S-releasers against chemotherapy-induced neuropathic pain: Role of Kv7 potassium channels. *Neuropharmacology* (2017) 121:49–59. doi: 10.1016/j.neuropharm.2017.04.029

141. Kida K, Marutani E, Nguyen RK, Ichinose F. Inhaled hydrogen sulfide prevents neuropathic pain after peripheral nerve injury in mice. *Nitric Oxide* (2015) 46:87–92. doi: 10.1016/j.niox.2014.11.014

142. Xue R, Hao DD, Sun JP, Li WW, Zhao MM, Li XH, et al. Hydrogen sulfide treatment promotes glucose uptake by increasing insulin receptor sensitivity and ameliorates kidney lesions in type 2 diabetes. *Antioxid Redox Signal* (2013) 19(1):5–23. doi: 10.1089/ars.2012.5024

143. Giovinazzo D, Bursac B, Sbodio JI, Nalluru S, Vignane T, Snowman AM, et al. Hydrogen sulfide is neuroprotective in alzheimer's disease by sulfhydrating GSK3beta and inhibiting tau hyperphosphorylation. *Proc Natl Acad Sci U.S.A.* (2021) 118(4):e2017225118. doi: 10.1073/pnas.2017225118

144. Hine C, Harputlugil E, Zhang Y, Ruckenstuhl C, Lee BC, Brace L, et al. Endogenous hydrogen sulfide production is essential for dietary restriction benefits. *Cell* (2015) 160(1-2):132–44. doi: 10.1016/j.cell.2014.11.048

145. Hine C, Mitchell JR. Calorie restriction and methionine restriction in control of endogenous hydrogen sulfide production by the transsulfuration pathway. *Exp Gerontol.* (2015) 68:26–32. doi: 10.1016/j.exger.2014.12.010

146. Whiteman M, Winyard PG. Hydrogen sulfide and inflammation: the good, the bad, the ugly and the promising. *Expert Rev Clin Pharmacol* (2011) 4(1):13–32. doi: 10.1586/ecp.10.134

147. Benavides GA, Squadrito GL, Mills RW, Patel HD, Isbell TS, Patel RP, et al. Hydrogen sulfide mediates the vasoactivity of garlic. *Proc Natl Acad Sci U S A.* (2007) 104(46):17977–82. doi: 10.1073/pnas.0705710104

148. Liang D, Wu H, Wong MW, Huang D. Diallyl trisulfide is a fast H2S donor, but diallyl disulfide is a slow one: The reaction pathways and intermediates of glutathione with polysulfides. *Org Lett* (2015) 17(17):4196–9. doi: 10.1021/acs.orglett.5b01962

149. Citi V, Martelli A, Testai L, Marino A, Breschi MC, Calderone V. Hydrogen sulfide releasing capacity of natural isothiocyanates: is it a reliable explanation for the multiple biological effects of brassicaceae? *Planta Med* (2014) 80(8-9):610–3. doi: 10.1055/s-0034-1368591

150. Lucarini E, Micheli L, Trallori E, Citi V, Martelli A, Testai L, et al. Effect of glucoraphanin and sulforaphane against chemotherapy-induced neuropathic pain: Kv7 potassium channels modulation by H2 s release in vivo. *Phytother Res* (2018) 32(11):2226–34. doi: 10.1002/ptr.6159

151. Lucarini E, Pagnotta E, Micheli L, Parisio C, Testai L, Martelli A, et al. Eruca sativa meal against diabetic neuropathic pain: An H2S-mediated effect of glucoerucin. *Molecules* (2019) 24(16):3006. doi: 10.3390/molecules24163006

152. Martelli A, Piragine E, Gorica E, Citi V, Testai L, Pagnotta E, et al. The H2S-donor erucin exhibits protective effects against vascular inflammation in human endothelial and smooth muscle cells. *Antioxid (Basel)* (2021) 10(6):961. doi: 10.3390/antiox10060961

153. Piragine E, Flori L, Di Cesare Mannelli L, Ghelardini C, Pagnotta E, Matteo R, et al. Eruca sativa mill. seed extract promotes anti-obesity and hypoglycemic effects in mice fed with a high-fat diet. *Phytother Res* (2021) 35(4):1983–90. doi: 10.1002/ptr.6941

154. Cai YR, Hu CH. Computational study of H2S release in reactions of diallyl polysulfides with thiols. *J Phys Chem B* (2017) 121(26):6359–66. doi: 10.1021/acs.jpcb.7b03683

155. Lin Y, Yang X, Lu Y, Liang D, Huang D. Isothiocyanates as H2S donors triggered by cysteine: Reaction mechanism and structure and activity relationship. *Org Lett* (2019) 21(15):5977–80. doi: 10.1021/acs.orglett.9b02117

156. Liu Y, Yang R, Liu X, Zhou Y, Qu C, Kikuiri T, et al. Hydrogen sulfide maintains mesenchymal stem cell function and bone homeostasis *via* regulation of

Ca(2+) channel sulfhydration. *Cell Stem Cell* (2014) 15(1):66–78. doi: 10.1016/j.stem.2014.03.005

157. Grassi F, Tyagi AM, Calvert JW, Gambari L, Walker LD, Yu M, et al. Hydrogen sulfide is a novel regulator of bone formation implicated in the bone loss induced by estrogen deficiency. *J Bone Miner Res* (2016) 31(5):949–63. doi: 10.1002/jbmr.2757

158. Lee SK, Chung JH, Choi SC, Auh QS, Lee YM, Lee SI, et al. Sodium hydrogen sulfide inhibits nicotine and lipopolysaccharide-induced osteoclastic differentiation and reversed osteoblastic differentiation in human periodontal ligament cells. *J Cell Biochem* (2013) 114(5):1183–93. doi: 10.1002/jcb.24461

159. Ma J, Shi C, Liu Z, Han B, Guo L, Zhu L, et al. Hydrogen sulfide is a novel regulator implicated in glucocorticoids-inhibited bone formation. *Aging (Albany NY).* (2019) 11(18):7537–52. doi: 10.18632/aging.102269

160. Zheng W, Li X, Zhang T, Wang J. Biological mechanisms and clinical efficacy of sulforaphane for mental disorders. *Gen Psychiatr* (2022) 35(2):e100700. doi: 10.1136/gpsych-2021-100700

161. Jiang X, Chen Y, Lu K, Zhang H, Fan X. GYY4137 promotes bone formation in a rabbit distraction osteogenesis model: a preliminary report. *J Oral Maxillofac Surg* (2015) 73(4):732.e1–6. doi: 10.1016/j.joms.2014.11.012

162. Sivapalan T, Melchini A, Saha S, Needs PW, Traka MH, Tapp H, et al. Bioavailability of glucoraphanin and sulforaphane from high-glucoraphanin broccoli. *Mol Nutr Food Res* (2018) 62(18):e1700911. doi: 10.1002/mnfr.201700911

163. Fahey JW, Wade KL, Stephenson KK, Panjwani AA, Liu H, Cornblatt G, et al. Bioavailability of sulforaphane following ingestion of glucoraphanin-rich broccoli sprout and seed extracts with active myrosinase: A pilot study of the effects of proton pump inhibitor administration. *Nutrients* (2019) 11(7):1489. doi: 10.3390/nu11071489

164. Lai RH, Miller MJ, Jeffery E. Glucoraphanin hydrolysis by microbiota in the rat cecum results in sulforaphane absorption. *Food Funct* (2010) 1(2):161–6. doi: 10.1039/c0fo00110d

165. Bheemreddy RM, Jeffery EH. The metabolic fate of purified glucoraphanin in F344 rats. *J Agric Food Chem* (2007) 55(8):2861–6. doi: 10.1021/jf0633544

166. Liu X, Wang Y, Hoeflinger JL, Neme BP, Jeffery EH, Miller MJ. Dietary broccoli alters rat cecal microbiota to improve glucoraphanin hydrolysis to bioactive isothiocyanates. *Nutrients* (2017) 9(3):262. doi: 10.3390/nu9030262

167. Davidson R, Gardner S, Jupp O, Bullough A, Butters S, Watts L, et al. Isothiocyanates are detected in human synovial fluid following broccoli consumption and can affect the tissues of the knee joint. *Sci Rep* (2017) 7 (1):3398. doi: 10.1038/s41598-017-03629-5

168. Oliviero T, Lamers S, Capuano E, Dekker M, Verkerk R. Bioavailability of isothiocyanates from broccoli sprouts in protein, lipid, and fiber gels. *Mol Nutr Food Res* (2018) 62(18):e1700837. doi: 10.1002/mnfr.201700837

169. Dinkova-Kostova AT, Fahey JW, Kostov RV, Kensler TW. KEAP1 and done? targeting the NRF2 pathway with sulforaphane. *Trends Food Sci Technol* (2017) 69(Pt B):257–69. doi: 10.1016/j.tifs.2017.02.002

170. Fahey JW, Wade KL, Wehage SL, Holtzclaw WD, Liu H, Talalay P, et al. Stabilized sulforaphane for clinical use: Phytochemical delivery efficiency. *Mol Nutr Food Res* (2017) 61(4). doi: 10.1002/mnfr.201600766

171. Gasparello J, Gambari L, Papi C, Rozzi A, Manicardi A, Corradini R, et al. High levels of apoptosis are induced in the human colon cancer HT-29 cell line by Co-administration of sulforaphane and a peptide nucleic acid targeting miR-15b-5p. *Nucleic Acid Ther* (2020) 30(3):164–74. doi: 10.1089/nat.2019.0825

172. Ullah MF. Sulforaphane (SFN): An isothiocyanate in a cancer chemoprevention paradigm. *Medicines (Basel).* (2015) 2(3):141–56. doi: 10.3390/medicines2030141

173. Zhang Z, Garzotto M, Davis EW2nd, Mori M, Stoller WA, Farris PE, et al. Sulforaphane bioavailability and chemopreventive activity in men presenting for biopsy of the prostate gland: A randomized controlled trial. *Nutr Cancer.* (2020) 72 (1):74–87. doi: 10.1080/01635581.2019.1619783

174. Jeon SM, Kim JE, Shin SK, Kwon EY, Jung UJ, Baek NI, et al. Randomized double-blind placebo-controlled trial of powdered brassica rapa ethanol extract on alteration of body composition and plasma lipid and adipocytokine profiles in overweight subjects. *J Med Food* (2013) 16(2):133–8. doi: 10.1089/jmf.2012.2249

175. Bahadoran Z, Tohidi M, Nazeri P, Mehran M, Azizi F, Mirmiran P. Effect of broccoli sprouts on insulin resistance in type 2 diabetic patients: a randomized double-blind clinical trial. *Int J Food Sci Nutr* (2012) 63(7):767–71. doi: 10.3109/09637486.2012.665043

176. Blekkenhorst LC, Bondonno CP, Lewis JR, Devine A, Zhu K, Lim WH, et al. Cruciferous and allium vegetable intakes are inversely associated with 15-year atherosclerotic vascular disease deaths in older adult women. *J Am Heart Assoc* (2017) 6(10):e006558. doi: 10.1161/JAHA.117.006558

177. Millen AE, Subar AF, Graubard BI, Peters U, Hayes RB, Weissfeld JL, et al. Fruit and vegetable intake and prevalence of colorectal adenoma in a cancer screening trial. *Am J Clin Nutr* (2007) 86(6):1754–64. doi: 10.1093/ajcn/86.5.1754

178. Traka MH, Melchini A, Coode-Bate J, Al Kadhi O, Saha S, Defernez M, et al. Transcriptional changes in prostate of men on active surveillance after a 12-mo glucoraphanin-rich broccoli intervention-results from the effect of sulforaphane on prostate CAncer PrEvention (ESCAPE) randomized controlled trial. *Am J Clin Nutr* (2019) 109(4):1133–44. doi: 10.1093/ajcn/nqz012

179. Matheson EM, Mainous AG3rd, Carnemolla MA. The association between onion consumption and bone density in perimenopausal and postmenopausal non-Hispanic white women 50 years and older. *Menopause* (2009) 16(4):756–9. doi: 10.1097/gme.0b013e31819581a5

180. Greco EA, Pietschmann P, Migliaccio S. Osteoporosis and sarcopenia increase frailty syndrome in the elderly. *Front Endocrinol (Lausanne).* (2019) 10:255. doi: 10.3389/fendo.2019.00255

181. Sim M, Blekkenhorst LC, Lewis JR, Bondonno CP, Devine A, Zhu K, et al. Vegetable and fruit intake and injurious falls risk in older women: a prospective cohort study. *Br J Nutr* (2018) 120(8):925–34. doi: 10.1017/S0007114518002155

182. Gu Y, Zhang S, Wang J, Chi VTQ, Zhang Q, Liu L, et al. Relationship between consumption of raw garlic and handgrip strength in a large-scale adult population. *Clin Nutr* (2020) 39(4):1234–41. doi: 10.1016/j.clnu.2019.05.015

183. Sim M, Blekkenhorst LC, Lewis JR, Bondonno CP, Devine A, Zhu K, et al. Vegetable diversity, injurious falls, and fracture risk in older women: A prospective cohort study. *Nutrients* (2018) 10(8):1081. doi: 10.3390/nu10081081

184. Zuhra K, Tome CS, Forte E, Vicente JB, Giuffre A. The multifaceted roles of sulfane sulfur species in cancer-associated processes. *Biochim Biophys Acta Bioenerg.* (2021) 1862(2):148338. doi: 10.1016/j.bbabio.2020.148338

185. Kolluru GK, Shen X, Kevil CG. Reactive sulfur species: A new redox player in cardiovascular pathophysiology. *Arterioscler Thromb Vasc Biol* (2020) 40 (4):874–84. doi: 10.1161/ATVBAHA.120.314084

186. Roda B, Zhang N, Gambari L, Grigolo B, Eller-Vainicher C, Gennari L, et al. Optimization of a monobromobimane (MBB) derivatization and RP-HPLC-FLD detection method for sulfur species measurement in human serum after sulfur inhalation treatment. *Antioxid (Basel)* (2022) 11(5):939. doi: 10.3390/antiox11050939

187. Chen K, Nakasone Y, Xie K, Sakao K, Hou DX. Modulation of allicin-free garlic on gut microbiome. *Molecules* (2020) 25(3):682. doi: 10.3390/molecules25030682

188. Chen K, Xie K, Liu Z, Nakasone Y, Sakao K, Hossain A, et al. Preventive effects and mechanisms of garlic on dyslipidemia and gut microbiome dysbiosis. *Nutrients* (2019) 11(6):1225. doi: 10.3390/nu11061225

189. Keirns BH, Lucas EA, Smith BJ. Phytochemicals affect T helper 17 and T regulatory cells and gut integrity: implications on the gut-bone axis. *Nutr Res* (2020) 83:30–48. doi: 10.1016/j.nutres.2020.08.006

190. Lee SH, Bang S, Jang HH, Lee EB, Kim BS, Kim SH, et al. Effects of allium hookeri on gut microbiome related to growth performance in young broiler chickens. *PloS One* (2020) 15(1):e0226833. doi: 10.1371/journal.pone.0226833

191. Pacifici R. Bone remodeling and the microbiome. *Cold Spring Harb Perspect Med* (2018) 8(4):a031203. doi: 10.1101/cshperspect.a031203

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Glossary

ACP5	Acid phosphatase 5, tartrate resistant
AG	Age-associated
AGE	Aged garlic extract
AITC	Allyl isothiocyanate
ALP	Alkaline phosphatase
ASCOs	*S*-alk(en)yl cysteine sulfoxides
AS	Allyl sulfide
ASVD	Atherosclerotic vascular disease
Atg5	Autophagy related 5
Atg12	Autophagy related 12
BITC	Benzyl isothiocyanate
BM	Bone marrow
BMD	Bone mineral density
BMM	Bone marrow macrophages
BMMSCs	Bone marrow-derived mesenchymal stem cells
BMP	Bone morphogenetic protein
BS/BV	Bone surface/bone volume
BSE	Broccoli sprout extract
BSP	Bone sialoprotein
BV	Bone volume
BVF	Bone volume fraction
BV/TV	Bone volume / trabecular volume
CAT	Catalase
CBS	Cystathionine beta synthase
Clcr	Calcitonin receptor-like receptor
CCK-8	Cell counting kit-8
c-fms	Colony-stimulating factor-1 receptor
Col I	Collagen I
CSE	Cystathionine-γ-lyase
CTR	Calcitonin receptor
CTX-I	Serum type I collagen breakdown product
CTSK	Cathepsin K
DADS	Diallyl disulfide
DATS	Diallyl trisulfide
DC-STAMP	Dendritic cell-specific transmembrane protein
DEXA	Dual-energy X-ray absorptiometry
Dlx5	Distal-Less Homeobox 5
DPDS	Dipropyl disulfide
ECM	Extracellular matrix
ER	Erucin
ERK	Extracellular signal-regulated kinase
EZ4U	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide-like assay
FcRγ	Fc receptor standard g chain
FOXO	Forkhead box O
GCLC	Glutamate cysteine ligase catalytic subunit
GCLM	Glutamate-cysteine ligase modifier subunit

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GH	Growth hormone
GLS	S-β-thioglucoside N-hydroxhysulfates; glucosinolates
GPCS	γ-glutamyl-*trans*-*S*-1-propenyl-L-cysteine sulfoxide
GRA	Glucoraphanin
GSAC	γ-glutamyl-*S*-allyl-L-cysteine
GSH	Glutathione
HBITC	4-hydroxybenzyl isothiocyanate
HO1	Heme oxygenase-1
HOMA-IR	Homeostatic Model Assessment for Insulin Resistance
H_2S	Hydrogen sulfide
IGF-1	Insulin-like growth factor 1
ITCs	Isothiocyanates
JAK2	Janus Kinase 2
JNK	Jun N-terminal kinases
KEAP-1	Kelch-like erythroid-cell-derived protein with CNC homology (ECH)-associated protein
LC3-II	Microtubule-associated protein 1A/1B-*light* chain 3
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase
M-CSF	Macrophage colony-stimulating factor
MMP-9	Matrix metallopeptidase 9
MDA	Malondialdehyde
MSC	Mesenchymal stromal cells
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NFATc1	Nuclear factor-activated T cells c1
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NQO1	NAD(P)H: quinone oxidoreductase 1
NOX-1	NADPH oxidase 1
NRF2	Nuclear factor erythroid-derived 2-related factor 2
OCN	Osteocalcin
OC-STAMP	Osteoclast-stimulatory transmembrane protein
OP	Osteoporosis
OPG	Osteoprotegerin
OPN	Osteopontin
OSCAR	Osteoclasts-specific activating receptor
OSCs	Organosulfur compounds
OSX	Osterix
P1NP	Procollagen 1 intact N-terminal propeptide
PeCSO	γ-glutamyl-propenyl-L-cysteine sulfoxide
pNPP	*p*-nitrophenyl phosphate
PPAR-γ	Proliferator-activated receptor-γ
PRDX-1	Peroxiredoxin 1
PTH	Parathormone
RAC1	RAC family small GTPase 1
RANK	Receptor activator of nuclear factor κ B
RANKL	Receptor activator for nuclear factor κ B ligand
RUNX-2	Runt-related transcription factor 2
ROS	Reactive oxygen species
RSS	Reactive sulfur species

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SAC	*S*-allylcysteine
SAMC	*S*-allylmercaptocysteine
SAMG	*S*-allylmercaptoglutathione
SFN	Sulforaphane
SIRT	Sirtuin
SMAD-1	SMAD family member 1
STAT3	Signal transducer and activator of transcription 3
Tb.N	Trabecular number
Tb.Th	Trabecular thickness
Tb.Sp	Trabecular space
TRAF-6	Tumor necrosis factor receptor-associated factor 6
TRAP	Tartrate-resistant acid phosphatase
TRAP5b	Tartrate-resistant acid phosphatase 5b
VOSCs	Volatile organosulfur compounds
WISP-1	WNT1-inducible-signaling pathway protein 1
Wnt	Wingless/Integrated
WST-8	Water-soluble tetrazolium-8

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Association between caffeine intake and lumbar spine bone mineral density in adults aged 20–49: A cross-sectional study

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Background: Many epidemiological studies have investigated the connection between coffee intake and bone mineral density (BMD), but the results are controversial. This study aimed to assess the association between caffeine consumption and lumbar BMD in adults aged 20–49.

Methods: From a cross-sectional study based on a large sample of the National Health and Nutrition Examination Survey 2011–2018. After controlling for confounders, the weighted multivariate linear regression model was created and stratified by age, gender, and race for subgroup analysis. In addition, we simultaneously stratified analysis by age and sex and divided caffeine intake into quartiles to assess the association between coffee intake and BMD.

Results: Caffeine intake was not significantly linked with lumbar BMD in this study of 7041 adults. In subgroup studies stratified by age, there was a significant correlation between lumbar BMD and caffeine consumption in participants aged 30–39 and 40–49. In females, there was a positive correlation between lumbar BMD and coffee consumption stratified by gender. When evaluated by race, the association between lumbar BMD and caffeine intake was independent of race. Consequently, when stratifying for age, sex, and coffee intake quartiles, a significant positive correlation was discovered between the fourth coffee intake quartile and lumbar BMD in females aged 30–39. In addition, a negative correlation was discovered between coffee consumption and lumbar BMD in males aged 40–49.

Conclusions: Our research indicates that drinking coffee may benefit 30–39 women's lumbar BMD, but it may adversely affect men aged 40–49.

KEYWORDS

caffeine intake, bone mineral density, NHANES, cross-sectional study, osteoporosis

Introduction

Osteoporosis (OP) is a degenerative disease of the bones that results in weakened bones, weakened microarchitecture, increased fragility, and increased fracture risk (1, 2). Owing to the development of an aging population, osteoporosis has become the most common bone-related chronic disease and the bone metabolic disease with the highest incidence. According to a worldwide survey by the International Society for Clinical Densitometry and the International Foundation for Osteoporosis, more than 70 million Americans will be diagnosed with osteoporosis or bone loss by 2030 (3). The economic burden of osteoporosis-related fractures is significant, costing approximately $17.9 billion annually in the United States (4). Therefore, clinical attention should be focused on identifying modifiable osteoporosis risk factors, such as coffee drinking.

Coffee is one of the most widely consumed beverages in the world nowadays. According to a survey conducted by the National Coffee Association, roughly 64% of adults in the United States drink coffee daily, and approximately 517 million cups of coffee are consumed daily (5). Therefore, researchers pay more attention to the effect of caffeine on human health. Epidemiological research (6–11) indicates that coffee consumption can prevent or decrease the risk of cardiovascular disease, chronic liver conditions, neurodegeneration, and cancer. However, coffee consumption can also adversely affect the human body, such as sleep disturbance, anxiety, and poor pregnancy outcomes (12). Coffee can significantly affect metabolic levels in adults and has been proven in numerous studies to affect metabolic diseases such as obesity and diabetes significantly (13–16).

In the past, a great deal of epidemiological research has been carried out in order to investigate the connection between coffee consumption and BMD. However, the conclusions have been inconsistent. In a Taiwanese longitudinal study, Chang et al. found drinking coffee to have a significantly beneficial relationship with BMD in both males and premenopausal women (17). However, Hallstrom et al. observed that high coffee intake in middle-aged and elderly Swedish women decreased BMD by 2%–4% compared to low coffee intake (18). It is puzzling that Demirbag et al. investigated 200 premenopausal individuals and found no correlation between coffee consumption and BMD (19). These contradictory results may be attributable to their demographic traits, small sample size, bias in data collection, and other aspects. Therefore, we used the National Health and Nutrition Examination Survey (NHANES) database from 2011 to 2018 to conduct a large-scale, broadly representative clinical study on the connection between coffee consumption and BMD to guide clinicians.

Methods

Data source and study population

This study is based on 2011–2018 NHANES data. The NHANES is a nationally representative sample database that assesses the physical and mental well-being of adults and children in the United States. The NHANES has a complicated, multi-stage stratified sample design that covers individuals from various life backgrounds and is representative of the population as a whole. In addition to demographic, socioeconomic, and nutritional questions, the survey also includes physiological measurements and laboratory tests. The information collected by NHANES is used by public health officials, legislators, and clinicians to estimate how common chronic diseases are and to create good public health policies, public health initiatives, and services that protect the health of the population.

We retrieved a total of 39,156 participants from the 2011–2018 NHANES database, and we excluded 11378 subjects older than 49 years and 16,539 subjects younger than 20. Ultimately, 7041 participants were included in our investigation after we eliminated 5493 adults who had missed two 24-hour dietary recall interviews about caffeine use (n = 2869) or lumbar BMD (n = 1329)(Figure 1).

Ethics statement

NHANES requires all survey participants to sign an informed consent form, which is evaluated and authorized by the National Center for Health Statistics Ethics Review Board. After privacy has been protected, the data are now available to the public. It is already feasible to convert data into an analyzed format. All statistics will be used for research methodology, and all research will comply with applicable laws and standards as long as we follow the research's data usage rules.

Covariates

In this study, we used two 24–hour dietary recall interviews to determine how much caffeine each participant drank daily. The first 24-hour dietary recall interview was conducted in person at the Mobile Examination Center (MEC), and the second 24–hour dietary recall interview was conducted by phone 3–10 days after the MEC. All participants were required to complete both interviews. The subjects were evaluated using dual-energy X-ray

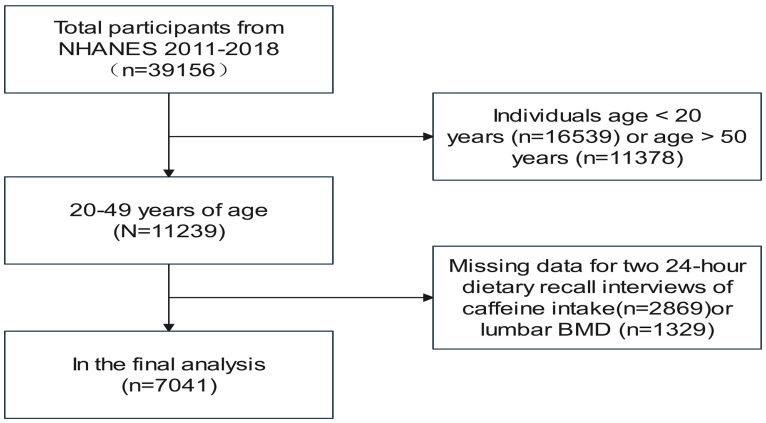

FIGURE 1
Flowchart of participants selection.

absorptiometry (DXA), performed on a Hologic Discovery Model A densitometer (Hologic, Inc., Bedford, Massachusetts) using Apex version 3.2 software. The radiographer who performed the evaluations was trained and certified. A whole-body scan was performed, and the subject's BMD was calculated (Containing lumbar spine BMD information but no femoral neck BMD). In the Body Composition Procedure Manual, which may be found on the NHANES website, there is further information regarding the DXA examination protocol. In addition, we incorporated gender, race/ethnicity, educational level, and moderate exercise as categorical variables in this study. Age, BMI, the ratio of family income to poverty, alkaline phosphatase, blood calcium, blood phosphorus, blood uric acid, total cholesterol, triglyceride, glycohemoglobin, blood urea nitrogen, serum creatinine, urinary albumin creatinine ratio, total albumin, average caffeine intake, lumbar BMD are continuous variables. Visit www.cdc.gov/nchs/nhanes/ to learn more about how covariate data were collected, how two 24-hour recall interviews were conducted, and how lumbar BMD outcome variables were measured.

Statistical analyses

All of the estimates in this investigation were computed using sample weights, following recommended by the NCHS. Continuous variables are shown as the Mean ± SD, and the weighted multiple linear regression model was utilized to determine whether there were differences between the various groupings of continuous variables. The chi-square test results were analyzed to see whether or not there was a significant difference between the categorical variable components. The results of the test are reported as %. Following the recommendations provided by Reporting on Observational Studies in Strengthening Epidemiology, three weighted multivariate linear regression models were created to acquire a more comprehensive understanding of the association between coffee consumption and BMD. Model 1 was left untouched. Model 2 has been tweaked to consider age, gender, and race/ethnicity. Model 3 adjusted for all confounders. We did subgroup analyses based on age, gender, and race to get the most out of these data and investigate further the association between coffee consumption and lumbar BMD. The EmpowerStats (http://www.empowerstats.com) and R (http://www.r-project.org) were utilized for every statistical analysis. In general, we regarded $P < 0.05$ as statistically meaningful.

Results

Table 1 provides the weighted characteristics of the study samples, which include demographic and medical characteristics. The research included 7041 subjects, with 3565 males and 3476 females participating. The average age of men was 33.65 ± 8.62 years old, and that of women was 34.42 ± 8.84 years old, and the difference was statistically meaningful. The daily caffeine intake of male participants was 0.15 ± 0.18 g, and that of female participants was 0.13 ± 0.15 g, and the difference was also statistically meaningful. Furthermore, the difference still existed in educational level, ratio of family income to poverty, moderate activity, alkaline phosphatase, blood calcium, total cholesterol, blood uric acid, triglyceride, blood urea nitrogen, blood creatinine, glycohemoglobin, lumbar BMD and total protein. No statistically significant differences were observed between the male and female participants in terms of,race or ethnicity, BMI, blood phosphorus, and the urine albumin creatinine to ratio.

Table 2 presents three weighted multivariate linear regression models. In Model 1, without adjustment for variables, there was a statistically negative association between caffeine consumption

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TABLE 1 Weighted characteristics of the study sample.

	Male (n=3565)	Female (n=3476)	*P* value
Age (years)	33.65 ± 8.62	34.42 ± 8.84	< 0.001
Race/ethnicity (%)			0.261
White	57.74	58.06	
Black	11.6	12.89	
Mexican American	12.4	11.61	
Other race	18.25	17.44	
Educational level, n (%)			< 0.001
Less than high school	12.6	11.37	
High school	23.4	17.96	
More than high school	64	70.67	
Ratio of family income to poverty (%)	2.90 ± 1.62	2.72 ± 1.61	< 0.001
Moderate activities (%)			<0.001
No	25.87	29.9	
Yes	74.13	70.1	
Body mass index (kg/m^2)	28.72 ± 6.22	28.99 ± 7.62	0.104
Alkaline phosphatase (u/l)	66.96 ± 21.76	63.42 ± 21.16	< 0.001
Serum calcium (mmol/l)	2.37 ± 0.08	2.32 ± 0.08	< 0.001
Serum phosphorus (mmol/l)	1.20 ± 0.19	1.21 ± 0.17	0.054
Serum uric acid (umol/l)	361.15 ± 71.95	272.74 ± 61.75	< 0.001
Total cholesterol (mmol/l)	4.92 ± 1.02	4.79 ± 0.90	< 0.001
Triglyceride (mmol/l)	1.93 ± 1.72	1.40 ± 1.20	< 0.001
Glycohemoglobin (%)	5.41 ± 0.77	5.37 ± 0.71	0.026
Blood urea nitrogen (mmol/l)	4.80 ± 1.39	4.04 ± 1.33	< 0.001
Serum creatinine (umol/l)	85.06 ± 16.67	65.22 ± 20.21	< 0.001
Urinary albumin creatinine ratio (mg/g)	18.81 ± 215.08	23.62 ± 123.91	0.257
Total protein (g/l)	72.30 ± 4.14	71.21 ± 4.06	< 0.001
Average caffeine intake (g/day)	0.15 ± 0.18	0.13 ± 0.15	< 0.001
Lumbar bone mineral density (g/cm^2)	1.03 ± 0.15	1.06 ± 0.14	< 0.001

Continuous variables are presented as Mean ± SD, P-value was calculated by a weighted linear regression model. Categorical variables are presented as %, P-value was calculated by chi-square test.

and lumbar BMD. As for Model 2 with partial adjustment for covariates and model 3 with adjustment for all variables, average caffeine intake and lumbar BMD were linked, but the link was not statistically significant. When stratified by age, average caffeine consumption was positively and significantly associated with lumbar BMD in adults aged 30–39 and 40–49. Average caffeine intake in female adults was significantly positively associated with lumbar BMD when stratified by gender. Coffee intake and lumbar BMD were not associated with race when evaluated by race.

Table 3 displays the correlation between average caffeine consumption and BMD stratified by age and sex. Caffeine consumption was found to be positively related to lumbar BMD in females aged 30–39 and negatively related to lumbar BMD in males aged 40–49.By further stratifying the average caffeine intake, we studied the relationship between caffeine intake and lumbar bone mineral density and took the lowest quartile of caffeine intake as the control group. The trend between the quartile of average caffeine intake and lumbar BMD remained significant in the two subgroups.Notably, in the subgroup of women aged 30 – 39 years, the fourth quartile was significantly different from the lowest group, but no difference was found among the four quartile in the subgroup of men aged 40 – 49 years.

Discussion

Coffee is the most popular beverage in the world. Osteoporosis is a common endocrine and metabolic disease. BMD is one of the most important diagnostic indexes of OP. Many epidemiological studies have been conducted to investigate the connection between coffee consumption and BMD, but the conclusions are controversial (17, 18, 20). As a result, we used the NHANES database to perform this extensive, representative cross-sectional study.

From the NHANES 2011–2018, we chose a representative sample of 9041 adults aged 20–49. In subgroups stratified by age, according to our data, a significant correlation existed between the consumption of coffee and lumbar BMD in

Table 2 Association between average caffeine intake (g/day) and lumbar bone mineral density (g/cm^2).

Exposure	Model 1, β (95% CI)	Model 2, β (95% Cl)	Model 3, β (95% CI)
Average caffeine intake (g/day)	-0.027 (-0.048, -0.007)**	-0.002 (-0.023, 0.019)	0.000 (-0.021, 0.021)
Stratified by age			
20–29 years old	-0.015 (-0.057, 0.028)	0.016 (-0.027, 0.058)	0.022 (-0.020, 0.064)
30–39 years old	0.009 (-0.028, 0.046)*	0.040 (0.002, 0.077)	0.040 (0.002, 0.077)*
40–49 years old	-0.054 (-0.086, -0.022)**	-0.035 (-0.068, -0.003)*	-0.033 (-0.065, -0.001)*
Stratified by gender			
Male	-0.055 (-0.082, -0.028)***	-0.022 (-0.050, 0.006)	-0.019 (-0.046, 0.009)
Female	0.036 (0.005, 0.068)*	0.039 (0.005, 0.072)*	0.037 (0.004, 0.070)*
Stratified by race			
White	-0.004 (-0.035, 0.027)	0.006 (-0.026, 0.038)	0.009 (-0.022, 0.040)
Black	0.026 (-0.063, 0.115)	0.016 (-0.075, 0.107)	0.010 (-0.081, 0.100)
Mexican American	-0.066 (-0.141, 0.009)	-0.027 (-0.104, 0.051)	-0.038 (-0.117, 0.041)
Other race	-0.026 (-0.064, 0.012)	-0.021 (-0.059, 0.017)	-0.024 (-0.062, 0.014)

Model 1: All variables were not adjusted.
Model 2: Age, sex, race were adjusted.
Model 3: All variables were adjusted.
The model is not adjusted for the stratification variable itself in the subgroup analysis.
*P<0.05, **P<0.01, ***P<0.001.

individuals aged 30–39 and 40–49. In subgroups stratified by gender, we found that association between caffeine intake and bone mineral density is also influenced by gender. The three models failed to find this connection in subgroups stratified by race. Since the association between the amount of caffeine consumed and the BMD of the lumbar spine varies depending on age and gender, we further analyzed and stratified gender and age simultaneously. We found that women aged 30–39 had a significant positive association between coffee consumption and BMD and that in this group. However, in 40–49 years old men subgroup, we found a negative correlation.

TABLE 3 Association between Average caffeine intake (g/day) and lumbar bone mineral density (g/cm^2), stratified by age and gender.

Quintiles of average caffeine intake (g/day)	Lumbar bone mineral density (g/cm^2), β (95% Cl)	
	Male	Female
20–29 years old	0.025 (-0.029, 0.078)	0.056 (-0.012, 0.125)
Lowest quartiles	reference	reference
2nd	0.004 (-0.017, 0.025)	-0.007 (-0.026, 0.012)
3rd	-0.003 (-0.024, 0.019)	0.006 (-0.013, 0.026)
4th	-0.019 (-0.042, 0.003)	0.014 (-0.008, 0.036)
P for trend	0.096	0.158
30–39 years old	0.023 (-0.024, 0.071)	0.080 (0.019, 0.142)*
Lowest quartiles	reference	reference
2nd	-0.006 (-0.033, 0.020)	0.009 (-0.014, 0.033)
3rd	-0.012 (-0.037, 0.012)	0.012 (-0.013, 0.036)
4th	0.003 (-0.020, 0.026)	0.025 (0.000, 0.050)*
P for trend	0.748	0.049
40–49 years old	-0.070 (-0.113, -0.026)**	0.007 (-0.041, 0.054)
Lowest quartiles	reference	reference
2nd	0.001 (-0.032, 0.033)	0.013 (-0.015, 0.040)
3rd	0.016 (-0.018, 0.049)	0.020 (-0.007, 0.047)
4th	-0.027 (-0.058, 0.004)	0.011 (-0.016, 0.037)
P for trend	0.026	0.553

All variables were adjusted.
*P<0.05, **P<0.01.

The effect of coffee consumption on BMD is controversial as one of the three most popular beverages in the world. In a clinical study of 4066 postmenopausal women, Choi et al. analyzed by multivariate logistic regression, adjusting for all confounding factors, that the highest coffee intake group had a 36% lower prevalence of osteoporosis than the lowest group ($P = 0.015$) (21). In a prospective study with up to 30 years of follow-up, researchers randomly selected 7495 men aged 46–56 and found that coffee consumption reduced the incidence of hip fractures. Researchers have found that males who drink coffee have a lower risk of fractures (22). Similarly, Yu et al. demonstrated in a large, community-based cross-sectional study that moderate coffee consumption decreases the prevalence of osteoporosis in men (23). In a recent meta-analysis of approximately 400,000 participants, researchers found that people with high coffee intake had a lower risk of osteoporosis than those with low coffee intake [OR (95% CI): 0.79 (0.65–0.92)] (24). A Hong Kong OP study showed that the serum metabolite levels of caffeine were significantly correlated with BMD, which to some extent, confirmed our findings (25). As for the potential pathophysiological aspects of the effect of coffee consumption on BMD. In a recent study, Berman et al. showed that caffeine regulates osteoblast/osteoclast differentiation through nonspecific antagonism of adenosine receptors (26). Ankita et al. suggested that the physiological release of purine in the extracellular space plays an important role in bone homeostasis, both by acting on membrane receptors directly affecting osteocytes and by synergistic stimulation with some osteoactive hormones (27). In addition, researchers also believe that caffeine affects bone metabolism through mechanisms such as regulating calcium and altering the lipid profile (28). Consistent with previous studies, we believe that young women who consume coffee can improve their lumbar spine BMD. However, the finding that men aged 40–49 years who drink coffee may have a potential risk of lumbar spine BMD contradicts previous studies, and we did not find an association after stratifying all variables only by sex, so the conclusions of this male-specific study need to be cautiously interpreted.

Nevertheless, there are some concerns with our study. People older than 50 years were not included in this study due to a scarcity of data, resulting in these subjects being excluded from the analysis, which severely limits the possibility of considering the basic endpoint of fragility fracture risk when referring to "bone health." This is one of the major limitations of this study. Because this was a cross-sectional study, it was not possible to determine whether or not there was a causal connection between the use of coffee and lumbar spine BMD. Due to the limitations of the questionnaire, personal eating habits and lifestyles could not be assessed, and the menstrual conditions, sex hormone levels, and drug use of female participants may have affected the study's findings. Levels of bioactive were unknown. The specific mechanism of coffee action is not involved in this study. This study has many covariates, and there may be some collinearity between them. In addition, hip femoral neck BMD was not included in this study due to data limitations. Despite these limitations, it is undeniable that our study's sample size is significant and that it represents a sophisticated, stratified, multi-stage probabilistic sample of the non-institutionalized American population. In terms of survey techniques and quality assurance, NHANES is of high caliber. Additionally, after adjusting for various confounders, we performed weighted multiple linear regression analysis and subgroup analyses stratified by age, gender, and ethnicity to assess the effect of coffee consumption on BMD. Most importantly, the findings of this study provide new information for patients with osteoporosis or low bone mass since they demonstrate that drinking coffee has a beneficial impact on BMD in young women. For future research on this topic, due to the lack of data on people over 50 years of age in this study, we suggest that future studies take more into account factors such as menopause and old age. Considering the limitations and shortcomings of our study, we recommend that more prospective studies be conducted in the future to confirm the causal relationship between caffeine and BMD. In addition, coffee contains various active ingredients, and future studies should fully consider the effects of other minerals and other factors on bone.

In conclusion, according to this study, drinking coffee may benefit women aged 30–39 lumbar BMD, but may negatively affect men aged 40–49. Although our study is focused on caffeine, in our daily life, we also need to pay attention to the differences in the content of minerals, lipids, proteins, and other substances in different coffee powders, which may also have an impact on bone health, although it is difficult to weight them because they are not easy to calculate and consider in observational studies.

Data availability statement

Publicly available datasets were analyzed in this study. The survey data can be found here: www.cdc.gov/nchs/nhanes/.

Ethics statement

NHANES protocols were approved by the Research Ethics Review Board of NCHS, and Written informed consent was obtained from each participant.

Author contributions

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

References

1. Gregson CL, Armstrong DJ, Bowden J, Cooper C, Edwards J, Gittoes N, et al. Correction: UK clinical guideline for the prevention and treatment of osteoporosis. *Arch Osteoporos* (2022) 17:80. doi: 10.1007/s11657-022-01115-8

2. Gonzalez RE, Debrach-Schneider AC, Lamy O. [Osteoporosis]. *Rev Med Suisse* (2022) 18:56–8. doi: 10.53738/REVMED.2022.18.764-65.56

3. Clynes MA, Westbury LD, Dennison EM, Kanis JA, Javaid MK, Harvey NC, et al. Bone densitometry worldwide: A global survey by the ISCD and IOF. *Osteoporos Int* (2020) 31:1779–86. doi: 10.1007/s00198-020-05435-8

4. Clynes MA, Harvey NC, Curtis EM, Fuggle NR, Dennison EM, Cooper C. The epidemiology of osteoporosis. *Br Med Bull* (2020) 133:105–17. doi: 10.1093/bmb/ldaa005

5. Feng J, Wang J, Jose M, Seo Y, Feng L, Ge S. Association between caffeine intake and all-cause and cause-specific mortality: An analysis of the national health and nutrition examination survey (NHANES) 1999-2014 database. *Nurs Rep* (2021) 11:901–12. doi: 10.3390/nursrep11040083

6. Mansour A, Mohajeri-Tehrani MR, Samadi M, Qorbani M, Merat S, Adibi H, et al. Effects of supplementation with main coffee components including caffeine and/or chlorogenic acid on hepatic, metabolic, and inflammatory indices in patients with non-alcoholic fatty liver disease and type 2 diabetes: A randomized, double-blind, placebo-controlled, clinical trial. *Nutr J* (2021) 20:35. doi: 10.1186/s12937-021-00694-5

7. Sartini M, Bragazzi NL, Spagnolo AM, Schinca E, Ottria G, Dupont C, et al. Coffee consumption and risk of colorectal cancer: A systematic review and meta-analysis of prospective studies. *Nutrients* (2019) 11:694. doi: 10.3390/nu11030694

8. Wijarnpreecha K, Thongprayoon C, Ungprasert P. Coffee consumption and risk of nonalcoholic fatty liver disease: A systematic review and meta-analysis. *Eur J Gastroenterol Hepatol* (2017) 29:e8–e12. doi: 10.1097/MEG.0000000000000776

9. Grioni S, Agnoli C, Sieri S, Pala V, Ricceri F, Masala G, et al. Espresso coffee consumption and risk of coronary heart disease in a large Italian cohort. *PloS One* (2015) 10:e0126550. doi: 10.1371/journal.pone.0126550

10. Wu L, Sun D, He Y. Coffee intake and the incident risk of cognitive disorders: A dose-response meta-analysis of nine prospective cohort studies. *Clin Nutr* (2017) 36:730–6. doi: 10.1016/j.clnu.2016.05.015

11. Liu D, Li ZH, Shen D, Zhang PD, Song WQ, Zhang WT, et al. Association of sugar-sweetened, artificially sweetened, and unsweetened coffee consumption with all-cause and cause-specific Mortality: A Large prospective cohort study. *Ann Intern Med* (2022) 175:909–17. doi: 10.7326/M21-2977

12. Wikoff D, Welsh BT, Henderson R, Brorby GP, Britt J, Myers E, et al. Systematic review of the potential adverse effects of caffeine consumption in healthy adults, pregnant women, adolescents, and children. *Food Chem Toxicol* (2017) 109:585–648. doi: 10.1016/j.fct.2017.04.002

13. Harty PS, Stratton MT, Escalante G, Rodriguez C, Dellinger JR, Williams AD, et al. Effects of bang® keto coffee energy drink on metabolism and exercise performance in resistance-trained adults: A randomized, double-blind, placebo-controlled, crossover study. *J Int Soc Sports Nutr* (2020) 17:45. doi: 10.1186/s12970-020-00374-5

14. Pechmann LM, Petterle RR, Moreira CA, Borba VZC. Osteosarcopenia and trabecular bone score in patients with type 2 diabetes mellitus. *Arch Endocrinol Metab* (2021) 65:801–10. doi: 10.20945/2359-3997000000418

15. Lee CY, Back GY, Lee SH. Relationship between type 2 diabetes mellitus and lumbar bone mineral density in postmenopausal women. *Asian Spine J* (2021) 15:721–7. doi: 10.31616/asj.2021.0099

16. Cherukuri L, Kinninger A, Birudaraju D, Lakshmanan S, Li D, Flores F, et al. Effect of body mass index on bone mineral density is age-specific. *Nutrition Metab Cardiovasc Dis* (2021) 31:1767–73. doi: 10.1016/j.numecd.2021.02.027

17. Chang HC, Hsieh CF, Lin YC, Tantoh DM, Ko PC, Kung YY, et al. Does coffee drinking have beneficial effects on bone health of Taiwanese adults? a longitudinal study. *BMC Public Health* (2018) 18:1273. doi: 10.1186/s12889-018-6168-0

18. Hallström H, Byberg L, Glynn A, Lemming EW, Wolk A, Michaëlsson K. Long-term coffee consumption in relation to fracture risk and bone mineral density in women. *Am J Epidemiol* (2013) 178:898–909. doi: 10.1093/aje/kwt062

19. Demirbag D, Ozdemir F, Ture M. Effects of coffee consumption and smoking habit on bone mineral density. *Rheumatol Int* (2006) 26:530–5. doi: 10.1007/s00296-005-0020-4

20. Rychter AM, Ratajczak AE, Szymczak-Tomczak A, Michalak M, Eder P, Dobrowolska A, et al. Associations of lifestyle factors with osteopenia and osteoporosis in polish patients with inflammatory bowel disease. *Nutrients* (2021) 13:1863. doi: 10.3390/nu13061863

21. Choi E, Choi KH, Park SM, Shin D, Joh HK, Cho E. The benefit of bone health by drinking coffee among Korean postmenopausal women: A cross-sectional analysis of the fourth & fifth Korea national health and nutrition examination surveys. *PloS One* (2016) 11:e0147762. doi: 10.1371/journal.pone.0147762

22. Trimpou P, Landin-Wilhelmsen K, Odén A, Rosengren A, Wilhelmsen L. Male Risk factors for hip fracture-a 30-year follow-up study in 7,495 men. *Osteoporos Int* (2010) 21:409–16. doi: 10.1007/s00198-009-0961-7

23. Yu Q, Liu ZH, Lei T, Tang Z. Subjective evaluation of the frequency of coffee intake and relationship to osteoporosis in Chinese men. *J Health Popul Nutr* (2016) 35:24. doi: 10.1186/s41043-016-0060-2

24. Zeng X, Su Y, Tan A, Zou L, Zha W, Yi S, et al. The association of coffee consumption with the risk of osteoporosis and fractures: A systematic review and meta-analysis. *Osteoporos Int* (2022) 33:1871–93. doi: 10.1007/s00198-022-06399-7

25. Chau Y-P, Au PCM, Li GHY, Sing C-W, Cheng VKF, Tan KCB, et al. Serum metabolome of coffee consumption and its association with bone mineral density: The Hong Kong osteoporosis study. *J Clin Endocrinol Metab* (2020) 105:dgz210. doi: 10.1210/clinem/dgz210

26. Berman NK, Honig S, Cronstein BN, Pillinger MH. The effects of caffeine on bone mineral density and fracture risk. *Osteoporos Int* (2022) 33:1235–41. doi: 10.1007/s00198-021-05972-w

27. Agrawal A, Jørgensen NR. Extracellular purines and bone homeostasis. *Biochem Pharmacol* (2021) 187:114425. doi: 10.1016/j.bcp.2021.114425

28. Xu H, Liu T, Hu L, Li J, Gan C, Xu J, et al. Effect of caffeine on ovariectomy-induced osteoporosis in rats. *Biomed Pharmacother* (2019) 112:108650. doi: 10.1016/j.biopha.2019.108650

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Evaluation of food and nutrient intake in a population of subjects affected by periodontal disease with different levels of bone mineral density

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Introduction: Both osteoporosis and periodontitis are pathologies characterized by an imbalance in the bone tissue. Vitamin C is an important factor involved in maintaining the health of the periodontium; its deficiency causes characteristic lesions to periodontal tissues such as bleeding and redness of the gums. Among the essential minerals for the health of the periodontium we find instead calcium.

Objectives of the study: The objectives of the proposed study are to study the association between the presence of osteoporosis and periodontal disease. We tried to identify the possible connections between particular dietary patterns and therefore the etiopathogenesis of periodontal disease and secondarily of osteoporosis.

Materials and methods: 110 subjects were recruited in a single-center observational cross-sectional study carried through the collaboration between the University of Florence and the private institute of dentistry Excellence Dental Network based in Florence, suffering of periodontitis, 71 osteoporotic/osteopenic and 39 non-osteoporotic/osteopenic. Anamnestic data and information on eating habits were collected.

Results: The population showed eating habits that do not meet the intake levels recommended by the L.A.R.N. Regarding the relationship between nutrient intake and plaque index, it appears that in the population, the higher the intake of vitamin C through food, the lower the plaque index value is. This result could reinforce the scientific evidence that there is a protective factor in the onset of periodontal disease by the consumption of vitamin C which to date is still the subject of investigation. In addition, the same type of trend would also have been observed for calcium intake, but a larger sample size would be required to make this effect significant.

Conclusions: The relationship between osteoporosis and periodontitis and the role of nutrition in influencing the evolution of these pathologies still seems to be

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deeply explored. However, the results obtained seem to consolidate the idea that there is a relationship between these two diseases and that eating habits play an important role in their prevention.

KEYWORDS

periodontitis, osteoporosis, nutrients intakes, vitamin C, plaque index (PI), food frequency questionnaire (FFQ), food intake

Introduction

Periodontitis is a chronic inflammatory disease in which bacterial infection of periodontal tissues is necessary and sufficient for the onset and progression of the oral pathology. Yet, numerous other factors negatively affect the course of the disease, like smoking, hormonal changes, endocrine or systemic comorbidities, and poor oral hygiene. It is a condition with poly-microbial etiology which specifically affects the supporting tissues of the teeth. According to the Global Burden of Disease 2010, the global prevalence of severe periodontitis between 1990 and 2010, standardized by age, was 11.2%, i.e. the sixth most widespread disease in the world (1). Age-standardized incidence of the more severe forms in 2010 was similar to that of 1990, with 701 cases per 100,000 individuals per year. Prevalence gradually increased with age, showing a large increase between the third and fourth decades of life, with a peak at about 38 years old.

Osteoporosis is a systemic skeletal disease characterized by a reduction in bone mass and qualitative changes in the material properties of the macro- and micro-architecture of the bones, this leading to an increased risk of fracture even in case of minor traumas (2). Nowadays, densitometry allows to accurately measure the bone mass and BMD (bone mineral density) in g/cm^2 of bone surface, a property responsible for 60-80% of the mechanical resistance of a bone. According to the WHO (World Health Organization), diagnosis of osteoporosis made by densitometry relies on the assessment of bone mineral density through dual-energy x-ray absorptiometry (DXA) and on its comparison with the average value in healthy adult subjects. Standard deviation from the average peak of bone mass (T-score) is used as unit of measurement. This procedure represents the diagnostic test for osteoporosis and risk of fracture (2). To this regard, it has been observed that risk of fracture starts to exponentially increase with densitometry values of T-score <-2.5 SD, which indeed represents the threshold for diagnosing the presence of osteoporosis.

QUS systems (calcaneus Ultrasonography) are techniques used more and more commonly for densitometry investigations within population groups. Through the measurement of ultrasound variables applied to the calcaneus, they provide a clinical parameter known as Stiffness Index. The Stiffness Index describes the risk of osteoporotic fracture for post-menopausal women comparable to the BMD measured by DXA of the spine or hip.

After reviewing several studies performed using the QUS systems, the FDA approved the use of this procedure for the prevention from the risk of hip fracture, comparable to the DEXA study of the hip/spine.

Based on these diagnostic criteria, approximately 6% of men and 22% of women between 50 and 84 years old in the EU have osteoporosis, i.e. 27.6 million people (3).

Both osteoporosis and periodontitis are characterized by an imbalance between bone tissue resorption and bone tissue neogenesis, which finally results in bone tissue loss. Furthermore, recent studies suggested the possibility that these two pathologies are inter-connected, playing a role as reciprocal risk factors (4) (5).

Evaluating the possible implications that different diets could have on the oral bacterial population is an aspect still poorly investigated.

A large number of nutrients impact periodontal health, among these macro and micro-elements must be distinguished.

With reference to periodontal disease, the most important macronutrients (carbohydrates, proteins and lipids) are carbohydrates, which are involved in the progressing of periodontal disease associated with dental caries. In fact, high carbohydrate intake seems to promote oral dysbiosis, while its reduction decreases gingival inflammation. Furthermore, diets rich in saturated fats, known to increase oxidative stress, should be avoided in order to prevent the onset of periodontitis (6). To this regard, a higher body fat content has been associated with an increased gingival bleeding in older patients. On the other hand, polyunsaturated fats (such as omega 3) have shown a positive effect on periodontium conditions (7). Other studies conducted instead on protein deprived animals have resulted in the rupture of periodontal ligaments, degeneration of gingival tissues, and resorption of the alveolar bone (8). Another study has finally suggested an inverse relationship between high protein intake and periodontitis (9).

Strong association has also been reported between periodontal disease and obesity (10), a problem that has long been underestimated. Obesity has been indeed identified as risk factor for a number of systemic diseases with inflammatory background. A longitudinal study identified a positive correlation between BMI and the incidence of periodontal disease (6).

The correlation between nutrition and periodontal disease is for various reasons rather uncertain. However, although dental bacterial plaque is accepted as the main causative agent, incorrect dietary can affect both onset and course of the disease.

Several studies have demonstrated the influence of dental plaque as the main etiological factor for gingival inflammation, noting worsening of gingivitis when study participants stopped oral hygiene procedures (11) (12) (13). The authors therefore concluded that the gingivitis experimental protocol is not applicable if the diet does not include refined carbohydrates. Diet seems then to have a

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strong influence on the gum and on the inflammatory reaction of the periodontal. These effects could affect both tissue repair and immune system mechanisms, which are affected by:

- Decrease in the phagocytic activity of granulocytes;
- Modification of the immune response;
- Alteration in the synthesis of prostaglandins;
- Epithelial changes.

However, although dietary imbalance is not sufficient to induce periodontal disease without the simultaneous presence of the bacterial plaque, it can likewise affect its severity and extension, altering the resistance of the host organism and the regenerative capability of the tissues.

Nutritional recommendations to keep the periodontium in health include reduction of carbohydrate consumption and supplementation of omega 3 fatty acids, vitamin C, vitamin D, antioxidants and fibers (14). *In vitro* studies have shown that vitamin C and D intake may play an important role in the prevention of gingivitis and periodontal inflammation (15).

Evidence in the recent literature reinforces the concept of how important is vitamin D for periodontal health. Periodontal disease seems to be correlated with low vitamin D serum levels; in a cohort of pregnant women over 20 weeks of gestation from the University Hospital "Maggiore della Carità", Novara, Italy, the authors assessed serum levels of vitamin D and oral health status through the following indexes: Oral Hygiene Index (OHI), Plaque Control Record (PCR), Gingival Bleeding Index (GBI), and Community Periodontal Index of Treatment Needs (CPTIN). They finally found strong correlation between low serum levels of vitamin D and the idexes that identify periodontal disease (16).

Another relevant study indicates that Breast cancer (BC) survivors treated with aromatase inhibitors (AIs) commonly show several pathological issues, including poor oral health, bone health impairment, and vitamin D deficiency. This study evaluates the correlation between oral health and vitamin D status in BC survivors undergoing treatment with AIs through a machine learning approach. It's showed a significant correlation between DMFT and vitamin D levels; the regression machine learning model showed that vitamin D status and the use of dental floss were the most relevant variables in terms of correlation with Filled Permanent Teeth Index (DMFT). Vitamin D deficiency, inadequate use of dental floss have a negative impact on oral health in BC women (17).

Vitamin C is an important factor for maintaining the health of the periodontium. Its deficiency causes characteristic lesions of the periodontal tissues, such as bleeding and redness of the gums. *In vitro* studies suggest that local applications of vitamin C and magnesium salt decrease inflammation at the level of the gingival fibroblasts (18).

Among the essential minerals for the health of the periodontium there is calcium, which is essential for calcified tissues such as bones and teeth. Deficiencies of this mineral can affect the health of the periodontium. A Danish study demonstrated that a higher dairy intake decreases the severity of periodontitis in adulthood (19).

The aim of this work is therefore to study the association between the presence of osteoporosis and periodontal pathology. We did it by identifying possible connections between specific diets and the etio-pathogenesis of periodontal disease, primarily, and osteoporosis, secondarily.

Materials and methods

In the scope of a monocentric cross-sectional observational study carried out from November 2019 to December 2021 through the collaboration between the University of Florence and the private dentistry institute Excellence Dental Network based in Florence, were recruited 110 subjects (36 males and 73 females) affected by periodontitis, 71 of which also affected by osteoporosis or osteopenia. Detailed protocol was submitted to and approved by the Ethics Committee.

Criteria for the inclusion in the study were (1) written and signed declaration of informed consent, (2) age ≥ 18 years old, for both sexes, and (3) the diagnosis of periodontitis.

Exclusion criteria were instead (1) to have done antibiotic therapies or (2) steroid therapies in the past 3 months prior to the beginning of the study, (3) presence of parathyroid diseases or (4) diseases related to bone metabolism (except osteoporosis), and (5) development of neoplastic pathologies in the last five years. Subjects presenting eating disorders or pregnancy were also excluded.

Participants, all attending the abovementioned dental institute and all respecting the above listed inclusion criteria, were introduced to the project and provided of the relative documentation (patient information, informed consent, privacy policy). Data was collected anonymously, through the assignment of a specific alphanumeric code to each participant, organized in an electronic database and stored for the purposes expressed in the following scientific research. Two questionnaires for the collection of anamnestic information were administered to the participants, one for information related to the existence of previous pathologies and risk factors for osteoporosis, one for information on eating habits, with particular attention to vitamins and minerals diet intake. An attendance questionnaire already validated by Montomoli and collaborators was also administered (20). Information about potential intake of supplements has also been recorded. The questionnaire consisted of sixteen questions related to nutrient intake. Food selection included in the questionnaire was based on data obtained by the Italian Institute of Nutrition, related to the composition of the Italian diet, to the frequency with which foods are consumed, and their relative importance as sources of calcium. Regarding cheese, more questions were asked to better identify the types of cheese consumed. Main food classes were included in the questionnaire: cereals (pasta, rice, bread and similar and potatoes), fish-meat, eggs, legumes, vegetables, and fruit. Two questions related to the consumption of sweets rich in calcium that are commonly consumed by the Italian population, such as milk-based ice cream and milk chocolate, were also present. Finally, contribution to calcium intake from drinking water was also carefully evaluated, as it can represent an important source of this mineral. A list of the most consumed calcium-rich mineral waters commercialized in Italy has been attached to the Food frequency Questionnaire (FFQ). For each question, participants were also asked to indicate the amount of

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product consumed, selecting between small, medium, or large portion. In addition to calcium intake, the questionnaire also aimed to estimate the participants' intake of other macro and micro nutrients important for the health of bones and teeth, i.e. carbohydrates, proteins, lipids, phosphorus, sodium, iron, magnesium, potassium, selenium, zinc, vitamin C, vitamin D, and vitamin B12.

All data collected by the qualified staff of the IRF institute in Microdentistry and the Biomolecular Diagnostic laboratory were transcribed and archived in a database suitably prepared based on the purposes of this study:

- bone mass: those patients who were advised by the dental staff for assessment of the level of bone mineral density performed an ultrasound analysis (QUS) during the same dental visit. Such analysis was used by the dental staff as a primary screening tool to evaluate whether to send the subject for further mineralometric investigations. In case patients were sent for mineralometric analysis (MOC), bone mineral density (BMD) and lumbar and femoral T-score were measured and recorded.
- grading of periodontal disease: the dental staff probed the depth of the periodontal pockets, gingival recession, subgingival tartar, pus and bleeding, dental motility, and plaque index. Subjects were classified as suffering from either aggressive or chronic periodontitis (21).

Statistical analysis was carried out using SPSS software and Microsoft Excel. Quantitative data derived from the analysis of parameters relating to osteoporosis, periodontal disease and dietary habits (t-score, bacterial concentrations, frequency of food consumption, etc.) were described through the use of statistical indices of central tendency mean and variability (minimum, maximum, range, standard deviation). For qualitative data, most suitable descriptive statistics (frequency distributions, relative frequencies, etc.) were presented.

After classifying the patients on the basis of the presence or absence of osteoporosis/ostepenia, groups were compared in relation to a series of quantitative variables (bacterial concentrations, daily calcium intake, etc.) using the most suitable statistical tests for independent samples (or an equivalent non-parametric test in the case of non-normal distributions). In addition, the association between osteoporosis/osteopenia and qualitative variables (presence of periodontitis, eating habits) was evaluated.

After having classified patients according to the severity of periodontal disease (mild, moderate, severe), groups were compared in relation to a series of quantitative variables (t-score, blood parameters of bone metabolism) by ANOVA and consequent *post-hoc* corrected with the Bonferroni method (or an equivalent non-parametric test in the case of non-normal distributions). In addition, the association between the severity of periodontal disease and qualitative variables (presence of osteoporosis/osteopenia, eating habits, etc.) was evaluated.

Results

Descriptive statistics of the study population

Over the three-year project, 181 individuals were asked to take part in the study, 110 of which agreed to participate. The others refused to participate because not interested.

All 110 participants reported data about the mineralometric and metabolic condition of their bones, genetic analyses and microbiological features of their periodontal.

Only 44 people were finally able to provide anamnestic information and eating habits, this likely because of the unfortunate ongoing pandemic situation, which prevented direct contact with the subjects. Participants were thus contacted by telephone in order to have answers to the questions. Many subjects, at the time of the call, were either not found or no longer interested in participating to the project.

The descriptive data collected are shown below. Overall, we obtained data from 110 subjects, 36 males and 74 females.

Average age and anthropometry

The average age across the whole sample was 55 years old, while in regard to the anthropometric characteristics the average weight was 67 kg, the average height 1.68 m, and BMI 23.65, i.e. normal weight (Table 1).

Bone turnover marker and bone mineralometry

Mineralometric data were collected from the entire sample of subjects, obtained through computerized bone mineralometry (MOC) or calcaneus ultrasound (QUS). When possible, data on blood concentration of 25OHD3, PTH, calcium, phosphatemia, alkaline phosphatase, and bone alkaline phosphatase, were also collected (Table 2).

TABLE 1 Average age and anthropometry.

Age and anthropometry	N	Minimum	Maximum	Average	std. deviation	Asymmetry
	Statistics	Statistics	Statistics	Statistics	Statistics	Statistics
Age	110	27	101	55,22	13,208	,478
Weight (Kg)	107	41,0	120,0	67,250	15,0090	,835
Height (m)	107	1,53	1,89	1,6832	,08513	,271
BMI	107	15,6226	41,9143	23,652578	4,6736212	1,449

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On average, the value detected for 25OHD3 is insufficient if compared to the reference values.

The values of PTH, calcium, phosphatemia, alkaline phosphatase and bone alkaline phosphatase are instead in the average when compared to the reference values.

The average t-scores detected in the lumbar and femoral area through MOC examination fall below the desirable values, while the values detected by ultrasonography at the heel are on average in an optimal range, albeit at the limit.

35.5% of the sample had normal mineralometric values, 48.2% had values tending to osteopenia and 16.4% had values testifying a condition of osteoporosis (Table 3).

TABLE 3 Mineralometric examination result.

Exam result	Frequency	Valid percentage
normal	39	35,5
osteopenia	53	48,2
osteoporosis	18	16,4
Total	110	100,0

Grading of periodontal disease

Classification, pocket depth (PPD), gingival recession (REC), plaque index

The classification of the severity of periodontal disease was obtained using the classification proposed by Amitage GC (21).

The analysis shows that patients always have generalized forms of periodontitis, in which chronic forms prevail over youthful/aggressive forms; in particular, 16.4% have a mild chronic form, 41.8% a moderate form and 32.7% a severe form (Table 4).

TABLE 4 Types of periodontitis found in the population.

Periodontitis (Amitage CG 1999)	Frequency	Valid percentage
Chronic, mild, generalized	18	16,4
Chronic, moderate, generalized	46	41,8
Chronic, severe, generalized	36	32,7
Aggressive, mild, generalized	1	,9
Aggressive, moderate, generalized	1	,9
Aggressive, severe, generalized	8	7,3
Total	110	100,0

The mean pocket depth (PPD) detected by the test was 5.54 mm, therefore beyond the values considered physiological, while the mean gingival recession was 0.59 mm. The average plaque index was found to be 31.16% (Table 5).

Food questionnaire

Consumption of the main foods important for the health of bones and teeth

To the subgroup of people interviewed by telephone was asked about eating habits for estimating calcium intake (20) and other nutrients important for bone and tooth health:

- 38.6% of the sample consumes cow's milk;
- 47.7% consume yogurt;
- 65.9% consumes hard cheeses;
- 32.6% consume semi-hard cheeses;
- 77.3% consume soft cheeses;
- 20.5% consume ricotta cheese;
- 95.5% consumes pasta/rice;
- 81.8% consume bread;
- 54.5% consume potatoes;
- 93.2% consumes meat/fish;
- 84.1% consume eggs;
- 77.3% consume legumes;
- 95.5% consume vegetables;
- 84.1% consume fresh fruit;
- 29.5% consume ice cream;
- 14.3% consumes milk chocolate;
- 18.2% intake water rich in calcium.

Tables 6 and 7 show the average weekly intake frequencies and the average portions of the various foods taken into consideration.

In subjects who have declared their intake, milk is consumed almost every day, yogurt more occasionally, cheese only 2-3 times a week on average.

Pasta and bread have an average consumption of 3-4 times a week.

Meat and fish are eaten on average 4 times a week.

Legumes are consumed on average 2 times a week and vegetables at least once a day.

TABLE 2 Mineralometric data.

Mineralometric data	N	Average	std. deviation
	Statistics	Statistics	Statistics
Tscore lumbar	15	-2,093	1,5917
Tscore femur tot	13	-1,823	,9671
Tscore femur neck	14	-2,157	,9788
Tscore right foot	99	-,961	1,2538
Tscore left foot	99	-,948	1,2966

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TABLE 5 Average PPD, REC and plaque index in the population.

	N	Minimum	Maximum	Average	Std.Deviation
	Statistics	Statistics	Statistics	Statistics	Statistics
PPD average (mm)	110	2,20	15,00	5,5414	1,42438
REC average (mm)	110	,00	2,40	,5877	,59648
Plaque Index (%)	97	0	90,48	31,1609	25,10672

About fruit consumption, the question asked how many fruits per week were consumed; 37 out of 44 subjects replied to consume at least one fruit a week, the rest said they did not consume fruit. The average consumption of fresh fruit in the population was found to be 11.89 fruits per week (standard deviation ± 1479.56), or 1.69 fruits/day.

Daily intake of the main nutrients useful for the health of bones and teeth

Table 8 shows the daily intake values of the main nutrients analyzed, which are important for the prevention of bone and dental health.

Considering the average age of the population (55 years), our data show that the intake of some nutrients in the population included in this study is below the values recommended by the Reference levels of nutrient and energy intake (L.A.R.N.) (22), in particular:

- Calcium;
- iron;
- magnesium;
- potassium;
- selenium;
- zinc;
- vit D;
- vit B12;

result insufficient compared to the recommended daily intake.

Inferencial statistics- significance

Study of the differences in food consumption and nutrient intake between subjects with normal bone density, osteopenic and osteoporotic

By carrying out a univariate ANOVA test and related *post hoc* tests, an attempt was made to investigate whether there are significant differences in the intake of certain foods and nutrients between the different study groups, i.e. subjects with normal, osteopenic and osteoporotic t-score values.

From the analyses shown in Table 9, in which only the analyses carried out that have produced significant results are shown, we have obtained:

- Significant difference in the portion and frequency of consumption of fresh vegetables between normal and osteopenic subjects (higher consumption);

TABLE 6 Frequency of consumption of the different food groups.

Weekly consumption frequency			
Foods	N	Average (time/week)	Std. Deviation
Cow's milk	17	5,94	1,56
Yogurt	21	3,86	2,032
Hard cheeses	29	3,1	1,934
Semi-hard cheeses	14	1,93	1,141
Soft cheeses	34	1,82	1,029
Ricotta cheese	9	1,89	1,054
Pasta/riso	42	4,31	2,054
Bread	36	5,33	2,138
Potatoes	24	1,71	0,751
meat/fish	41	4,2	1,860
eggs	37	2,68	1,547
Legumes	34	2,41	1,598
Vegetables	42	9,29	4,397
Ice cream	13	2,08	1,038
Milk chocolate	6	1,83	1,329

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TABLE 7 Average portions of the different food groups.

Medium portion			
Foods/Aliments	N	Avarage (g)	St.deviation
Cow's milk	17	207,35	59,794
Yogurt	21	129,05	37,504
Hard cheeses	29	64,66	35,955
Semi-hard cheeses	14	62,86	30,237
Soft cheeses	34	91,47	34,455
Ricotta cheese	9	102,78	8,333
Pasta/rice	42	90,95	25,644
Bread	36	95,28	32,38
Potatoes	24	250	106,322
Meat/Fish	41	135,37	30,091
Eggs	37	133,78	77,329
Legumes	34	121,47	47,106
Vegetables	42	176,9	68,876
Ice cream	13	84,62	37,553
Milk chocolate	6	41,67	30,277

• Significant difference in the portion and frequency of vegetable consumption between normal and osteoporotic subjects (higher consumption);

• Significant difference in the frequency of consumption of legumes between normal and osteopenic subjects (higher consumption);

• Significant difference in the frequency of consumption of legumes between normal and osteoporotic subjects (higher consumption). The other analyzes did not give significant results.

As regards the intake of the different nutrients in the three different categories of subjects, the following was found:

- Significant difference in Fe intake between normal and osteopenic subjects (higher intake);
- Significant difference in Mg intake between normal subjects and osteopenic (higher) intake;
- Significant difference in K intake between normal and osteopenic subjects (higher intake);
- Significant difference in vitamin C intake between normal and osteopenic subjects (higher intake);
- Significant difference in vitamin C intake between normal and osteoporotic subjects (higher intake);

TABLE 8 Average intakes of the analyzed nutrients.

	N	Minimum	Maximum	Average	Std. Deviation
	Statistics	Statistics	Statistics	Statistics	Statistics
Ca/die mg	44	218,9843	1507,5456	760,717584	321,6166047
P/die mg	44	385,6909	1482,6531	931,571488	237,3435570
Na/die mg	44	439,9885	1921,2101	973,602366	346,1657990
Fe/die mg	44	2,0775	12,7557	7,893709	2,5206753
Mg/die mg	44	60,8884	254,7129	171,823180	53,2108933
K/die mg	44	550,0149	3386,0009	2105,757209	667,6191528
Se/die mcg	44	11,4049	43,4157	27,981607	8,0220100
Zn/die mg	44	3,6157	14,5086	8,410503	2,2191760
VitC mg	44	4,8416	230,3100	132,337146	60,4172013
VitD mcg	44	,4771	3,1066	1,710894	,7033637
VitB12 mcg	44	1,1937	7,2377	3,843221	1,4569935

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TABLE 9 ANOVA univariata, study of the differences in food consumption between subjects with normal bone density, osteopenic e osteoporotic subjects.

Dependent Variable	Result	Result	Sig.
Frequency LEGUME	Normal	Osteopenia	0,032
		Osteoporosis	0,332
	Osteopenia	Normal	0,032
		Osteoporosis	0,998
	Osteoporosis	Normal	0,332
		Osteopenia	0,998
Portion VEGETABLES	Normal	Osteopenia	0,01
		Osteoporosis	0,038
	Osteopenia	Normal	0,01
		Osteoporosis	0,852
	Osteoporosis	Normal	0,038
		Osteopenia	0,852
Frequency VEGETABLES	Normal	Osteopenia	0,559
		Osteoporosis	0,122
	Osteopenia	Normal	0,559
		Osteoporosis	0,379
	Osteoporosis	Normal	0,122
		Osteopenia	0,379

The p values are shown in Table 10.

The other analyses did not give significant results.

Evaluation of the correlations between the plaque index and nutrient intake (calcium, carbohydrates and vitamin C)

Using the Spearman rank correlation coefficient, it was investigated whether there was a correlation between the intake values of certain nutrients important for bone and tooth health (calcium, carbohydrates and vitamin C) and the plaque index values (Table 11):

- as the vitamin C intake value decreases, the plaque index value in the entire population increases;
- for Calcium and Carbohydrates, no correlation is observed for too low a sample size.

Discussion

Descriptive evaluations

The data collected show us that the population involved in this study is made up of a group of subjects tending to belong to the over 50 age group, with BMI values within the recommended range, for both the male and female population. With regard to the subgroup to which it was possible to administer the anamnestic questionnaire, a population that tends to be "healthy" emerged. The data relating to bone turnover and mineralometric values describe a population with blood values of PTH, calcemia and phosphatemia tendentially in line with the desirable values, but with values of 25OHD3 below the optimal value. Furthermore, the t-score values of both QUS and MOC, when available, identify a population mainly made up of fragile subjects from the point of view of the risk of fracture and osteoporosis, with a smaller number of subjects with values tending to normal, which they were then compared for the variables under study with the more frail subjects.

Periodontal and bone evaluations

The assumption on which subjects were recruited for participation to the study was that of being affected by periodontal disease. On this regard, sample analysis shows that the most represented forms have a chronic rather than an aggressive nature, especially of moderate or advanced type, with high gingival recession values and periodontal pocket depths in the tested sites.

Nutritional evaluations

The population that provided answer to the food questionnaire showed eating habits that do not meet the intake levels recommended by the L.A.R.N. Nutrients important for bone health and relevant for the prevention of periodontal disease and mouth health in general, such as calcium, iron, magnesium, potassium and fiber, were found to be below the recommended values. It is no coincidence that these nutrients are mainly taken from foods of plant origin, such as fruit, vegetables and legumes. Foods that have proved to be deficient in the consumption habits of the population, both in terms of frequency of consumption and in amount consumed per portion. About the calcium intake, it is interesting to note that in our population this nutrient mainly derives from the intake of cheese, which also bring nutrients that are dangerous for health when consumed in excess (cholesterol and triglycerides), rather than from leaner foods, (milk and yogurt) which seem to be consumed much less frequently on average. This situation brings the attention on the need for a more efficient dietary education of the patients. To this regard, the dental staff could play an important intermediary role between patients and nutrition specialists, like nutritionists and dieticians, in order to limit and prevent nutritional imbalances predisposing to a whole series of pathologies like the periodontitis itself, osteoporosis, dismetabolisms and hypertension. Quite relevant results emerged from the analysis of significance and correlation; Specifically, it seems that subjects with lower t-score values, and therefore predisposed to a greater risk of bone fragility, show unsatisfactory but still higher intakes on average for many nutrients important for bone health among those that we have already mentioned (Fe, Mg, K, vit C, fibers). This result could be explained by the fact that a subject already defined at risk of developing pathology has already been sensitized, at least in part, to the importance of nutrition in the prevention of these pathologies by their generic physician or likewise by health personnel previously encountered.

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TABLE 10 ANOVA univariata study of the differences in food consumption between subjects with normal bone density, osteopenic e osteoporotic subjects.

Dependent variable	Result mineralometry	Result mineralometry	Error std.	Sig.
Ca/die_mg	Normal	Osteopenia	103,5798	0,847
		Osteoporosis	161,6607	0,477
	Osteopenia	Normal	103,5798	0,847
		Osteoporosis	162,5093	0,697
	Osteoporosis	Normal	161,6607	0,477
		Osteopenia	162,5093	0,697
P/die_mg	Normal	Osteopenia	76,30852	0,436
		Osteoporosis	119,0975	0,914
	Osteopenia	Normal	76,30852	0,436
		Osteoporosis	119,7227	0,921
	Osteoporosis	Normal	119,0975	0,914
		Osteopenia	119,7227	0,921
Na/die_mg	Normal	Osteopenia	111,4268	0,472
		Osteoporosis	173,9078	0,824
	Osteopenia	Normal	111,4268	0,472
		Osteoporosis	174,8207	0,986
	Osteoporosis	Normal	173,9078	0,824
		Osteopenia	174,8207	0,986
Fe/die_mg	Normal	Osteopenia	0,717718	0,004
		Osteoporosis	1,120168	0,233
	Osteopenia	Normal	0,717718	0,004
		Osteoporosis	1,126048	0,849
	Osteoporosis	Normal	1,120168	0,233
		Osteopenia	1,126048	0,849
Mg/die_mg	Normal	Osteopenia	15,55401	0,013
		Osteoporosis	24,27571	0,167
	Osteopenia	Normal	15,55401	0,013
		Osteoporosis	24,40314	0,999
	Osteoporosis	Normal	24,27571	0,167
		Osteopenia	24,40314	0,999
K/die_mg	Normal	Osteopenia	197,1462	0,015
	Normal	Osteoporosis	307,6932	0,381
	Osteopenia	Normal	197,1462	0,015
		Osteoporosis	309,3084	0,858
	Osteoporosis	Normal	307,6932	0,381
		Osteopenia	309,3084	0,858
Se/die_mcg	Normal	Osteopenia	2,515653	0,158
		Osteoporosis	3,92627	0,756
	Osteopenia	Normal	2,515653	0,158

(Continued)

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TABLE 10 Continued

Dependent variable	Result mineralometry	Result mineralometry	Error std.	Sig.
		Osteoporosis	3,946881	0,881
	Osteoporosis	Normal	3,92627	0,756
		Osteopenia	3,946881	0,881
Zn/die_mg	Normal	Osteopenia	0,69102	0,178
		Osteoporosis	1,0785	0,333
	Osteopenia	Normal	0,69102	0,178
		Osteoporosis	1,084161	0,96
	Osteoporosis	Normal	1,0785	0,333
		Osteopenia	1,084161	0,96
VitC_mg	Normal	Osteopenia	17,35689	0,013
		Osteoporosis	27,08952	0,046
	Osteopenia	Normal	17,35689	0,013
		Osteoporosis	27,23173	0,844
	Osteoporosis	Normal	27,08952	0,046
		Osteopenia	27,23173	0,844
VitD_mcg	Normal	Osteopenia	0,225023	0,5
		Osteoporosis	0,351201	0,912
	Osteopenia	Normal	0,225023	0,5
		Osteoporosis	0,353045	0,502
	Osteoporosis	Normal	0,351201	0,912
		Osteopenia	0,353045	0,502
VitB12_mcg	Normal	Osteopenia	0,472399	0,81
		Osteoporosis	0,737289	0,88
	Osteopenia	Normal	0,472399	0,81
		Osteoporosis	0,74116	0,659
	Osteoporosis	Normal	0,737289	0,88
		Osteopenia	0,74116	0,659

Real primary prevention, the one intended for healthy subjects, would therefore be an element of greater criticality, as people who still do not perceive a problem would tend to be less attentive to their eating habits. For this reason, it would be essential in the future to increase awareness of food choices even in healthy subjects, in order to prevent any nutrient shortcomings and future pathological developments.

Another result of great interest was obtained about the relationship between nutrient intake and plaque index, a factor that describes the severity of periodontal disease. From the correlation analyzes carried out, it appears that across the population, the higher the intake of vitamin C through food, the lower the plaque index value is. This result could reinforce the scientific evidence that there is a protective factor in the onset of periodontal disease from the consumption of vitamin C, which is currently subject of investigation and debate). Systematic reviews on this aspect were performed in latest years; in 2019 Akio et al. selected 14 articles corresponding to inclusion criteria after a full revision of 716 articles. The vitamin C intake and blood levels were negatively related to periodontal disease in all seven cross-sectional studies. The subjects who suffer from periodontitis presented a lower vitamin C intake and lower blood-vitamin C levels than the subjects without periodontal disease in the two case-control studies. The patients with a lower dietary intake or lower blood level of vitamin C showed a greater progression of periodontal disease than the controls. The intervention using vitamin C administration improved gingival bleeding in gingivitis, but not in periodontitis. Alveolar bone absorption was also not improved. The present systematic review suggested that vitamin C contributes to a reduced risk of periodontal disease (23). In 2021 Hytham N. et al. performed another systematic review in which they found six studies fulfilled the inclusion criteria. Vitamin C supplementation helped improve bleeding indices in gingivitis but did not significantly lead to reduction of probing depths or clinical attachment gain for periodontitis. In this case, administration of vitamin C as an adjunct to non-surgical

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TABLE 11 Spearman's correlation, evaluation of the correlations between the plaque index and nutrient intake (calcium, carbohydrates and Vit C).

			Plaque Index
Rho di Spearman	Plaque Index	Correlation coefficient	1,000
		Sig. (2-code)	
		N	97
	VitC_mg	Correlation coefficient	-,385
		Sig. (2-code)	,025
		N	34
	Ca/die_mg	Correlation coefficient	-,199
		Sig. (2-code)	,259
		N	34

periodontal therapy did not result in clinically significant improvements in pocket probing depths at 3 months in periodontitis patients. With the limited evidence available, no recommendation can be made for supplementation of vitamin C in conjunction with initial periodontal therapy for subjects with periodontitis to improve primary treatment outcome measures. (24).

Once this aspect will be consolidated, we could then focus on the role that this vitamin plays in the prevention of the osteoporotic disease (25), another aspect currently under investigation, and define its importance in the prevention from the two diseases. Furthermore, a similar trend would have also been observed for calcium intake, but a larger sample size would be required to make this effect significant. In the future, we hope to be able to deepen the relationship of this nutrient with the prevention of periodontal disease, which is already fundamental in the prevention of bone health.

Conclusions

The relationship between osteoporosis and periodontitis and the role of nutrition in influencing the course of these pathologies seems still to be extensively explored. However, our results consolidate the idea that there is a relationship between these two diseases, and that eating habits play an important role in their prevention. The analyses presented here may be of great interest for the development of future studies aiming to expand the sample size and to reduce the confounding factors present at the level of the studied populations.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding authors.

Ethics statement

The studies involving human participants were reviewed and approved by Comitato etico regionale per la sperimentazione clinica della regione Toscana. The patients/participants provided their written informed consent to participate in this study.

Author contributions

LG: Main author and performer of the study. LC: Supervisor of the project. BP: Revisor of the main article. FT: organized the collaboration and the cooperation between F.I.R.M.O. and EDN. FM: Main Chief of EDN. TI: organized the collaboration and the cooperation between F.I.R.M.O. and University of Florence. MB: Main Chief of F.I.R.M.O. All authors contributed to the article and approved the submitted version.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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References

1. Kassebaum NJ, Bernabé E, Dahiya M, Bhandari B, Murray CJ, Marcenes W. Global burden of severe periodontitis in 1990-2010: a. *J Dent Res* (2014) 93(11):1045–53.

2. Adami G, Fassio A, Rossini M, Caimmi C, Giollo A, Orsolini G, et al. Osteoporosis in rheumatic diseases. *Int J Mol Sci* (2019) 20(23):5867.

3. Hernlund E, Svedbom A, Ivergård M, Compston J, Cooper C, Stenmark J, McCloskey EV, et al. Osteoporosis in the European union: medical management, epidemiology and economic burden. *Arch Osteoporosis* (2013).

4. Tezal M, Wactawski-Wende J, Grossi SG, Dmochowski J, Genco RJ. Periodontal disease and the incidence of tooth loss in postmenopausal woman. *J Periodontol* (2005) 76 (7):1123–8. doi: 10.1902/jop.2005.76.7.1123

5. Straka M, Straka-Trapezanlidis M, Deglovic J, Varga I. Periodontitis and osteoporosis. *Neuroendocrinol Lett* (2015) 36(5):401–6.

6. Varela-López A, Giampieri F, Bullón P, Battino M, Quiles JL. Role of lipids in the onset, progression and treatment of periodontal disease. a systematic review of studies in humans. *Int J Mol Sci* (2016) 17:1–13. doi: 10.3390/ijms17081202.

7. Schifferle RE. Periodontal disease and nutrition: separating the evidence from current fads. *Periodontol 2000* (2009) 50:78–89. doi: 10.1111/j.1600-0757.2008.00297.x

8. Chawla TN, Glickman I. Protein deprivation and the periodontal structures of the albino rat. *Oral Surg Oral Med Oral Pathol* (1951) 4:578–602. doi: 10.1016/0030-4220(51) 90052-7

9. Stahl SS, Sandler HC, Cahn L. The effect of protein deprivation upon the oral tissues of the rat and particularly upon periodontal structures under irritation. *Oral Surg Oral Med Oral Pathol* (1955) 8:760–8. doi: 10.1016/0030-4220(55)90040-2

10. Park JB, Nam G. Obesity in relation to oral health behaviors: an analysis of the Korea national health and nutrition examination survey. *Exp Ter Med* (2016) 12(5):3093–100. doi: 10.3892/etm.2016.3697

11. Loe H, Theilade E, Jensen SB. Experimental gingivitis in man. *J Periodontol* (1965). doi: 10.1902/jop.1965.36.3.177

12. Brecx MC, Fröhlicher I, Gehr P, Lang NP. Stereological observations on long-term experimental gingivitis in man. *J Clin Periodontol* (1988) 15(10):621–7. doi: 10.1111/j.1600-051X.1988.tb02262.x

13. Baumgartner S, Imfeld T, Schicht O, Rath C, Persson RE, Persson GR. The impact of the stone age diet on gingival conditions in the absence of oral hygiene. *Periodontol* (2009) 80(5):759–68. doi: 10.1902/jop.2009.080376

14. Nielsen SJ, Trak-Fellermeier MA, Joshipura K, Dye BA. Dietary fiber intake is inversely associated with periodontal disease among US adults. *J Nutr* (2016) 146 (12):2530–6. doi: 10.3945/jn.116.237065

15. Najeeb S, Zafar MS, Khurshid Z, Zohaib S, Almas K. The role of nutrition in periodontal health: an update. *Nutrients* (2016) 8(9):530. doi: 10.3390/nu8090530

16. Ferrillo M, Migliario M, Roccuzzo A, Molinero-Mourelle P, Falcicchio G, Umano GR, et al. Periodontal disease and vitamin d deficiency in pregnant. *J Clin Med* (2021) 10 (19):4578. doi: 10.3390/jcm10194578

17. Ferrillo M, Migliario M, Marotta N, Lippi L, Antonelli A, Calafiore D, et al. Oral health in breast cancer women with vitamin D deficiency: A machine learning study. *J Clin Med* (2022) 11(16):4662. doi: 10.3390/jcm11164662

18. Rekha C, Poornima G, Manasa M, Abhipsa V, Pavithra Devi J, Vijay Kumar HT, et al. Ascorbic acid, total phenol content and antioxidant activity of fresh juices of four ripe and unripe citrus fruits. *Chem Sci Trans* (2012) 1:303–10. doi: 10.7598/cst2012.182

19. Adegboye ARA, Chrisrtensen LB, Heitmann BL. Intake of dairy products in relation to periodontitis in older danish adults. *Nutrients* (2012) 4:1219–29. doi: 10.3390/nu4091219

20. Montomoli M, Gonnelli S, Giacchi M, Mattei R, Cuda C, Rossi S, Gennari C. Validation of a food frequency questionnaire for nutritional calcium intake assessment in Italian women. *Eur J Clin Nutr* (2002) 56:21–30. doi: 10.1038/sj.ejcn.1601278

21. Armitage GC, Jeffcoat MK, Chadwick DE, Taggart EJ Jr, Numabe Y, Landis JR, et al. Longitudinal evaluation of elastase as a marker for the progression of periodontitis. *J Periodontol* (1994) 65(2):120–8. doi: 10.1902/jop.1994.65.2.120

22. SINU. *Livelli di assunzione di riferimento di nutrienti ed energia per una sana popolazione italiana*. (2014), Melzo (MI): SICS Editore Srl.

23. Miura H, Tada A. The relationship between vitamin c and periodontal diseases: A systematic review. *Int J Environ Res Public Health* (2019) 16(14):2472.

24. Hytham N, Fageeh HI. Efficacy of vitamin c supplementation as an adjunct in the non-surgical management of periodontitis: a systematic review. *Syst Rev* (2021) 10:5. doi: 10.1186/s13643-020-01554-9

25. Olga Brzezińska ZŁ. Role of vitamin c in osteoporosis development and treatment-a literature review. *Nutrients* (2020) 12(8):2394.

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High-fat diet causes undesirable bone regeneration by altering the bone marrow environment in rats

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Objective: Diet structure has changed greatly over the last few decades, and high-calorie diets have become an integral part of people's daily diet, as well as the important cause of obesity in society. Several organ systems, including the skeletal system, are seriously affected by high-fat-diets (HFD) in the world. There is, however, still a lack of knowledge about the effects of HFD on bone regeneration and the possible mechanisms involved. In this study, the difference in bone regeneration between rats under HFD and low-fat-diets (LFD) was evaluated by monitoring the process of bone regeneration in distraction osteogenesis (DO) model animals, as well as the possible mechanisms.

Methods: A total of 40 Sprague Dawley (SD) rats (5 weeks old) were randomly divided into HFD group (n=20) and LFD group (n=20). Except for feeding methods, there were no differences between the two groups in terms of treatment conditions. All animals received the DO surgery eight weeks after starting to feed. After a delay of 5 days (latency phase), the active lengthening phase was performed for 10 days (0.25 mm/12 h), and the consolidation phase followed for 42 days. An observational study of bone included radioscopy (once a week), micro-computed tomography (CT), general morphology, biomechanics, histomorphometry, and immunohistochemistry.

Result: The results showed that HFD group had a higher body weight than LFD group after 8, 14, and 16 weeks of feeding. Furthermore, at the final observation, there were statistically significant differences between LFD group and HFD group in terms of total cholesterol (TC), triglycerides (TG), low-density lipoprotein (LDL), and high-density lipoprotein (HDL) levels. Additionally, observations on bone regeneration showed a slower regeneration and a lower biomechanical strength in HFD group than LFD group, based on radiography, micro-CT, general morphology, biomechanics, histomorphometry, and immunohistochemistry.

Conclusion: In this study, HFD resulted in elevated blood lipids, increased adipose differentiation at the bone marrow level, and delayed bone regeneration. The pieces of evidence are beneficial to better understand the association between diet and bone regeneration and to adjust the diet optimally for fracture patients.

KEYWORDS

adipose tissue, bone regeneration, distraction osteogenesis, high-fat-diet, obesity

1 Introduction

Over the years, diet-induced obesity has become more common in society, and researchers have paid a lot of attention to it. It is well known that a habit of high-fat diet (HFD) has a profound impact on the metabolism of various organ systems in the human body, including the skeletal system. HFD and bone, however, have been the subject of widespread controversy, with many researchers coming to different conclusions about the effects of HFD on bones. According to a widely accepted view in past research, high body mass was associated with increased mechanical loading, which could benefit bone health (1–3). However, recent evidence suggests that children and adolescents who were obese were more likely to suffer from bone fractures (4). According to Hou J et al., there is also a complex link between obesity and bone health through different mechanisms that may be involved in leptin, adiponectin, and many pro-inflammatory factors (5).

Some related studies have observed that mice fed with a HFD suffer from low bone mass, but the changes in osteoclast and osteoblast function were not always consistent *in vitro* (6–8). Generally, low bone mass is associated with an increase in osteoclasts and a decrease in osteoblasts. However, there are a number of hormones and circulating cytokines involved in regulating the formation and apoptosis of osteoblasts and osteoclasts, which is an extremely complex process. (5, 9–11). Moreover, bone marrow mesenchymal stem cells (BMSCs) can differentiate into osteoblasts or adipocytes (12, 13). Hence, osteogenesis may be decreased by excessive differentiation of BMSCs into bone marrow adipocytes. While HFD has been studied in animal and clinical experiments, the majority of studies used non-traumatic bones to analyze its effects on bone. Hence, it is essential to study the effects of HFD on newly formed bone regeneration and consolidation following trauma.

Distraction osteogenesis (DO) which was used to make animal models in this study is a surgical technique that stimulates bone tissue regeneration by stretching tension forces on severed bone tissue (14). DO is a bone-regeneration process in which two vascularized bones are generated by gradual distraction, and as the bone segments are gradually distracted, new bone tissue is generated between them (14). Bone undergoes regeneration under controlled mechanical conditions owing to its intrinsic ability to do so (14). Additionally, DO is considered the best method for generating bone tissue *in vivo* and can be used to create a large new segment of bone tissue (15), which newly regenerated bone is more convenient to be observed and studied for the internal structural changes of bone regeneration than normal fracture. Hence, DO is widely used in experimental research of bone regeneration and clinical treatment that includes limb discrepancy, bone non-union, bone infection, bone defect, and malformation (16–20). Furthermore, in the process of bone regeneration, BMSCs differentiate into chondrocytes, fibroblasts, or osteoblasts to form fracture calluses, essential for healing fractured bones (21, 22). Hence, this study uses DO technique to establish an animal model that will enable us to observe bone regeneration more clearly following trauma.

According to our hypothesis, HFD has the potential to slow the rate and reduce the quality of bone tissue regeneration after trauma, and this result may be related to changes in the bone marrow microenvironment. To verify this hypothesis, a series of observations and tests were conducted in the fractured segments to assess the impact of HFD on bone regeneration and consolidation.

2 Materials and methods

2.1 Animals

In this study, forty male Sprague Dawley (SD) rats (5 weeks old) were randomly divided into HFD group (n=20) and LFD group (n=20) as study subjects. The animals were raised at a temperature of 20-25°C and a humidity of 50-60% with free access to water and a pelleted diet of high-fat (HFD group, 60% kcal, D12492) or low-fat (LFD group, 4.5% kcal, General maintenance feed) (Beijing Boaigang Biotechnology Co. Ltd, Beijing, China) starting at 5 weeks of age. The time-dependent nodes of dietary and surgical observation have been shown in Figure 1A. All experimental procedures were approved by the Animal Ethics Committee of Xinjiang medical university (IACUC-202003318-82).

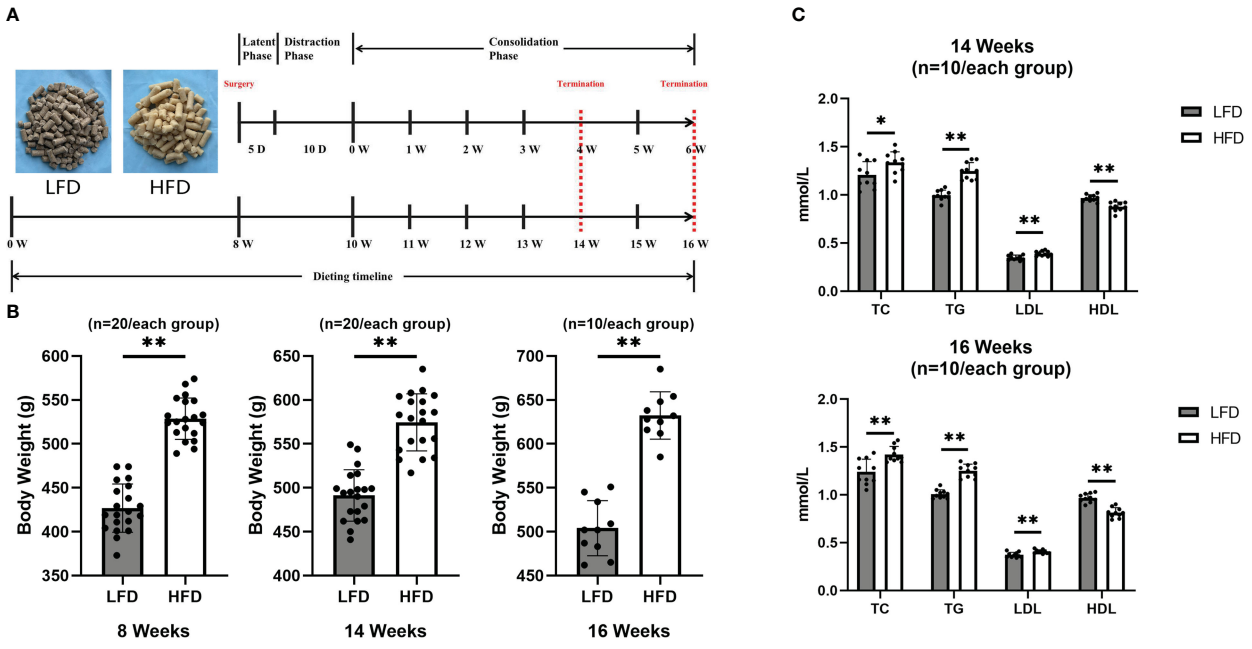

FIGURE 1
HFD-induced obese rats. **(A)**Dietary and surgical observation timeline. **(B)** Body weight values were higher in the HFD group at 8, 14, and 16 weeks than in the LFD group. **(C)** At 14 and 16 weeks of feeding, quantitative evaluations showed that TC, TG, and LDL levels in HFD were significantly higher than those in LFD while HDL levels were higher in LFD group. (*P<0.05, **P<0.01).

2.2 Surgical procedures and DO procedures

All surgical operations and postoperative procedures were performed by the same skilled surgical team. Eight weeks of feeding were administered to 40 rats before surgery. During the operation, anesthesia was administered using 2% pentobarbital sodium (3 mg/100 g) to each rat. For infection prevention, the preoperative administration of benzyl penicillin was carried out. Under sterile conditions, four stainless steel self-tapping screws were used to install a monolateral distraction external fixator (Designed and manufactured by this research team) on the right femur in rat, and then a mid-diaphysis transverse osteotomy was performed with the miniature bone saw (23; Figure 2).

With the antibiotic solution, daily pin site care was performed. Each experimental rat received daily intramuscular injections of benzyl penicillin for three days following surgery to prevent infection. Each rat was housed in a cage and allowed to move freely. Water and chow were provided (diets were the same as before surgery).

The DO procedure consisted of three phases (23, 24): a latency phase of 5 days, an active lengthening phase of 10 days (0.25 mm/12 h), and a consolidation phase of 42 days (six weeks).

Four and six weeks after consolidation, rats were randomly selected for sacrifice (n = 10 per group). Cardiac blood samples were drawn for lipid analysis. Bone samples were harvested from both femurs for further analysis.

2.3 Body weight and blood lipid analysis

Using a standard scale, the body weight was collected and analyzed in rats at the critical time points of 8, 14, and 16 weeks of feeding.Blood lipids including total cholesterol (TC), triglycerides (TG), low-density lipoprotein (LDL), and high-density lipoprotein (HDL) were determined by extracting heart blood from rats after 14 (four weeks of consolidation) and 16 (six weeks of consolidation) weeks of feeding.

2.4 Digital radiographic analysis

In order to monitor bone regeneration of the distraction zone, each rat was subjected to an anteroposterior (AP) radiographic examination weekly after isoflurane anesthesia until sacrifice using the same digital radiographic apparatus (HF400VA, MIKASA X-RAY Co., Ltd., Tokyo, Japan) and conditions (44kV, 4.5mAs). According to digital radiographic analysis, a callus formation stage is characterized by high fracture density, fuzzy fracture lines, and amorphous bone around the fracture. At this stage of healing, the callus disappears, fracture lines disappear, and trabeculae pass through the fractured end.

2.5 Micro-computed tomography (CT) analysis

Microstructural change within the distraction zone (bone regeneration) was quantitatively assessed using micro-CT imaging

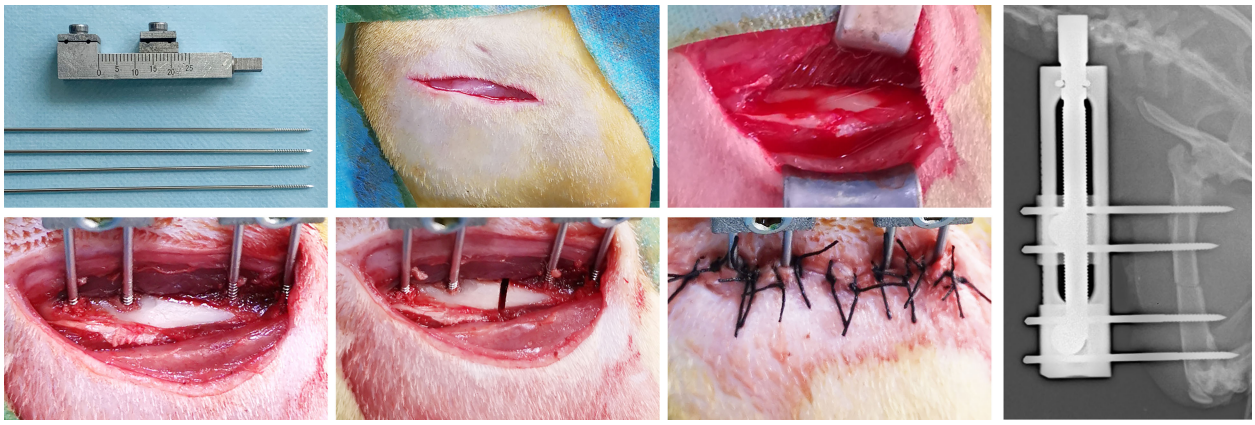

FIGURE 2
The surgical procedures for the rat right femur model of DO.

(80 kV, 313μA for 0.203 s, voxel size 18 μm; SkyScan 1176, Bruker, America) on the representative femur specimens (n=3 per group) that were collected at the 6-weeks of consolidation. Skyscan NRecon software was used to optimize and recompute the scanned images, and Skyscan CTAn software was used for three-dimensional (3D) analysis based on the manufacturer's instructions. There was a definition of an area of interest (ROI) as the distraction zone surrounded by the periosteum at its proximal and distal ends (25). Bone mineral density (BMD) and bone volume/total tissue volume (BV/TV) measurements were made only on bone within the ROI.

2.6 Biomechanical test

In order to evaluate the strength of bone regeneration and repair, mechanical properties were used (n = 3 per group). In this procedure, samples of six-weeks consolidation without external fixators and screws were evaluated by a three-point bending test (RGM-3005T, ShenZhen Reger Instrument Co., Ltd., China), and control femurs consisted of unoperated femurs. In the experiments, with an 18mm span, the femur long axis was perpendicular to the blades. 0.5mm/min was constantly applied in the distraction zone with the AP direction until failure was achieved. Several indexes were measured on both the healthy and damaged femurs, including ultimate load, modulus of elasticity (E-modulus), energy to failure, and stiffness.

2.7 Histomorphometry in calcified tissue

For further analysis, a 10% formalin buffer was applied to all specimens for 48 hours, followed by an ethanol solution of 75%. Each group's specimens (n = 3 per group) were successively dehydrated and fattened with xylene and embedded in methyl methacrylate following termination at each time point. With the help of a hard tissue microtome, sections 10μm thick were cut. In order to observe the histomorphometric appearance, Von Kossa, Masson Trichrome, Goldner Trichrome, and Safranin O staining were used.

2.8 H&E and immunohistochemistry in decalcified tissue

Four specimens per group were decalcified over a 4-week period using 10% ethylenediaminetetraacetic acid solution for evaluation of decalcified tissues. Following that, paraffin embedding was performed. For H&E and immunohistochemistry staining, five-mm sections were cut using a microtome. Observations were conducted on three ROI fields randomly selected for each section.

According to a standard protocol, the specimens were deparaffinized in xylene, rehydrated in gradient alcohol, and immunohistochemistry was administrated. For 20 minutes, the endogenous peroxidase activity was quenched with 0.3% hydrogen peroxide. An antigen retrieval solution of 0.4% pepsin was used for 25 minutes at 37°C, followed by a blocking solution containing 5% goat serum for 30 minutes at 37°C. Subsequently, anti-runt-related transcription factor 2 (RUNX2) (1:100, sc390351, Santa Cruz, CA, USA), anti-osterix (Osx) (1:400, ab209484, Abcam, Cambridge, UK), anti-osteocalcin (OCN) (1:100, 23418-1-AP, Proteintech, Wuhan, China), and anti-osteopontin (1:100, 22952-1-AP, Proteintech, Wuhan, China) primary antibodies were incubated overnight at 4°C on sections. The signals were detected with a horseradish peroxidase-streptavidin system (ZLI-9019, ZSGB-BIO, Beijing, China) after incubation in a secondary antibody (PV6000, ZSGB-BIO, Beijing, China) for 1h at 37°C, followed by hematoxylin counterstaining. For the analysis of each section, three fields of ROI were randomly selected and observed at a magnification of 200×. With Image Pro Plus 6.0 software, the same pixel value (brown) was set to calculate the positively stained areas in all specimens, and the proportion of positive area to total area was calculated by a semi-quantitative analysis.

2.9 Statistical analysis

OpenEpi V2 open source calculator was used to calculate sample size. A study with a two-sided 95% Confidence Interval and an 80% power required 20 animals per group. Statistical analysis was conducted using SPSS 22.0. A three-time calculation was performed under the same conditions for each data set to be analyzed. Throughout this paper, all continuous variables have been expressed as mean ± standard deviation (SD). An analysis of the Shapiro-Wilk test was conducted in order to determine the normality of the data. We evaluated the statistical differences between two specific groups by using the independent-samples t-test or the Mann-Whitney U test. $P < 0.05$ was considered a statistically significant difference. Based on GraphPad Prism v.6.0, graphs were created.

3 Results

3.1 Body weight and blood lipid analysis

Approximately eight weeks after feeding, there was a significant difference between groups in terms of body weight. Furthermore, the final results showed that the body weight was higher in the HFD group by 25% in 14 weeks and 30% in 16 weeks of feeding, compared with the LFD group (Figure 1B). Additionally, at the final observation, a blood lipid test was performed and showed that the HFD group was significant higher in TC, TG, and LDL than LFD group at 14 and 16 weeks of feeding (Figure 1C).

3.2 Sequential digital radiographs

In these experiments, there were no deaths among the rats, and they all recovered from the surgery and survived until the end of the experiment. There were no significant difficulties with daily activities for any of the rats, as they all achieved normal ambulation.

Digital radiographs were taken weekly to monitor the consolidation progress of distraction regeneration, as shown in Figure 3. During the first two weeks of the consolidation phase, high fracture density, fuzzy fracture lines, and amorphous bone around the fracture were presented in both two groups (Figure 3A). Nevertheless, bone regeneration in the LFD group was greater than in the HFD group after consolidation for three weeks. During the bone consolidation at week four and five, callus decrease and fracture lines disappear were observed in the LFD group but not in the HFD group. Additionally, in the distraction zone, there was a fuzzy fracture line in the HFD group after 6 weeks of consolidation, and proximal and distal fracture ends remained unhealed. However, the LFD group did not show this phenomenon, and the trabeculae pass through the fractured ends. Similarly, the general examination of dissected specimens and micro-CT examination after six weeks of consolidation revealed similar results (Figures 3B, 4).

3.3 Three-dimensional (3D) microstructure of bone regeneration

Following 6 weeks of consolidation, the representative micro-CT images revealed almost complete recanalization of the marrow cavity in the LFD group, but a narrow closure of the bone marrow cavity was observed in HFD group (Figure 4). Additionally, a lower BMD was found in the HFD group (361.33 ± 16.71mg/cm^3) compared to the LFD group (404.51 ± 10.54mg/cm^3) (P=0.019) (Figure 4B). Similarly, there was a significant difference in BV/TV result between the two groups (30.67 ± 3.06% in HFD group vs. 43.67 ± 2.52% in LFD group) (P<0.01) (Figure 4C). According to the results, we hypothesized that in the HFD group, bone mineralization was significantly delayed and numerous non-mineralized tissues were presented in the distracted segments,

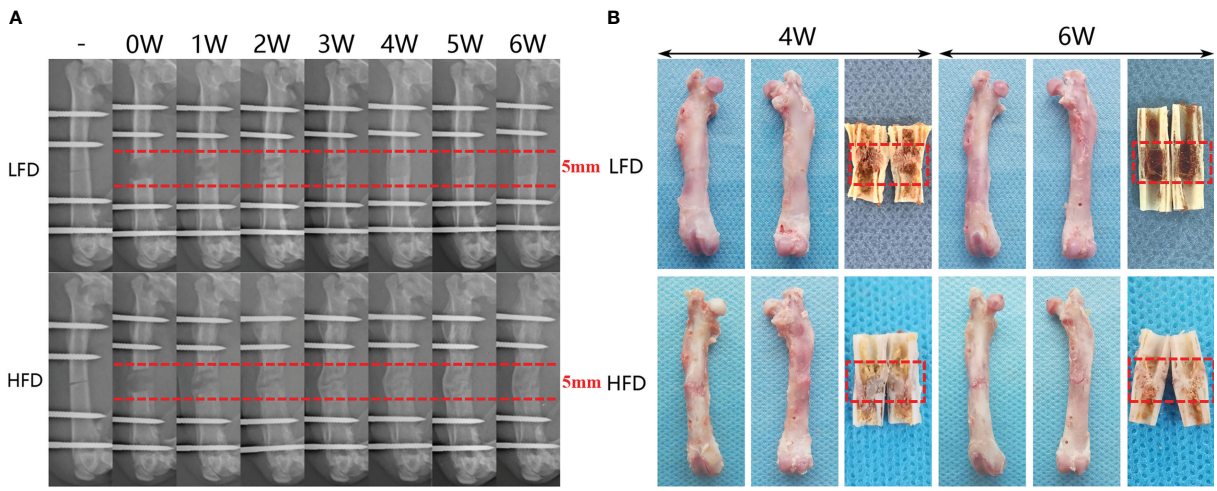

FIGURE 3
Distraction osteogenesis effects bone regeneration differently based on diet. **(A)** There is a six-week consolidation duration for the distraction X-ray images that regenerate each week. **(B)** After four- and six-week consolidation, a general image of the specimens can be seen.

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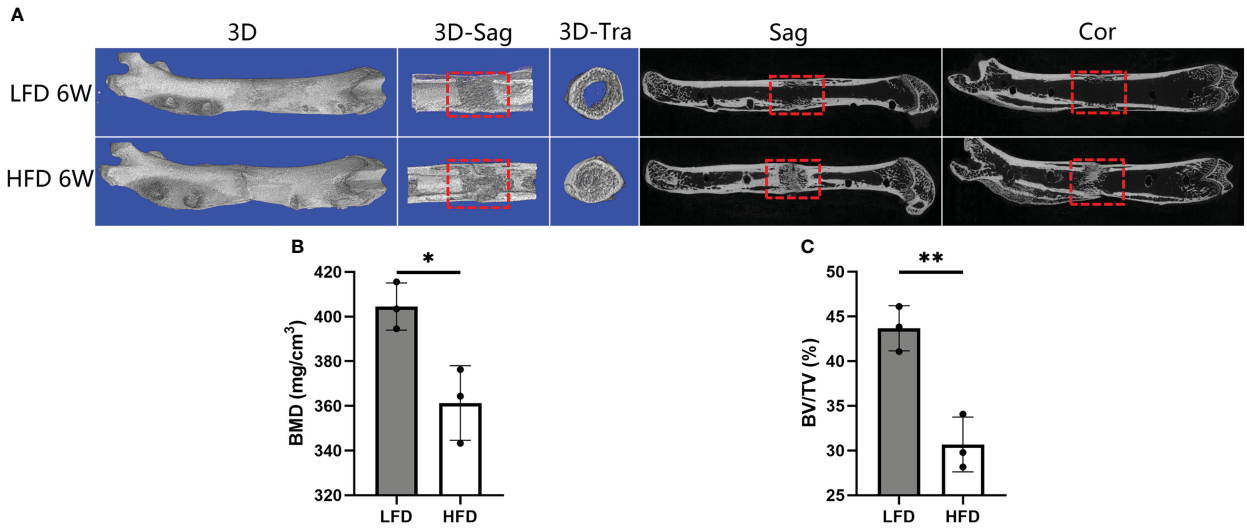

FIGURE 4
Results of micro-CT evaluation showing decreased regenerate quality after rats were fed a high-fat diet. **(A)** Representative three-dimensional (3D) micro-CT images of the distraction zone at the termination of the 6-week consolidation. **(B, C)** Quantitative evaluation of BMD and BV/TV, manifesting both two values in LFD group were significantly higher than those in HFD group. (*P<0.05, **P<0.01).

which may contribute to a lower BMD and BV/TV in the HFD group compared with the LFD group.

3.4 Mechanical properties of regenerated bone

Following 6 weeks of consolidation, the mechanical properties of collected samples were evaluated using a three-point bending test. The results showed that there were better outcomes in LFD group with a higher E-modulus (49.35 ± 4.84% in LFD group vs. 37.49 ± 4.57% in HFD group) and energy to failure (54.37 ± 2.32% in LFD group vs. 45.41 ± 3.65% in HFD group). However, no significant difference was observed in ultimate load (41.86 ± 1.42% in LFD group vs. 36.91 ± 3.28% in HFD group) and stiffness (48.19 ± 3.9% in LFD group vs. 43.36 ± 6.23% in HFD group) between the two groups (Figure 5).

3.5 Histomorphometry in calcified samples

Several histomorphological characteristics were examined in calcified samples, including Von Kossa, Masson, Goldner Trichrome, Safranin O & Fast Green. During Von Kossa staining, calcified tissue is black in color. In the HFD group, we can find an apparent gap in the interested area after 4 weeks of bony consolidation based on Von Kossa staining. Moreover, in the HFD group, Safranin O staining showed that there were numerous chondrocytes (stained red) in the interested area after 4 weeks of bony consolidation and indicated that there was still incomplete mineralization of regenerated bone here. However, in the LFD group, newly regenerated calluses were of higher quality and volume (Figure 6). At the 6 weeks of bony consolidation, Von Kossa staining showed that in both the LFD and HFD groups, the fracture space has disappeared, and the regenerated cortical bone is continuous. Complete reconstruction and recanalization of the bone marrow cavity were performed in the LFD group. However, in the HFD group, it has not yet been completely recanalized and there was still a small amount of callus in the marrow cavity. Similarly, according to Safranin O, Goldner Trichrome, and Masson staining, the observed results demonstrated that HFD significantly slows bone formation in DO (Figure 6).

3.6 Histological assessments in decalcified samples

At the end point of observation (6-weeks consolidation), H&E staining was used to assess the adipocytes in the bone marrow, and the qualitative results showed a significant difference between the HFD (128.8 ± 6.5 N/mm^2) and LFD groups (45.8 ± 3.9 N/mm^2). There is a higher level of adipose differentiation in the HFD group, according to the observation (Figure 7).

In the immunohistochemical analysis, the expression of Runx2, Osterix, OCN, and OPN was lower in the HFD group at 4 weeks of consolidation compared with LFD group (P=0.039 or P<0.01). Interestingly, the aforementioned indicators were lower in the LFD group than in the HFD group at the end of the six-week consolidation period (P<0.01). According to our hypothesis, this may be because the regenerated trabeculae in the LFD group were more mature than those in the HFD group, and therefore, fewer osteogenic factors and proteins were produced in their place (Figure 8).

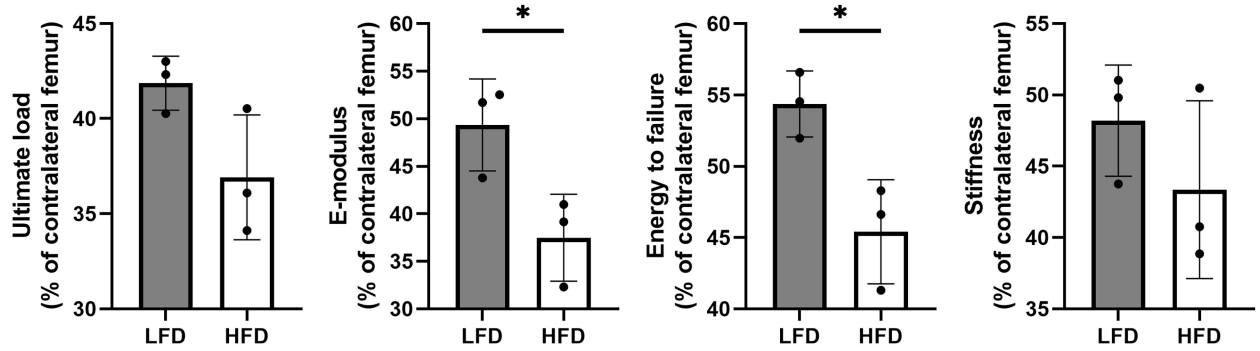

FIGURE 5
Results of mechanical properties and values were normalized to the contralateral femur. (*P<0.05).

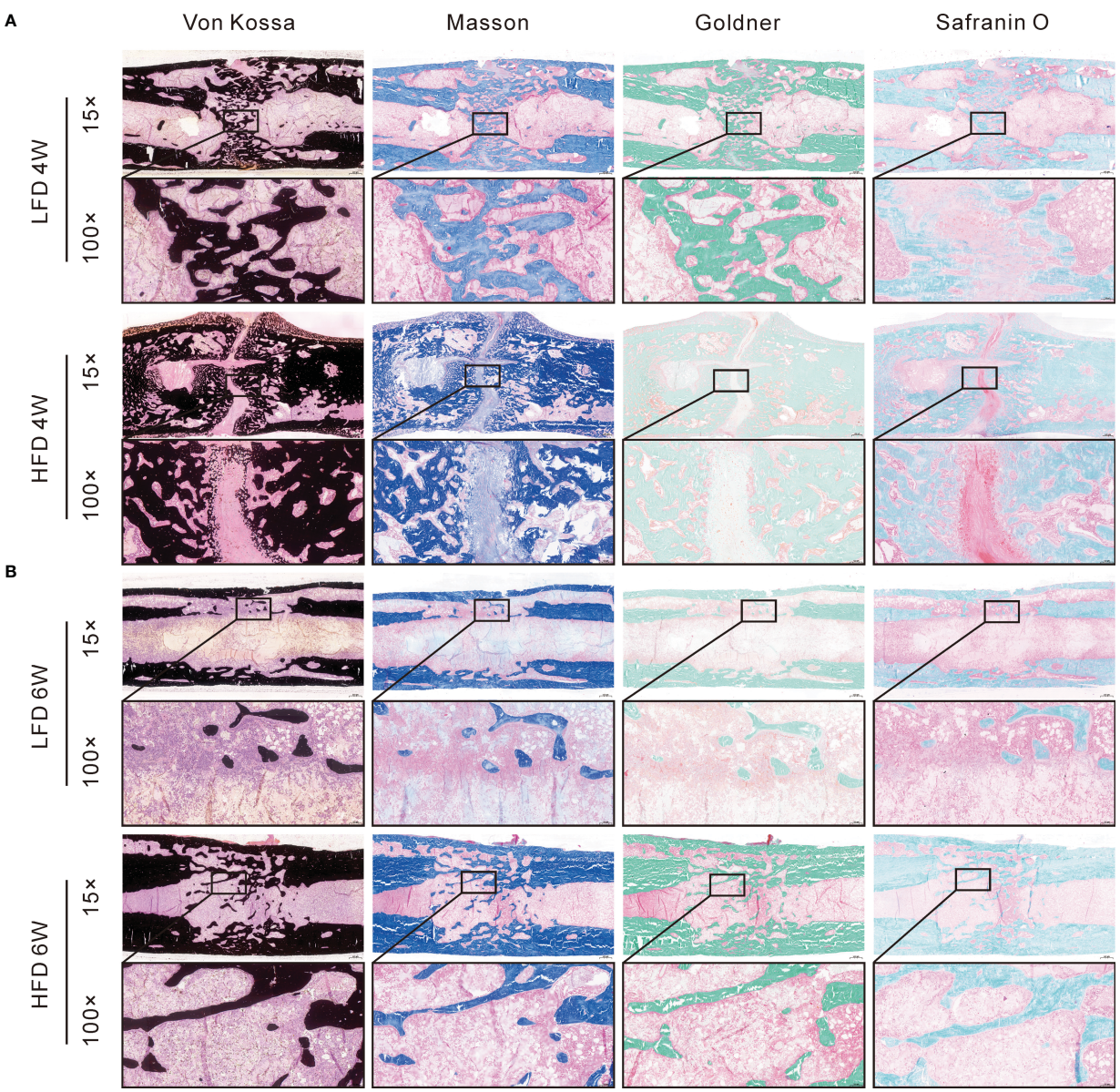

FIGURE 6
Histomorphological analysis of bone regeneration during the consolidation period. Von Kossa, Masson, Goldner Trichrome, and Safranin O & Fast Green staining indicated the decreased bone regeneration in HFD group. **(A)** 4-weeks consolidation. **(B)** 6-weeks consolidation.

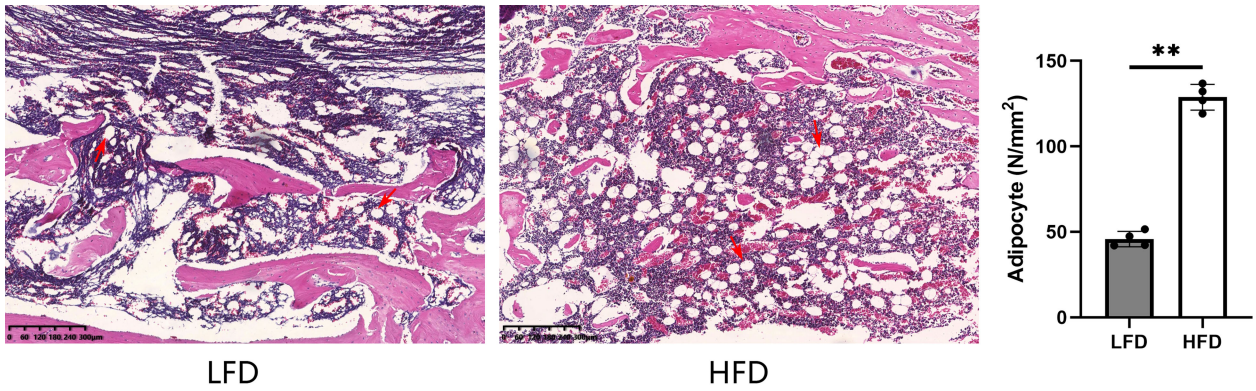

FIGURE 7
By H&E staining, the HFD group demonstrated significantly higher values than the LFD group when it came to bone marrow adipocytes. (**P<0.01, adipocytes were marked with red arrows).

4 Discussion

The increased bone-forming activity that results from distraction is attributed to the stimulatory effect of tension on blood vessel formation and on the recruitment and proliferation of bone progenitor cells (26). In numerous previous studies, it has been shown that diet can affect the angiogenesis of regenerated bone tissue and the recruitment and proliferation of bone progenitor cells (6–8, 27). Additionally, there is evidence that HFD-induced high body mass and increased mechanical loading may benefit bone health (1–3). However, as a result of conflicting reports in previous studies on adipose tissue and bone regeneration, it has been difficult to assess the consequences of HFD on bone health (6–8, 27). In the present study, we compared the rate of regeneration of the femoral shaft in rats fed with different feeding methods and analyzed possible mechanisms behind it. As a result of HFD-rats, blood lipid levels rise, bone marrow adipose tissue (BMAT) is increased, and bone regeneration is reduced in the distraction gap.

According to previous research, HFD often results in weight gain, which undoubtedly benefits weight-bearing bones. As research has advanced, this view has gradually been refuted, and more and more researchers have confirmed that HFD is detrimental to bone development. Studies indicate that the effects of HFD on bone health increase with the duration of the diet. A study reported a significant increase in bone mass within 8 weeks of HFD in C57BL/6J mice, but a reduction in bone mass after 16 and 24 weeks of HFD (28). In addition, other studies showed that mice that were fed HFD for an extended period had a decreased bone mass, and that they had a poor recovery ability after LFD was performed (29, 30). The same results showed that HFD increased BMAT formation (as measured by increased volume, number, and size) and decreased bone mass (30–36). As Fazeli et al. confirmed, bone marrow adipocytes and osteoblasts had a negative correlation, so the

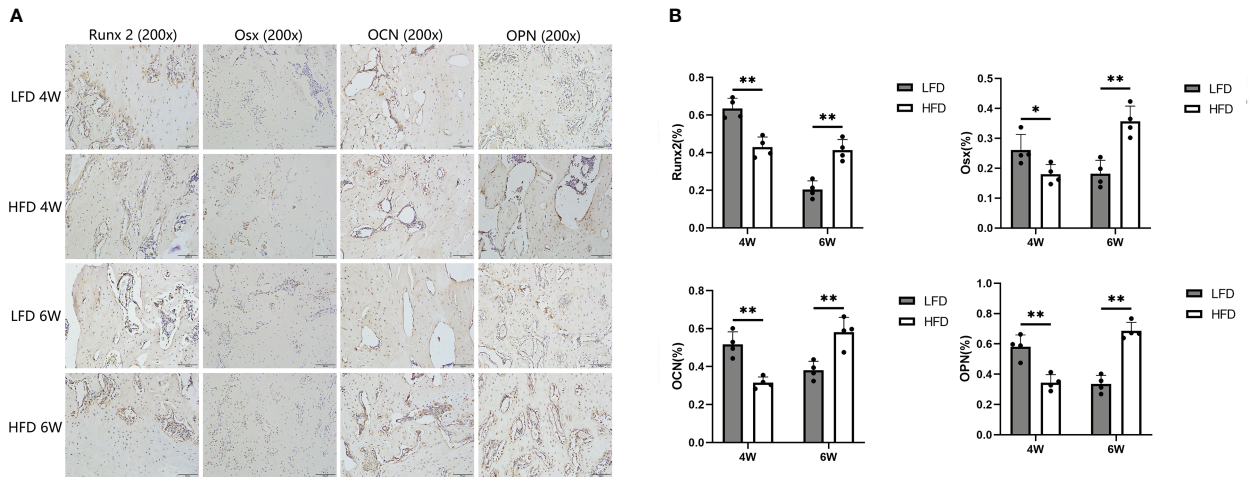

FIGURE 8
Immunohistochemical analysis. **(A)** Immunohistochemistry images of Runx 2, Osterix, OCN, and OPN in the two groups at the termination of 4-week and 6-week consolidation. **(B)** The semiquantitative measurements showed the 4 markers were highly expressed in LFD group compared to HFD group after 4 weeks of consolidation. At the termination of the 6-week consolidation, these indicators were lower expressed in LFD group compared to HFD group. (*P<0.05, **P<0.01).

differentiation direction of bone marrow mesenchymal stem cells (BMSCs) is crucial for bone regeneration. (37). In addition, other researchers demonstrated that bone marrow adipocytes secrete pro-inflammatory mediators (TNFα and IL-6) and adipokines, which can reduce the differentiative capacity of BMSCs in bone cells, but promote adipogenesis (38, 39).

Our study found that bone marrow adipocytes in the HFD group were significantly more numerous than those in the LFD group. However, it was observed that bone regeneration was delayed in the HFD group after 4 and 6 weeks of consolidation. The digital radiograph demonstrated that the bone regeneration after trauma was delayed in the HFD group. Furthermore, like radiographic results, the micro-CT examination revealed that LFD significantly improved bone regeneration and recanalization of the medullary cavity compared with HFD. Additionally, quantitative analyses concluded that bone quality is clearly impaired by HFD by decreasing BMD, BV/TV, and mechanical properties in regenerated segments. A slower bone regeneration was also observed in the HFD group when compared with the LFD group based on histomorphological assessment. In DO, HFD had detrimental effects on bone regeneration, as evidenced by the aforementioned compelling findings.

Bone health has been shown to be adversely affected by high-energy diets in previous studies (7, 30, 40–43). Similarly, our results showed that rats in the HFD group had less bone mass, slower bone regeneration, and higher BMAT volumes, suggesting that bone mass loss and slow bone regeneration are related to BMSCs differentiation and cytokines they secrete. Many researchers have reported an inverse relationship between bone formation and BMAT based on pathophysiological studies and suggested that enhanced differentiation of BMSCs into adipocytes reduces differentiation into osteoblasts, reducing bone formation as a result (32, 33, 44, 45). Our data support this conclusion under HFD, as we observed increased BMAT volume and quantity and delayed bone regeneration in the distraction zone in HFD rats.

Furthermore, hyperlipidemia load (TC, TG, and LDL) in the circulation increases lipid uptake by the skeleton and promotes the differentiation of BMSCs into adipocytes (46, 47). On the other hand, studies have shown that oxidation products of LDL-C can regulate the metabolic process of bone by affecting osteoblasts (48, 49). It has been shown that mildly modified LDL inhibits osteoblast differentiation by increasing extracellular oxidative stress responses among osteoblasts and BMSCs (50). Moreover, the oxidation products of LDL-C have also been found to stimulate the transformation of mouse BMSCs into adipocytes *in vitro* (51). Further, LDL-C and oxidized LDL can also reduce bone regeneration by stimulating p53 to cause osteoblast apoptosis (52). As a result of the above findings, the increased levels of LDL in the circulating blood appear to contribute to the delayed bone regeneration and mineralization in the distraction zone, which reduce the number and differentiation of osteoblasts and increase the number and activity of osteoclasts.

It is well known that bone tissue regeneration results from the joint regulation of bone formation and bone resorption. During bone formation, osteoblast differentiation plays a crucial role. Observation of osteogenic markers during bone regeneration can help determine osteoblast differentiation. In this study, these markers were analyzed and showed that a significant reduction in the expression of early and terminal osteogenic markers (Runx2 and Osx in early expression; OPN and OCN in a terminal expression) in the HFD group was observed. However, after 6 weeks of consolidation, the expression of these markers was reversed between the two groups, and there was a higher expression in the HFD group. According to the evaluation mentioned earlier, HFD significantly reduced bone formation. Hence, we speculated that in comparison to HFD group, less osteogenic relative factors and proteins were produced in the more mature regenerate trabeculae in LFD group at 6-week consolidation.

In bone formation, intramembranous and endochondral ossification play an important role, which requires a high blood supply (53). As bones develop and regenerate, blood supply and osteogenesis are tightly intertwined (54–57). As well as providing oxygen for bone development and regeneration, an adequate blood supply can also activate BMSCs and stimulate them to differentiate into osteoblasts (57). According to previous studies, increased BMAT impaired hematopoiesis, including depletion of B lymphocytes and inhibition of proliferation and differentiation of hematopoietic stem cells (58–60). Additionally, hyperlipidemia and the accumulation of lipid droplets in distraction zones may slow and obstruct local blood flow, impacting bone regeneration. (61). As a consequence of the above studies, the increase in BMAT observed in the distraction zone of rats receiving HFD may further affect bone regeneration by affecting the blood supply there.

Although our study yielded promising results, it also had several limitations. First of all, the present study only implemented one style of HFD, and future investigations are needed to identify whether additional styles will produce different results. In addition, different from humans, rats are walking with four limbs and receive less weight-bearing and mechanical stimulation on the femur. Therefore, further experimental methods need to be developed and optimized to avoid this difference. Moreover, in this study, an evaluation of the effectiveness of regeneration was based on histological and morphological characteristics of the regenerated bone, there may be future directions focusing on the molecular mechanisms underlying the effects observed. In summary, the observed results demonstrated that a relationship between HFD and bone regeneration may be mediated by the bone marrow microenvironment and dyslipidemia.

5 Conclusion

As a result of the establishment of a DO model in this study, the adverse effects of a high-fat diet on bone health were magnified and observed, and the mechanism may be related to the differentiation of BMSCs into adipose tissue under a high-fat diet. The result differs from traditional beliefs that high-fat diets are good for bone health because they cause weight gain. In addition, elevated lipid levels caused by a high-fat diet may affect the blood supply of new bone tissue, something that needs to be verified by further research.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding authors.

Ethics statement

The animal study was reviewed and approved by the Animal Ethics Committee of Xinjiang medical university.

Author contributions

Conceptualization, FC, AY, YSL, and YL; methodology, FC and AY; software, FC; investigation, FC, AY, and KL; data analysis, FC, KL, WC, and RZ; writing original draft preparation, FC; review and editing, AY, KL, WC, RZ, YSL, and YL; supervision, YSL and YL; funding acquisition, AY, and YL. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

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References

1. Albala C, Yanez M, Devoto E, Sostin C, Zeballos L, Santos JL. Obesity as a protective factor for postmenopausal osteoporosis. *Int J Obes Relat Metab Disord* (1996) 20(11):1027–32.

2. Looker AC, Flegal KM, Melton LR. Impact of increased overweight on the projected prevalence of osteoporosis in older women. *Osteoporos Int* (2007) 18(3):307–13. doi: 10.1007/s00198-006-0241-8

3. Dimitri P, Bishop N, Walsh JS, Eastell R. Obesity is a risk factor for fracture in children but is protective against fracture in adults: a paradox. *Bone* (2012) 50(2):457–66. doi: 10.1016/j.bone.2011.05.011

4. Rana AR, Michalsky MP, Teich S, Groner JI, Caniano DA, Schuster DP. Childhood obesity: a risk factor for injuries observed at a level-1 trauma center. *J Pediatr Surg* (2009) 44(8):1601–5. doi: 10.1016/j.jpedsurg.2008.11.060

5. Hou J, He C, He W, Yang M, Luo X, Li C. Obesity and bone health: A complex link. *Front Cell Dev Biol* (2020) 8:600181. doi: 10.3389/fcell.2020.600181

6. Cao JJ, Gregoire BR, Gao H. High-fat diet decreases cancellous bone mass but has no effect on cortical bone mass in the tibia in mice. *Bone* (2009) 44(6):1097–104. doi: 10.1016/j.bone.2009.02.017

7. Cao JJ, Sun L, Gao H. Diet-induced obesity alters bone remodeling leading to decreased femoral trabecular bone mass in mice. *Ann N Y Acad Sci* (2010) 1192:292–7. doi: 10.1111/j.1749-6632.2009.05252.x

8. Cao JJ. Effects of obesity on bone metabolism. *J Orthop Surg Res* (2011) 6:30. doi: 10.1186/1749-799X-6-30

9. Shoelson SE, Herrero L, Naaz A. Obesity, inflammation, and insulin resistance. *Gastroenterology* (2007) 132(6):2169–80. doi: 10.1053/j.gastro.2007.03.059

10. Halade GV, El JA, Williams PJ, Fajardo RJ, Fernandes G. Obesity-mediated inflammatory microenvironment stimulates osteoclastogenesis and bone loss in mice. *Exp Gerontol.* (2011) 46(1):43–52. doi: 10.1016/j.exger.2010.09.014

11. Le P, Kawai M, Bornstein S, Demambro VE, Horowitz MC, Rosen CJ. A high-fat diet induces bone loss in mice lacking the Alox5 gene. *Endocrinology* (2012) 153(1):6–16. doi: 10.1210/en.2011-0082

12. Li CJ, Cheng P, Liang MK, Chen YS, Lu Q, Wang JY, et al. MicroRNA-188 regulates age-related switch between osteoblast and adipocyte differentiation. *J Clin Invest.* (2015) 125(4):1509–22. doi: 10.1172/JCI77716

13. Li CJ, Xiao Y, Yang M, Su T, Sun X, Guo Q, et al. Long noncoding RNA bmncr regulates mesenchymal stem cell fate during skeletal aging. *J Clin Invest.* (2018) 128 (12):5251–66. doi: 10.1172/JCI99044

14. Ilizarov GA. The tension-stress effect on the genesis and growth of tissues. part i. the influence of stability of fixation and soft-tissue preservation. *Clin Orthop Relat Res* (1989) 238):249–81.

15. Makhdom AM, Hamdy RC. The role of growth factors on acceleration of bone regeneration during distraction osteogenesis. *Tissue Eng. B: Rev* (2013) 19:442–53. doi: 10.1089/ten.TEB.2012.0717

16. Paley D, Maar DC. Ilizarov bone transport treatment for tibial defects. *J Orthop Trauma.* (2000) 14(2):76–85. doi: 10.1097/00005131-200002000-00002

17. Parsons B, Strauss E. Surgical management of chronic osteomyelitis. *Am J Surg* (2004) 188(1A Suppl):57–66. doi: 10.1016/S0002-9610(03)00292-7

18. Spiegelberg B, Parratt T, Dheerendra SK, Khan WS, Jennings R, Marsh DR. Ilizarov principles of deformity correction. *Ann R Coll Surg Engl* (2010) 92(2):101–5. doi: 10.1308/003588410X12518836439326

19. Wen G, Xu J, Wu T, Zhang S, Chai Y, Kang Q, et al. Functionalized Polycaprolactone/Hydroxyapatite composite microspheres for promoting bone consolidation in a rat distraction osteogenesis model. *J Orthop Res* (2020) 38(5):961–71. doi: 10.1002/jor.24542

20. Yang Y, Pan Q, Zou K, Wang H, Zhang X, Yang Z, et al. Administration of allogeneic mesenchymal stem cells in lengthening phase accelerates early bone consolidation in rat distraction osteogenesis model. *Stem Cell Res Ther* (2020) 11 (1):129. doi: 10.1186/s13287-020-01635-5

21. Wang X, Wang Y, Gou W, Lu Q, Peng J, Lu S. Role of mesenchymal stem cells in bone regeneration and fracture repair: a review. *Int Orthop* (2013) 37(12):2491–8. doi: 10.1007/s00264-013-2059-2

22. Jin YZ, Lee JH. Mesenchymal stem cell therapy for bone regeneration. *Clin Orthop Surg* (2018) 10(3):271–8. doi: 10.4055/cios.2018.10.3.271

23. Xu J, Wu T, Sun Y, Wang B, Zhang J, Lee WY, et al. Staphylococcal enterotoxin C2 expedites bone consolidation in distraction osteogenesis. *J Orthop Res* (2017) 35 (6):1215–25. doi: 10.1002/jor.23372

24. Xu J, Wang B, Sun Y, Wu T, Liu Y, Zhang J, et al. Human fetal mesenchymal stem cell secretome enhances bone consolidation in distraction osteogenesis. *Stem Cell Res Ther* (2016) 7(1):134. doi: 10.1186/s13287-016-0392-2

25. Perrien DS, Nicks KM, Liu L, Akel NS, Bacon AW, Skinner RA, et al. Inhibin a enhances bone formation during distraction osteogenesis. *J Orthop Res* (2012) 30:288–95. doi: 10.1002/jor.21501

26. Ai-Aql ZS, Alagl AS, Graves DT, Gerstenfeld LC, Einhorn TA. Molecular mechanisms controlling bone formation during fracture healing and distraction osteogenesis. *J Dent Res* (2008) 87:107–18. doi: 10.1177/154405910808700215

27. Cao JJ, Picklo MS. Involuntary wheel running improves but does not fully reverse the deterioration of bone structure of obese rats despite decreasing adiposity. *Calcif Tissue Int* (2015) 97(2):145–55. doi: 10.1007/s00223-015-9992-6

28. Tian L, Wang C, Xie Y, Wan S, Zhang K, Yu X. High fructose and high fat exert different effects on changes in trabecular bone micro-structure. *J Nutr Health Aging.* (2018) 22(3):361–70. doi: 10.1007/s12603-017-0933-0

29. Inzana JA, Kung M, Shu L, Hamada D, Xing LP, Zuscik MJ, et al. Immature mice are more susceptible to the detrimental effects of high fat diet on cancellous bone in the distal femur. *Bone* (2013) 57(1):174–83. doi: 10.1016/j.bone.2013.08.003

30. Scheller EL, Khoury B, Moller KL, Wee NK, Khandaker S, Kozloff KM, et al. Changes in skeletal integrity and marrow adiposity during high-fat diet and after weight loss. *Front Endocrinol (Lausanne).* (2016) 7:102. doi: 10.3389/fendo.2016.00102

31. Halade GV, Rahman MM, Williams PJ, Fernandes G. High fat diet-induced animal model of age-associated obesity and osteoporosis. *J Nutr Biochem* (2010) 21 (12):1162–9. doi: 10.1016/j.jnutbio.2009.10.002

32. Bredella MA, Torriani M, Ghomi RH, Thomas BJ, Brick DJ, Gerweck AV, et al. Vertebral bone marrow fat is positively associated with visceral fat and inversely associated with IGF-1 in obese women. *Obes (Silver Spring).* (2011) 19(1):49–53. doi: 10.1038/oby.2010.106

33. Bredella MA, Gill CM, Keating LK, Torriani M, Anderson EJ, Punyanitya M, et al. Assessment of abdominal fat compartments using DXA in premenopausal women from anorexia nervosa to morbid obesity. *Obes (Silver Spring).* (2013) 21(12):2458–64. doi: 10.1002/oby.20424

34. Doucette CR, Horowitz MC, Berry R, Macdougald OA, Anunciado-Koza R, Koza RA, et al. A high fat diet increases bone marrow adipose tissue (MAT) but does not alter trabecular or cortical bone mass in C57BL/6J mice. *J Cell Physiol* (2015) 230 (9):2032–7. doi: 10.1002/jcp.24954

35. Bornstein S, Moschetta M, Kawano Y, Sacco A, Huynh D, Brooks D, et al. Metformin affects cortical bone mass and marrow adiposity in diet-induced obesity in Male mice. *Endocrinology* (2017) 158(10):3369–85. doi: 10.1210/en.2017-00299

36. Styner M, Pagnotti GM, Mcgrath C, Wu X, Sen B, Uzer G, et al. Exercise decreases marrow adipose tissue through ss-oxidation in obese running mice. *J Bone Miner. Res* (2017) 32(8):1692–702. doi: 10.1002/jbmr.3159

37. Fazeli PK, Horowitz MC, Macdougald OA, Scheller EL, Rodeheffer MS, Rosen CJ, et al. Marrow fat and bone–new perspectives. *J Clin Endocrinol Metab* (2013) 98 (3):935–45. doi: 10.1210/jc.2012-3634

38. Horowitz MC, Berry R, Holtrup B, Sebo Z, Nelson T, Fretz JA, et al. Bone marrow adipocytes. *Adipocyte* (2017) 6(3):193–204. doi: 10.1080/21623945.2017.1367881

39. Singh L, Tyagi S, Myers D, Duque G. Good, bad, or ugly: the biological roles of bone marrow fat. *Curr Osteoporos Rep* (2018) 16(2):130–7. doi: 10.1007/s11914-018-0427-y

40. Goulding A, Taylor RW, Jones IE, Mcauley KA, Manning PJ, Williams SM. Overweight and obese children have low bone mass and area for their weight. *Int J Obes Relat Metab Disord* (2000) 24(5):627–32. doi: 10.1038/sj.ijo.0801207

41. Patsch JM, Kiefer FW, Varga P, Pail P, Rauner M, Stupphann D, et al. Increased bone resorption and impaired bone microarchitecture in short-term and extended high-fat diet-induced obesity. *Metabolism* (2011) 60(2):243–9. doi: 10.1016/j.metabol.2009.11.023

42. Junior IF, Cardoso JR, Christofaro DG, Codogno JS, de Moraes AC, Fernandes RA. The relationship between visceral fat thickness and bone mineral density in sedentary obese children and adolescents. *BMC Pediatr* (2013) 13:37. doi: 10.1186/1471-2431-13-37

43. Fajardo RJ, Karim L, Calley VI, Bouxsein ML. A review of rodent models of type 2 diabetic skeletal fragility. *J Bone Miner. Res* (2014) 29(5):1025–40. doi: 10.1002/jbmr.2210

44. Rzonca SO, Suva LJ, Gaddy D, Montague DC, Lecka-Czernik B. Bone is a target for the antidiabetic compound rosiglitazone. *Endocrinology* (2004) 145(1):401–6. doi: 10.1210/en.2003-0746

45. Bredella MA, Fazeli PK, Miller KK, Misra M, Torriani M, Thomas BJ, et al. Increased bone marrow fat in anorexia nervosa. *J Clin Endocrinol Metab* (2009) 94 (6):2129–36. doi: 10.1210/jc.2008-2532

46. Greenberg AS, Obin MS. Obesity and the role of adipose tissue in inflammation and metabolism. *Am J Clin Nutr* (2006) 83(2):461S–5S. doi: 10.1093/ajcn/83.2.461S

47. Bartelt A, Koehne T, Todter K, Reimer R, Muller B, Behler-Janbeck F, et al. Quantification of bone fatty acid metabolism and its regulation by adipocyte lipoprotein lipase. *Int J Mol Sci* (2017) 18(6). doi: 10.3390/ijms18061264

48. Lee SJ, Kang SW, Do HJ, Han I, Shin DA, Kim JH, et al. Enhancement of bone regeneration by gene delivery of BMP2/Runx2 bicistronic vector into adipose-derived stromal cells. *Biomaterials* (2010) 31(21):5652–9. doi: 10.1016/j.biomaterials.2010.03.019

49. Lozano D, Sanchez-Salcedo S, Portal-Nunez S, Vila M, Lopez-Herradon A, Ardura JA, et al. Parathyroid hormone-related protein (107-111) improves the bone regeneration potential of gelatin-glutaraldehyde biopolymer-coated hydroxyapatite. *Acta Biomater.* (2014) 10(7):3307–16. doi: 10.1016/j.actbio.2014.03.025

50. Navab M, Berliner JA, Watson AD, Hama SY, Territo MC, Lusis AJ, et al. The yin and yang of oxidation in the development of the fatty streak. a review based on the 1994 George Lyman duff memorial lecture. *Arterioscler Thromb Vasc Biol* (1996) 16 (7):831–42. doi: 10.1161/01.atv.16.7.831

51. Parhami F, Jackson SM, Tintut Y, Le V, Balucan JP, Territo M, et al. Atherogenic diet and minimally oxidized low density lipoprotein inhibit osteogenic and promote adipogenic differentiation of marrow stromal cells. *J Bone Miner. Res* (1999) 14 (12):2067–78. doi: 10.1359/jbmr.1999.14.12.2067

52. Srinivasan K, Viswanad B, Asrat L, Kaul CL, Ramarao P. Combination of high-fat diet-fed and low-dose streptozotocin-treated rat: a model for type 2 diabetes and pharmacological screening. *Pharmacol Res* (2005) 52(4):313–20. doi: 10.1016/j.phrs.2005.05.004

53. Karsenty G. The complexities of skeletal biology. *Nature* (2003) 423(6937):316–8. doi: 10.1038/nature01654

54. Danis A. [Mechanism of bone lengthening by the ilizarov technique]. *Bull Mem Acad R Med Belg.* (2001) 156(1-2):107–12.

55. Wan C, Gilbert SR, Wang Y, Cao X, Shen X, Ramaswamy G, et al. Activation of the hypoxia-inducible factor-1alpha pathway accelerates bone regeneration. *Proc Natl Acad Sci U S A.* (2008) 105(2):686–91. doi: 10.1073/pnas.0708474105

56. Schipani E, Maes C, Carmeliet G, Semenza GL. Regulation of osteogenesis-angiogenesis coupling by HIFs and VEGF. *J Bone Miner. Res* (2009) 24(8):1347–53. doi: 10.1359/jbmr.090602

57. Maes C, Carmeliet G, Schipani E. Hypoxia-driven pathways in bone development, regeneration and disease. *Nat Rev Rheumatol* (2012) 8(6):358–66. doi: 10.1038/nrrheum.2012.36

58. Naveiras O, Nardi V, Wenzel PL, Hauschka PV, Fahey F, Daley GQ. Bone-marrow adipocytes as negative regulators of the haematopoietic microenvironment. *Nature* (2009) 460(7252):259–63. doi: 10.1038/nature08099

59. Ambrosi TH, Scialdone A, Graja A, Gohlke S, Jank AM, Bocian C, et al. Adipocyte accumulation in the bone marrow during obesity and aging impairs stem cell-based hematopoietic and bone regeneration. *Cell Stem Cell* (2017) 20(6):771–784.e6. doi: 10.1016/j.stem.2017.02.009

60. Zhou BO, Yu H, Yue R, Zhao Z, Rios JJ, Naveiras O, et al. Bone marrow adipocytes promote the regeneration of stem cells and haematopoiesis by secreting SCF. *Nat Cell Biol* (2017) 19(8):891–903. doi: 10.1038/ncb3570

61. Insua A, Monje A, Wang HL, Miron RJ. Basis of bone metabolism around dental implants during osseointegration and peri-implant bone loss. *J Biomed Mater Res A.* (2017) 105(7):2075–89. doi: 10.1002/jbm.a.36060

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Dietary intervention reprograms bone marrow cellular signaling in obese mice

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Objectives: The current study aimed to investigate the pathogenesis of obesity-induced impaired bone mass accrual and the impact of dietary intervention on bone density in the mouse model of obesity.

Methods: Mice were fed with chow diet (CD) for 10 months, high-fat-diet (HFD) for 10 months, or HFD for 6 months then transferred to chow diet for 4 months (HFDt).

Results: Weight loss and decreased intrahepatic lipid accumulation were observed in mice following dietary intervention. Additionally, HFD feeding induced bone mass accrual, while diet intervention restrained trabecular bone density. These changes were further reflected by increased osteogenesis and decreased adipogenesis in HFDt mice compared to HFD mice. Furthermore, HFD feeding decreased the activity of the Wingless-related integration site (Wnt)-β-Catenin signaling pathway, while the Wnt signaling was augmented by diet intervention in the HFDt group.

Conclusions: Our findings suggest that a HFD inhibits bone formation and that dietary intervention reverses this inhibition. Furthermore, the dietary intervention was able to compensate for the suppressed increase in bone mass to a level comparable to that in the CD group. Our study suggests that targeting the Wnt signaling pathway may be a potential approach to treat obesity-induced impaired bone mass accrual.

KEYWORDS

obesity, bone formation, bone mass accrual, diet intervention, Wnt signaling pathway

1 Introduction

The global prevalence of obesity is estimated to reach 18% in men and 21% in women by 2025, representing a substantial health risk and economic burden (1). Obesity is commonly associated with dysregulated glucose, energy and fat metabolism, which can lead to an array of tissue-specific declines and dysfunctions (2, 3). Although current treatments for obesity often include bone mineral density decline as a side effect, this phenomenon is further exacerbated by the development of osteoporosis (OP) (4). Characterized by weakened bone microarchitecture and a decrease in bone density, OP is the most common bone disease worldwide, affecting an estimated 200 million people (5), and is a risk factor for various secondary health issues and mortality (6). Both obesity and OP have been found to have associations with nutrition, energy intake and sedentary lifestyles (7, 8).

Increasing evidence has shown that obesity, particularly due to excessive dietary fat intake, is associated with a heightened risk of fracture through disruption of bone remodeling and accelerated bone aging (9, 10). Studies have demonstrated a 33% body fat threshold at which visceral fat may produce a range of molecules with deleterious effects on the bone microenvironment (11, 12). Potential mechanisms underlying this phenomenon may include oxidative stress (13), elevated production of inflammatory cytokines (14), increased expression of peroxisome proliferator-activated receptor gamma (*PPARγ*) in bone marrow adipocytes (15), and disruption of Wnt signaling (16). The Wnt pathway is a key regulator of bone quantity and remodeling, largely due to its promotion of osteoblast differentiation and indirect control of osteoclastogenesis (17). The Wnt signaling pathway, comprising key molecules (Wnt3a, Wnt5a), has been identified as playing a pivotal role in bridging intrinsic processes associated with bone remodeling and energy metabolism, thus providing a promising avenue for the management of bone mass accrual (18).

Dietary interventions for weight loss are a well-established technique for managing obesity, with lifestyle modifications, such as dietary changes to decrease energy intake, being amongst the most widely investigated approaches (19). Thus, it is pertinent to ask whether the metabolic impairments in bone caused by a HFD can be reversed through a shift to a normal diet. Recent studies have exhibited a reversal of metabolic alterations, as well as a decrease in body weight, improved glucose tolerance, and decreased adiposity (20, 21), but little is known about the effects of changing diet on bone density. Scheller et al. demonstrated in a descriptive study that while a switch from a HFD to a CD could lead to weight loss, it could not fully prevent reduced bone formation. To better understand the effects of obesity-related reduced bone formation and the impact of a long-term dietary intervention on bone density, our study used mouse models of obesity and dietary restriction of fat components to induce weight loss.

Abbreviations: CD, chow diet; HFD, high-fat-diet; HFDt, high-fat diet-transfer; Wnt, Wingless-related integration site; OP, osteoporosis; PPARγ, peroxisome proliferator-activated receptor gamma; Micro-CT, micro-computed tomography; ELISA, enzyme linked immunosorbent assay; PINP, procollagen I N-terminal propeptide; EDTA, ethylenediaminetetraacetic acid; OCN, osteocalcin; TRAP, tartrate-resistant acid phosphatase; SDS-PAGE, SDS-polyacrylamide gel electrophoresi; TBST, TBS-Tween 20; HRP, horseradish peroxidase; ECL, enhanced chemilumescent; BM, bone marrow; BMSCs, bone marrow mesenchymal stem cells; DMEM, Dulbecco's modified Eagle's medium; PFA, paraformaldehyde; TG, total triglyceride; TC, total cholesterol; LDL-C, low density liptein cholesterol cholesterol; HDL-C, high density liptein cholesterol cholesterol; RBG, random blood glucose; FBG, fasting blood glucose; Ctsk, cathepsin K; Col1a1, collagen type I A I; ALP, alkaline phosphatase; Runx2, runt-related transcription factor 2; Adipoq, adiponectin; CD36, cluster of differentiation 36; TGFβ, transforming growth factor beta-1; Ror1/2, receptor tyrosine kinase-like orphan receptor 1/2; GSK3, glycogen synthase kinase 3.

2 Methods

2.1 Experimental animals

C57BL/6 male mice (6 weeks old) were obtained from the Laboratory Animal Center of Fudan University and housed in a specific pathogen-free environment (temperature: 23 ± 1°C; light-dark cycle: 12/12 hrs). Autoclaved food and water were provided ad libitum. Following a one-week acclimatization period, the animals were randomly divided into three groups: chow diet (CD; n=10), high-fat diet (HFD; n=12), and high-fat diet-transfer (HFDt; n=10). The CD group was fed a standard chow diet (Research Diets, D12450B, 10% cal% fat) for 10 months; the HFD group was fed a HFD (Research Diets, D12492, 60% cal% fat) for 10 months; and the HFDt group was fed a HFD for 6 months and then transferred to a chow diet for 4 months. HFDt group mice gradually increased body weight in the first 6 months, and then rapidly decreased to the same body weight as the CD group in the following 4 months. After 10 months of treatment, mice were fasted for 12 h and humanely euthanized under general anesthesia. To enrich the data on the relationship between male obesity and osteoporosis and reduce the confounding factors in the results, we used male mice for the model. All experimental protocols were approved by the Animal Care and Use Committee of Zhongshan Hospital, Fudan University.

2.2 Analysis of bone microstructure by micro-computed tomography

Following sacrifice, femurs were detached and fixed in 4% paraformaldehyde for subsequent micro-CT scanning and analysis. High-resolution imaging of the bone microstructure was carried out using a Skyscan 1176 system [software=Version 1.1 (build 6), Bruker, Kontich, Belgium] with a resolution of 8.96 microns. Trabecular bone mass was assessed by contouring a region 1.0 mm wide, positioned 500 microns from the proximal end of the distal femoral growth plate, and a threshold of 66–255 permille was applied. For the femoral cortical bone, a 500-micron-wide region was contoured starting 4.0 mm from the proximal end of the distal femoral growth plate, with a threshold of 114–255

permille. Three-dimensional reconstructions were created from the two-dimensional images acquired from the contoured regions.

2.3 Enzyme linked immunosorbent assay

Measurement of serum PINP in mice was conducted using the mouse procollagen I N-terminal propeptide (PINP) ELISA Kit (MU30602, Bioswamp) in accordance with the manufacturer's instructions. Measurement of serum CTX-1 in mice was conducted using the mouse type I collagen C-terminal peptide (CTX-1) ELISA Kit (Jining Shiye, China) in accordance with the manufacturer's instructions

2.4 Histology and immunostaining

Femurs and tibias were fixed in 4% paraformaldehyde for 48 h and incubated in 20% ethylenediaminetetraacetic acid (EDTA) solution for decalcification. Embedding in paraffin, dehydration, and cutting of 5 mm longitudinal sections were performed prior to staining with H&E, osteocalcin (OCN), and tartrate-resistant acid phosphatase (TRAP) (Sigma, Merck, Germany). After preparation of paraffin sections of the femur, they were first stained with hematoxylin staining solution, followed by eosin staining solution after dehydration with alcohol, and then dehydrated and sealed. The number of adipocytes per field of view was quantified using the Image J program. It is important to note that this method of fixing femoral adipocytes resulted in the degradation of a portion of the adipocytes and resulted in their inability to be identified. Immunohistochemistry following the IHC paraffin protocol (Abcam) with a OCN antibody (Proteintech, 23418-1-AP, 1:200) was performed and the proportion of positive cells in each field was quantified using the Image J program.

2.5 Quantitative RT-PCR analysis

Trizol reagent (Invitrogen) was used to extract total RNA from tibia, and cDNA was subsequently synthesized using a PrimeScript RT Reagent Kit (TaKaRa, Tokyo, Japan), following the manufacturer's instructions. Quantitative real-time PCR was subsequently performed using SYBR Green Premix Ex Taq (Takara, Japan) and Light Cycler 480 (Roche, Switzerland), with the $2^{-\Delta\Delta Ct}$ method used for data analysis and GAPDH as an internal control for normalization. The sequences of oligonucleotides employed for RT-PCR are listed in Table S1.

2.6 Western blot analysis

Western blotting was employed to assess protein expression levels in bones. Equal protein concentrations were subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto PVDF membranes (Milipore, Darmstadt, Germany). Subsequently, the membranes were blocked with 5% skim milk-PBS-Tween 20 for 1 h at room temperature and incubated with specific antibodies to PPARγ (1:1,000, P37231, CST), Runx2 (1:1000, AF2593, Beyotime), Wnt5a (1:1000, AF8358, Beyotime), Wnt3a (1:1000, AF8352, Beyotime), β-catenin (1:1000, AF0066, Beyotime), GAPDH (1:1000, AB_2736879, Abclonal), and β-Actin (1:1000, AB_2768234, Abclonal) overnight at 4°C. The blots were then washed with TBS-Tween 20 (TBST) and incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (1:5000) for 1 h at room temperature. Lastly, the blots were washed again and incubated with enhanced chemilumescent (ECL) substrates (Bio-rad) for 1 min and the Image J software was applied to analyze the blots.

2.7 Bone marrow cellularity, isolation, and culture of bone marrow mesenchymal stem cells

Isolation of bone marrow stromal cells (BMSCs) was achieved through flushing with Dulbecco's modified Eagle's medium (DMEM) containing low glucose (Vitrocell, Brazil) supplemented with 10% fetal calf serum (Vitrocell, Brazil), 100 IU/ml sodium penicillin G (Sigma-Aldrich, USA), and 100 μg/ml streptomycin (Sigma-Aldrich, USA) at 37°C in a 5% CO2-95% humidity atmosphere. BMSCs were isolated based on their capacity to adhere to plastic surfaces in cell cultures using the aforementioned low-glucose medium. Cells of passages 3–10 were used for differentiation assays, which involved seeding BMSCs in six-well plates and treating them with an osteogenic medium composed of 50 μg/ml ascorbic acid, 5 mM β-glycerophosphate, and 100 nM dexamethasone (all from Sigma). After seven days of differentiation, the cells were washed with PBS, fixed in 4% paraformaldehyde (PFA) for 2 min, and assayed with ALP Staining Kit (Beyotime, C3206).

2.8 Serum and liver biochemical assays

Serum total triglyceride (TG), total cholesterol (TC), low density liptein cholesterol cholesterol (LDL-C), and high density liptein cholesterol cholesterol (HDL-C) levels were detected using commercial kits (Jiancheng, China). Liver TG and TC levels were detected using content assay kits (Applygen, China).

2.9 Statistical analysis

Statistical analyses were conducted using GraphPad 8.0. One-way ANOVA was employed to assess the impacts of CD, HFDt, and HFD. Subsequent to the ANOVA, Tukey's multiple comparison test was utilized to further investigate the outcomes. When the homogeneity of variance assumption is violated for a one-way ANOVA, Welch's ANOVA can be conducted instead, and Games-Howell's multiple comparisons test was utilized to further investigate the outcomes.

We conducted a power analysis on the study model. Our effect size was 0.40, alpha error was 0.05, total sample size was 30, the

number of groups was 3, The analysis method was one-way ANOVA, and the power obtained by G.power software was 0.44.

3 Results

3.1 Diet intervention reprograms whole body metabolism in mice

To examine whether dietary intervention could reverse the phenotype of long-term HFD induced OP in mice, we established HFD, HFDt, and CD groups to assess phenotypes of tibia, femur, and BMSCs (Figure 1A). In contrast to the HFD mice, the body weight of HFDt mice was significantly reduced (P<0.0001) after the 4 months of dietary intervention, and there was no significant difference (P>0.05) between the HFDt group and the CD group. At the end of the 10th month, the mean body weight (± standard deviation) of the HFD mice was 57.92 ± 2.99 g, and that of the HFDt and CD groups was 36.13± 3.48g and 36.91± 1.62g, respectively (Figure 1B). During the 4 months of diet intervention, caloric intake was lower in the HFDt group than in the CD group (Figure S1A). Liver weight (P<0.01), liver weight/body weight (P<0.05), eWAT (P<0.001), and iWAT (P<0.01) showed that HFD mice had a significantly greater tissue mass than HFDt and CD mice, indicating a normalization of their mass due to weight loss in HFDt mice. (Figures 1C–F). Serum TG levels were similar in all groups. Further examination of the levels of serum TC, HDL-C, and LDL-C revealed that the HFD group had higher levels than both the CD and HFDt groups (Figures S1B-E). Analysis of liver TG levels in mice exposed to a HFD demonstrated that TG levels were significantly higher in the HFD group than in both the CD (P<0.01) and HFDt (P<0.05) groups (Figure S1G). There were no differences in random blood glucose (RBG) between all groups (Figure S1H). Fasting blood glucose (FBG) levels were higher in HFDt mice than in CD mice, but significantly lower than in HFD mice (P<0.05), indicating that dietary intervention could improve blood glucose in HFD mice (Figure S1I). Liver tissues from HFD mice revealed an increased area of adipocytes and lipid droplets, suggesting that long-term HFD caused severe fatty liver, while dietary intervention ameliorated long-term HFD-induced fat accumulation (Figures 1G, H). Collectively, these results suggest that HFD feeding leads to obesity and metabolic disorders in mice, with dietary intervention offering a solution.

3.2 Diet intervention improves bone microarchitecture and promotes bone formation

To further explore whether dietary intervention improved bone microarchitecture in HFD mice, we performed micro-CT in three groups of mice. Analysis of micro-CT scans in three groups of mice indicated that femur microarchitecture was detrimentally impacted by HFD, with loose trabeculae and disordered structure

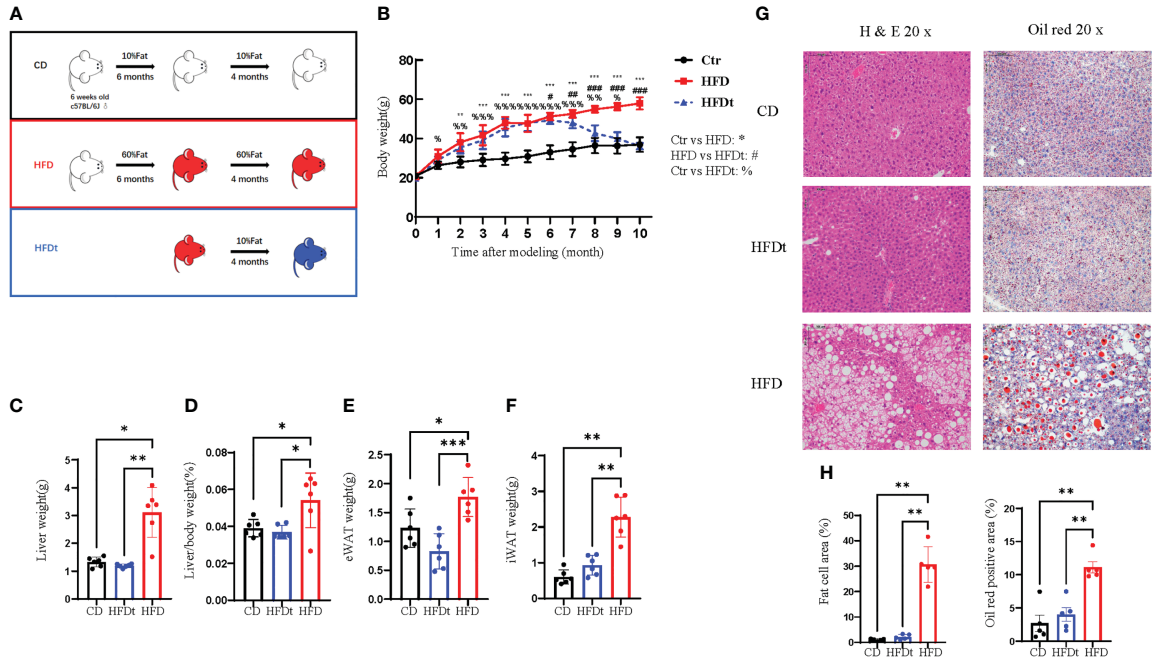

FIGURE 1
Diet intervention reprograms whole body metabolism in mice. **(A)** Experimental design: 6-week-old male C57BL/6 mice received a control diet (CD) (n = 10) or high-fat diet (HFD) (n = 20) ad libitum for 6 months. At the end of this period, half of the HFD group was switched to a chow diet for 4 months, referred to as the dietary intervention group (HFDt) (n = 10). **(B)** Body weight of mice after dietary intervention (n=10/group). **(C-F)** Liver weight, Liver weight/Body weight, Epididymal white adipose tissue (eWAT) weight, inguinal white adipose tissue (iWAT) weight (n = 6/group). **(G)** Histopathological analysis of H&E and oil red stained liver sections after CD, HFDt, or HFD treatment. **(H)** Quantification of H&E staining (left) and oil red staining (right) liver sections from the CD, HFDt, or HFD treated mice. All data shown were obtained from male animals. Significance was determined using one-way ANOVA or Welch's ANOVA. *P < 0.05, **P < 0.01, ***P < 0.001, $^{\%}$P < 0.05, $^{\%\%}$P < 0.01, $^{\%\%\%}$P < 0.001, $^{\#}$P < 0.05, $^{\#\#}$P < 0.01, $^{\#\#\#}$P < 0.001.

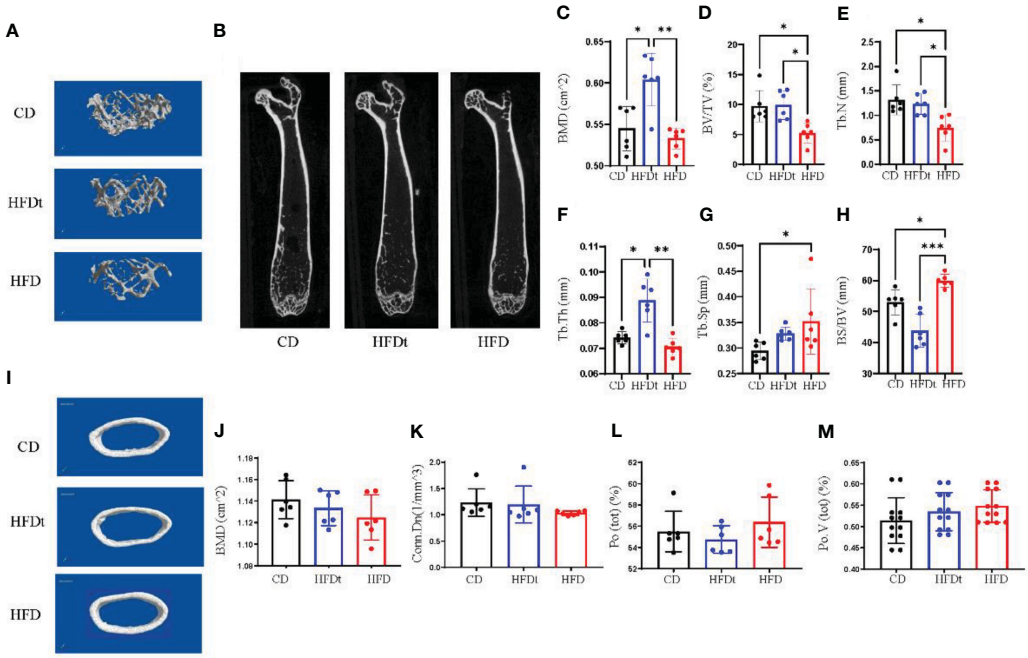

FIGURE 2
Diet intervention improves bone microarchitecture and promotes bone formation. **(A)** Micro-CT 3D reconstruction of representative images of tibial trabecular bone. **(B)** Sections of tibial trabecular bone from three groups. **(C-H)** Trabecular bone parameters at the distal femoral metaphysis, including BMD, BV/TV, Tb.N, Tb.Th, Tb.Sp and BS/BV after CD HFD or HFDt treatment (n = 6). **(I)** Micro-CT 3D reconstruction of representative images of tibial cortical bone. **(J-M)** Cortical bone parameters of the distal metaphysis of the femur were measured, including BMD, Conn.Dn, Ct.Po, Po.V (tot) after CD, HFD or HFDt treatment (n = 6). BMD, (Bone Mineral Density); BV/TV, trabecular bone volume per tissue volume; Tb. N, trabecular number; Tb.Th, trabecular thickness; Tb.Sp, trabecular separation; BS/BV, trabecular surface area per bone volume; Conn.Dn, connectivity density; Po (tot), Ct.Po, cortical porosity; Po.V (tot), total volume of pore space. Significance was determined using one-way ANOVA or Welch's ANOVA. *$P < 0.05$, **$P < 0.01$.

(Figures 2A, B, I). In contrast, bone microarchitecture was maintained in the diet intervention group, with regular and closely arranged trabecular bone structure resembling that of the control group. This demonstrated significant improvement of bone microarchitecture in HFD mice post-dietary intervention. Subsequent trabecular bone parameters analysis with three software sets revealed that BMD ($P<0.001$), BV/TV ($P<0.05$), Tb.N ($P<0.05$), Tb.Th ($P<0.01$) and BS/BV ($P<0.001$) were significantly improved in the HFDt group compared to the HFD group, with most indexes similar or even better than those in the control group ($P < 0.05$). Our findings suggest that long-term HFD-induced disruption of bone microarchitecture in mice is evident, and can be effectively ameliorated by dietary intervention (Figures 2C–H). In the analysis of cortical bone, no significant difference was found between the HFDt group and CD group ($P>0.05$) (Figures 2J–M). The results of cancellous and cortical bone analysis further demonstrate that most parameters of the HFDt mice tibia were fully restored following dietary intervention.

3.3 Dietary intervention promotes bone formation by enhancing osteoblast activity

In the bone marrow microenvironment, the dynamic balance of adipogenesis and osteogenesis, and the number of osteoblasts and osteoclasts all have a major impact on bone microarchitecture. The femurs of three groups of mice were stained to identify adipose, OCN and TRAP in order to evaluate the effect of the dynamic balance of adipogenesis and osteogenesis on bone microarchitecture. Results indicated that long-term HFD had significantly increased number of adipocytes per field in the distal femur, compared to CD ($P<0.05$) and HFDt ($P<0.05$) mice, suggesting that dietary intervention ameliorated the accumulation of adipocytes caused by long-term HFD (Figures 3A, D). We further examined the osteoblast and osteoclast biomarkers OCN and TRAP (Figures 3B, C). TRAP staining of femur revealed no difference ($P>0.05$) in the number of osteoclasts between the three groups of mice (Figure 3E). However, OCN exhibited significant variances between the three groups. OCN content was significantly lower in HFD mice than in CD ($P<0.05$) and HFDt ($P<0.05$) mice (Figure 3F). Serum PINP levels in HFD mice were significantly lower than in CD mice, whereas those in the HFDt group returned to normal (Figure 3G). There was no significant difference in serum CTX-1 levels between the three groups ($P>0.05$) (Figure 3H). These results suggest that HFD feeding perturbs the equilibrium between osteogenic and adipogenic differentiation in mouse bone marrow, which leads to reduced bone formation. Dietary intervention, however, appears to restore the balance of osteogenic and adipogenic processes in mouse bone marrow, thus restoring its osteogenic potential.

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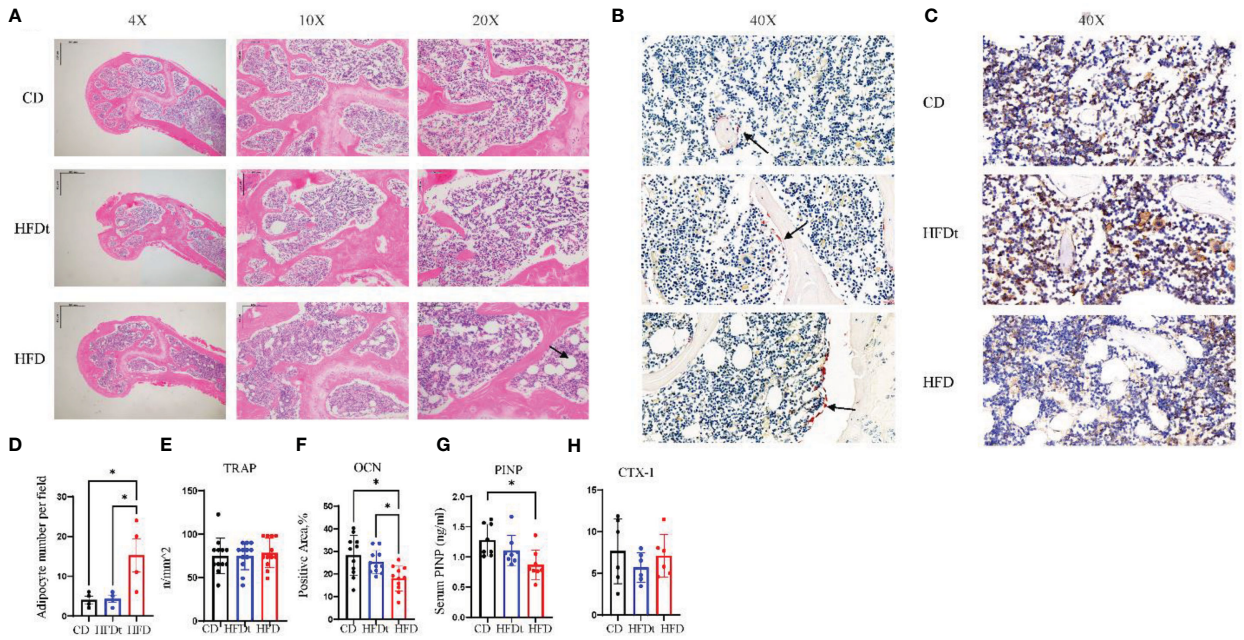

FIGURE 3
Dietary intervention promotes bone formation by enhancing osteoblast activity. **(A)** Representative images of femoral bone sections stained with H & E after CD, HFDt, or HFD treatment (Arrow indicates adipocytes). **(B)** Representative images of IHC staining for detecting the tartrate-resistant acid phosphatase (TRAP) expression, which reflects the osteoclast activity (Arrow indicates osteoclast). **(C)** Representative images of IHC staining for detecting the osteocalcin (OCN) expression, which reflects the osteoblast activity. **(D)** Analysis of adipocyte per field. **(E)** Analysis of trap-positive cell number per bone surface. **(F)** Analysis of Ocn-positive cell number per bone surface; **(G)** Levels of serum marker of bone formation. **(H)** Levels of serum marker of bone resorption. Significance was determined using one-way ANOVA or Welch's ANOVA. *$P < 0.05$.

3.4 Dietary intervention restores adipogenic and osteogenic balance in tibia and BMSCs

Analysis of mRNA expression in the tibia of mice was conducted to explore the metabolic mechanisms of dietary intervention on bone homeostasis. Relative expression of osteoclastic genes, *TRAP* and Cathepsin K (*Ctsk*), did not differ between HFD and HFDt mice ($P>0.05$) (Figure 4A). Conversely, relative expression of osteogenic genes, Collagen type I A I (*Col1a1*), alkaline phosphatase (*ALP*), and Runt-related transcription factor 2 (*Runx2*), were decreased in BMCs of HFD mice (Figure 4B). Moreover, relative expression of adipogenic genes, *PPARy*, adiponectin (*Adipoq*), and cluster of differentiation 36 (*CD36*), were increased in BMSCs of HFD mice (Figure 4C). Notably, relative expression of the inflammatory gene transforming growth factor beta-1 (*TGFβ*) was significantly increased in BMCs of HFDt mice compared to CD ($P<0.0001$) and HFD ($P<0.0001$) mice (Figure 4D). Further, the protein levels of *Runx2* and *PPARγ* in the tibia of mice were examined. *Runx2* protein expression was significantly higher ($P<0.05$) in HFDt mice than in HFD mice, while *PPARγ* protein expression was higher in HFD mice than in the other two groups (Figures 4H–J). The osteogenic and adipogenic balance of tibia is regulated by BMSCs, we further analyzed the mRNA expression in BMSCs. Results in mRNA expression in BMSCs revealed that, following HFD exposure, the relative expression of osteogenic genes (*Col1a1* and *Runx2*) was decreased (Figure 4E), while the relative expression of adipogenic genes (*CD36*, *PPARγ*) was increased (Figure 4F). Notably, the relative expression of the inflammatory gene (*TGFβ*) was significantly increased in BMSCs of HFD mice compared to CD ($P<0.01$) and HFD ($P<0.05$) mice (Figure 4G). These results indicate that HFD disrupts the balance of osteogenic and adipogenic differentiation in tibia and blunts bone formation, leading to bone loss. However, following dietary intervention, the balance of osteogenic and adipogenic differentiation in tibia was restored, which in turn restored the osteogenic potential. Furthermore, the results of BMSCs demonstrate the underlying mechanism by which dietary intervention ameliorates HFD-induced bone loss: dietary intervention could promote the differentiation of BMSCs toward the osteogenic direction, and inhibit the adipogenic capacity of BMSCs.

3.5 Dietary intervention improves local Wnt signaling pathway

Previous studies have indicated that HFD leads to bone loss and consumption may be associated with reduced activity of Wnt signaling pathways (22). Subsequent to these findings, our research evaluated the skeletal expression of both canonical and noncanonical Wnt signaling pathways in different groups of mice. mRNA and protein expression levels of *Wnt5a*, *Wnt3a*, β-catenin, and nuclear effectors *Tcf7l2* and *Tcf7* of the Wnt signaling pathway, as well as *LRP6*, were measured. Results showed that HFD feeding decreased skeletal *Wnt5a*, *Wnt3a*, and β-catenin mRNA expression

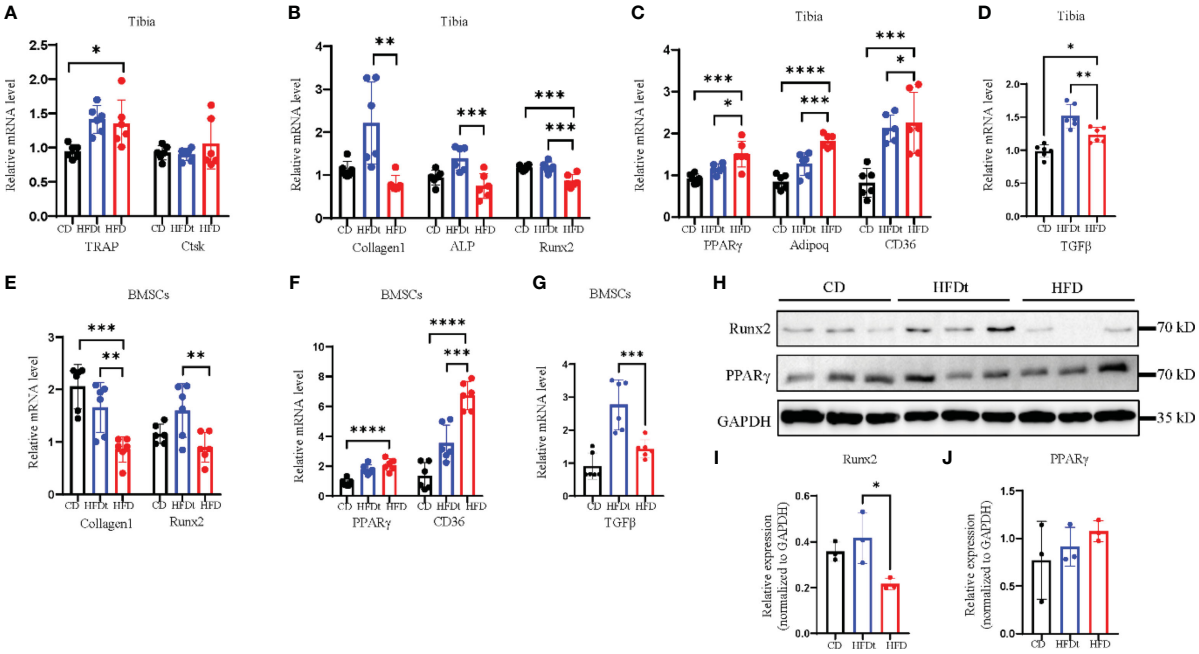

FIGURE 4
Dietary intervention restores adipogenic and osteogenic balance in tibia and BMSCs. **(A)** mRNA levels of osteoclastic genes in BMCs of CD, HFDt, or HFD mice (n = 6). **(B)** mRNA levels of osteoblastic genes in BMCs of CD, HFDt, or HFD mice (n = 6). **(C)** mRNA levels of adipogenic genes in BMCs of CD, HFDt, or HFD mice (n = 6). **(D)** mRNA levels of inflammatory genes in BMCs of CD, HFDt, or HFD mice (n = 6). **(E)** Immunoblot of Runx2, PPARγ protein expression in the bone of CD, HFDt, and HFD mice: each lane contains samples pooled from three mice, GAPDH is shown as a loading control. **(F, G)** The relative protein expression levels of Runx2, PPARγ. **(H)** mRNA levels of osteoblastic genes in BMSCs of CD, HFDt, or HFD mice (n = 6). **(I)** mRNA levels of adipogenic genes in BMSCs of CD, HFDt, or HFD mice (n = 6). **(J)** mRNA levels of inflammatory genes in BMSCs of CD, HFDt, or HFD mice (n = 6). Significance was determined using one-way ANOVA or Welch's ANOVA. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$; $^{****}P < 0.0001$.

and protein expression, compared to the control group (Figures 5A–J). However, in HFDt mice, Wnt signaling was augmented by dietary intervention, as indicated by increased expression of downstream signaling molecules.

4 Discussion

The global prevalence of obesity is on the rise, associated with a variety of metabolic disorders and complications, among which OP is particularly salient (23). Non-invasive dietary interventions may offer potential for ameliorating the effects of obesity on health. Despite the number of evidence demonstrating the deleterious effects of obesity on bone health, very few studies have comprehensively explored the implications of dietary interventions for obesity-induced OP (24, 25). The present study revealed that 10 months of HFD consumption led to increased body weight, liver weight, femoral trabecular bone loss, and dyslipidemia in mice. Subsequent 4 months of switching from HFD to a regular diet caused a notable improvement in bone microarchitecture and bone formation. The molecular mechanisms of HFD-induced impaired bone mass accrual and its reversal by diet intervention were further explored. It was observed that HFD caused downregulation of osteogenic genes (*Col1a1* and *Runx2*) and upregulation of adipogenesis genes (*CD36* and *PPARγ*) as well as downregulation of genes and proteins in the Wnt/β-catenin signaling pathway. Remarkably, diet intervention reversed these implications of HFD on bone, and restoration of local Wnt/β-catenin signaling may explain the potential mechanism. The findings of this study demonstrate the efficacy of diet intervention in regulating obesity-induced osteoporosis and suggest that it may serve as a promising non-invasive approach to ameliorating metabolic disorders associated with obesity.

Evidence has suggested a link between obesity and OP (26), with aged populations being particularly susceptible to the simultaneous presence of both syndromes (27). Despite numerous attempts to control obesity through pharmacological treatments, several initially approved anti-obesity drugs have been withdrawn due to serious adverse effects, including bone loss (28, 29). In light of this, it is essential to determine the relationship and underlying mechanisms between obesity and OP. This study assesses the impact of HFD on bone formation in obesity mice, demonstrating a decrease in the transcription factor Runx2, which plays a fundamental role in osteogenesis. Moreover, HFD was found to have a suppressive effect on *Runx2* at the transcriptional level in tibia and BMSCs, as well as at the protein level in mice tibia. Furthermore, HFD increased the expression of *PPARγ*, a marker of adipogenesis, suggesting a role of HFD in impairing bone formation and enhancing bone marrow adipogenesis. These findings provide insight into the potential effects of obesity on bone health, and the involvement of the Wnt signaling pathway (30). It is worth noting that some indexes of the HFDt group were higher than those of the CD group. The reason for this phenotype is unknown, but we analyzed that HFDt may activate

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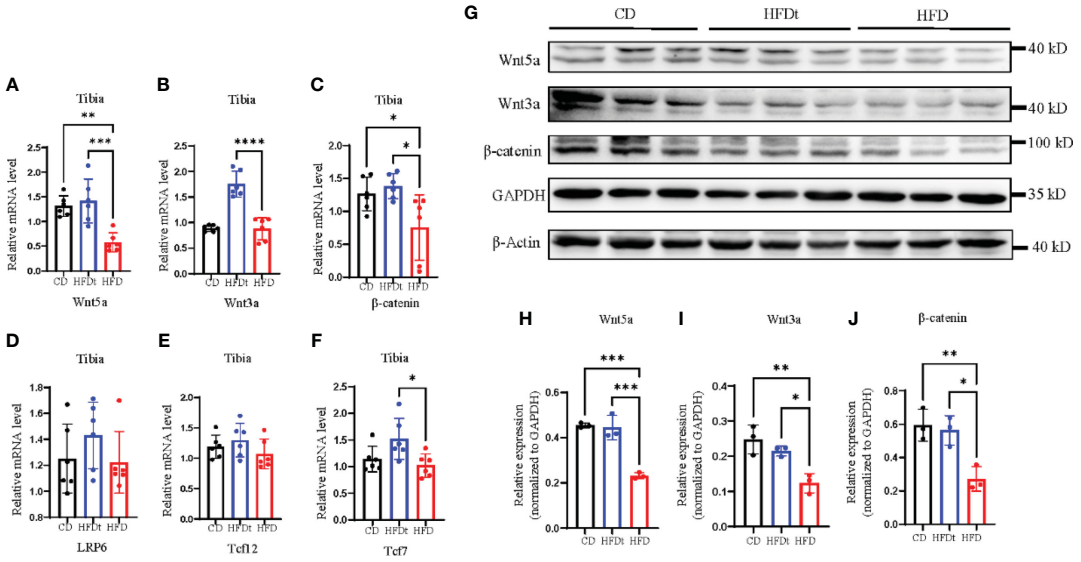

FIGURE 5
Dietary intervention improves local Wnt signaling pathway. **(A-F)** Tibia mRNA expression of the Wnt signaling molecules. **(G-J)** Immunoblot of Wnt signaling pathway protein expression in the bone of CD, HFDt, and HFD mice: each lane contains samples pooled from three mice, GAPDH is shown as a loading control. Significance was determined using one-way ANOVA or Welch's ANOVA. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$; ****$P < 0.0001$.

inflammatory path-related genes such as TGF-β, which can play a synergistic role with Runx2 to promote bone mass increase (31).

Previous research has demonstrated that obesity can lead to increased differentiation of BMSCs into adipocytes and reduced differentiation of BMSCs into osteoblasts (32). This observation has been attributed to the attenuation of the Wnt signaling pathway, which is a crucial regulator of bone formation, affecting the formation and function of osteoblasts, adipocytes, and osteoclasts (16, 33, 34). The canonical Wnt pathway is activated by ligands such as Wnt1 and Wnt3a and is mediated by β-catenin, which, upon activation, can translocate to the nucleus to induce the expression of target genes (16, 33, 34). Wnt5a, a noncanonical Wnt ligand, has been observed to activate both the canonical and noncanonical pathways. It does this by binding to canonical Wnt and modulating Wnt–β-catenin signaling in osteoblasts and certain stromal cell lines, as well as binding to receptor tyrosine kinase-like orphan receptor 1/2 (Ror1/2) to stimulate cell migration and polarization (35). In the Wnt signaling cascade, activated Wnt blocks glycogen synthase kinase 3 (GSK3)-catalyzed phosphorylation of β-catenin (36), leading to the subsequent translocation of unphosphorylated β-catenin into the nucleus and up-regulation of Runx2 expression (37). In the current study, we observed a downregulation of the canonical Wnt pathway components *Wnt3a*, *p-GSK*, and β-catenin in the skeleton of mice on a HFD, with a subsequent restoration of the canonical Wnt/β-catenin pathway following diet intervention (38). Wnt5a activates both the canonical and non-canonical Wnt pathways and has been observed to suppress adipogenesis, thereby promoting the differentiation of BMSCs into osteoblast lineage cells (39, 40). Further, we observed that Wnt5a suppressed transactivation of *PPARγ* and induced the expression of *Runx2*, leading to the promotion of osteogenesis (41). Interestingly, we also observed decreased Wnt5a signaling in the HFD group and a regained activity in the HFDt group. Our findings are consistent with previous work that revealed an increase in bone marrow adiposity and adipogenesis genes, as well as decreased bone density and osteogenesis genes, in HFD mice compared to HFDt and CD mice.

So far, lifestyle changes are considered the mainstay of the management of obesity. Dietary restriction has been shown to reduce diet-induced obesity and diabetes (42), reduced adipose tissue inflammation (43), intrahepatic lipid accumulation (44), and improved behavioral impairments (20). To our knowledge, little is known whether diet-induced weight loss may improve obesity-induced impaired bone mass accrual. As expected, weight loss reduced bone weight gain, liver weight, intrahepatic lipid accumulation, hyperlipidemia, glucose tolerance, which corresponds with previous studies. In support of this, our study showed that a 4-month dietary intervention reversed the impairments of HFD-induced bone density. These results provide further understanding of the relationship between diet-induced weight loss and bone health. Scheller et al. previously studied the effects of HFD and weight loss on male C57BJ/L mice, with duration of 12, 16, or 20 weeks and a group of mice fed HFD for 12 weeks and then on CD for 8 weeks to mimic weight loss. Contrary to results in our study, no statistically significant differences in femur trabecular morphology were found between the weight loss group and HFD group. We designed a longer duration of diet intervention, 16 weeks, and observed improved femur bone microarchitecture in the HFDt group. This difference may be attributed to the duration of weight loss, since Scheller's study was limited to 8 weeks of diet intervention. Although no statistically significant changes were observed, a tendency of recovery was still present in Scheller's work.

In this study, we demonstrate a link between obesity and bone formation, characterized by an increase in adipogenesis and a decrease in osteogenesis. Furthermore, these changes in bone architecture were reversed by dietary intervention, highlighting the potential of weight loss as a therapeutic strategy. Additionally, our

findings suggest that obesity-induced impaired bone mass accrual may be due to the suppression of the Wnt signaling pathway, and that dietary intervention may restore its activity. The results of this study provide a basis for further exploration of the mechanisms that underlie obesity-induced impared bone mass accrual, and future research should focus on interventions that augment the Wnt signaling pathway to prevent or ameliorate this condition.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found within the article/Supplementary Material.

Ethics statement

The animal study was reviewed and approved by Animal Care Committee of Zhongshan Hospital, Fudan University.

Author contributions

YZ, JY, XZ: Methodology, investigation, data analysis, writing—original draft. HC, ZW: Data analysis, conceptualization. XL: Funding acquisition. QW: Conceptualization, supervision. BZ: Methodology, resources, supervision. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2023.1171781/full#supplementary-material

References

1. Di Cesare M, Bentham J, Stevens GA, Zhou B, Danaei G, Lu Y, et al. Trends in adult body-mass index in 200 countries from 1975 to 2014: a pooled analysis of 1698 population-based measurement studies with 19·2 million participants. *Lancet* (2016) 387(10026):1377–96. doi: 10.1016/S0140-6736(16)30054-X

2. Singla P, Bardoloi A, Parkash AA. Metabolic effects of obesity: a review. *World J Diabetes* (2010) 1(3):76–88. doi: 10.4239/wjd.v1.i3.76

3. Longo M, Zatterale F, Naderi J, Parrillo L, Formisano P, Raciti GA, et al. Adipose tissue dysfunction as determinant of obesity-associated metabolic complications. *Int J Mol Sci* (2019) 20(9):2358. doi: 10.3390/ijms20092358

4. Klibanski A, Adams-Campbell L, Bassford T, Blair SN, Boden SD, Dickersin K, et al. Osteoporosis prevention, diagnosis, and therapy. *JAMA* (2001) 285(6):785–95. doi: 10.1001/jama.285.6.785

5. Qaseem A, Forciea MA, McLean RM, Denberg TD, Barry MJ, Cooke M, et al. Treatment of low bone density or osteoporosis to prevent fractures in men and women: a clinical practice guideline update from the American college of physicians. *Ann Intern Med* (2017) 166(11):818–39. doi: 10.7326/M15-1361

6. Altkorn D, Cifu AS. Screening for osteoporosis. *JAMA* (2015) 313(14):1467–8. doi: 10.1001/jama.2015.1064

7. Ilich JZ, Kelly OJ, Inglis JE, Panton LB, Duque G, Ormsbee MJ. Interrelationship among muscle, fat, and bone: connecting the dots on cellular, hormonal, and whole body levels. *Ageing Res Rev* (2014) 15:51–60. doi: 10.1016/j.arr.2014.02.007

8. Núñez NP, Carpenter CL, Perkins SN, Berrigan D, Jaque SV, Ingles SA, et al. Extreme obesity reduces bone mineral density: complementary evidence from mice and women. *Obes (Silver Spring)* (2007) 15(8):1980–7. doi: 10.1038/oby.2007.236

9. Bredella MA, Torriani M, Ghomi RH, Thomas BJ, Brick DJ, Gerweck AV, et al. Determinants of bone mineral density in obese premenopausal women. *Bone* (2011) 48 (4):748–54. doi: 10.1016/j.bone.2010.12.011

10. Tu KN, Lie JD, Wan CKV, Cameron M, Austel AG, Nguyen JK, et al. Osteoporosis: a review of treatment options. *P T* (2018) 43(2):92–104.

11. Rasouli N, Kern PA. Adipocytokines and the metabolic complications of obesity. *J Clin Endocrinol Metab* (2008) 93(11 Suppl 1):S64–73. doi: 10.1210/jc.2008-1613

12. Zhao L-J, Jiang H, Papasian CJ, Maulik D, Drees B, Hamilton J, et al. Correlation of obesity and osteoporosis: effect of fat mass on the determination of osteoporosis. *J Bone Miner Res* (2008) 23(1):17–29. doi: 10.1359/jbmr.070813

13. Ambrogini E, Que X, Wang S, Yamaguchi F, Weinstein RS, Tsimikas S, et al. Oxidation-specific epitopes restrain bone formation. *Nat Commun* (2018) 9(1):2193. doi: 10.1038/s41467-018-04047-5

14. Li Y, Lu L, Xie Y, Chen X, Tian L, Liang Y, et al. Interleukin-6 knockout inhibits senescence of bone mesenchymal stem cells in high-fat diet-induced bone loss. *Front Endocrinol (Lausanne)* (2020) 11:622950. doi: 10.3389/fendo.2020.622950

15. Walczak R, Tontonoz P. PPARadigms and PPARadoxes: expanding roles for PPARγ in the control of lipid metabolism. *J Lipid Res* (2002) 43(2):177–86. doi: 10.1016/S0022-2275(20)30159-0

16. Yao Q, Yu C, Zhang X, Zhang K, Guo J, Song L. Wnt/β-catenin signaling in osteoblasts regulates global energy metabolism. *Bone* (2017) 97:175–83. doi: 10.1016/j.bone.2017.01.028

17. Marini F, Giusti F, Palmini G, Brandi ML. Role of wnt signaling and sclerostin in bone and as therapeutic targets in skeletal disorders. *Osteoporosis Int* (2022) 34(2):213–238. doi: 10.1007/s00198-022-06523-7

18. Zhu M, Fan Z. The role of the wnt signalling pathway in the energy metabolism of bone remodelling. *Cell Prolif* (2022) 55(11):e13309. doi: 10.1111/cpr.13309

19. Knowler WC, Barrett-Connor E, Fowler SE, Hamman RF, Lachin JM, Walker EA, et al. Reduction in the incidence of type 2 diabetes with lifestyle intervention or metformin. *N Engl J Med* (2002) 346(6):393–403. doi: 10.1056/NEJMoa012512

20. Braga SP, Delanogare E, Machado AE, Prediger RD, Moreira ELG. Switching from high-fat feeding (HFD) to regular diet improves metabolic and behavioral impairments in middle-aged female mice. *Behav Brain Res* (2021) 398:112969. doi: 10.1016/j.bbr.2020.112969

21. Hatzidis A, Hicks JA, Gelineau RR, Arruda NL, De Pina IM, O'Connell KE, et al. Removal of a high-fat diet, but not voluntary exercise, reverses obesity and diabetic-like symptoms in male C57BL/6J mice. *Hormones (Athens Greece)* (2017) 16(1):62–74. doi: 10.14310/horm.2002.1720

22. Beier EE, Inzana JA, Sheu T-J, Shu L, Puzas JE, Mooney RA. Effects of combined exposure to lead and high-fat diet on bone quality in juvenile Male mice. *Environ Health Perspect* (2015) 123(10):935–43. doi: 10.1289/ehp.1408581

23. Pagnotti GM, Styner M, Uzer G, Patel VS, Wright LE, Ness KK, et al. Combating osteoporosis and obesity with exercise: leveraging cell mechanosensitivity. *Nat Rev Endocrinol* (2019) 15(6):339–55. doi: 10.1038/s41574-019-0170-1

24. Scheller EL, Khoury B, Moller KL, Wee NKY, Khandaker S, Kozloff KM, et al. Changes in skeletal integrity and marrow adiposity during high-fat diet and after weight loss. *Front Endocrinol (Lausanne)* (2016) 7:102. doi: 10.3389/fendo.2016.00102

25. Lu L, Tang M, Li J, Xie Y, Li Y, Xie J, et al. Gut microbiota and serum metabolic signatures of high-Fat-Induced bone loss in mice. *Front Cell Infect Microbiol* (2021) 11:788576. doi: 10.3389/fcimb.2021.788576

26. Leslie WD, Morin SN, Majumdar SR, Lix LM. Effects of obesity and diabetes on rate of bone density loss. *Osteoporosis Int* (2018) 29(1):61–7. doi: 10.1007/s00198-017-4223-9

27. Ormsbee MJ, Prado CM, Ilich JZ, Purcell S, Siervo M, Folsom A, et al. Osteosarcopenic obesity: the role of bone, muscle, and fat on health. *J cachexia sarcopenia Muscle* (2014) 5(3):183–92. doi: 10.1007/s13539-014-0146-x

28. Narayanaswami V, Dwoskin LP. Obesity: current and potential pharmacotherapeutics and targets. *Pharmacol Ther* (2017) 170:116–47. doi: 10.1016/j.pharmthera.2016.10.015

29. Pilitsi E, Farr OM, Polyzos SA, Perakakis N, Nolen-Doerr E, Papathanasiou AE, et al. Pharmacotherapy of obesity: available medications and drugs under investigation. *Metabol: Clin Exp* (2019) 92:170–92. doi: 10.1016/j.metabol.2018.10.010

30. Takada I, Kouzmenko AP, Kato S. Wnt and PPARgamma signaling in osteoblastogenesis and adipogenesis. *Nat Rev Rheumatol* (2009) 5(8):442–7. doi: 10.1038/nrrheum.2009.137

31. Tian Y, Mu H, Dong Z, Wang Y, Gao Y, Gao Y, et al. The synergistic effects of TGF-β1 and RUNX2 on enamel mineralization through regulating ODAPH expression during the maturation stage. *J Mol Histol* (2022) 53(2):483–92. doi: 10.1007/s10735-022-10060-2

32. Pierce JL, Begun DL, Westendorf JJ, McGee-Lawrence ME. Defining osteoblast and adipocyte lineages in the bone marrow. *Bone* (2019) 118:2–7. doi: 10.1016/j.bone.2018.05.019

33. Chen JR, Lazarenko OP, Wu X, Tong Y, Blackburn ML, Shankar K, et al. Obesity reduces bone density associated with activation of PPARγ and suppression of wnt/β-catenin in rapidly growing male rats. *PloS One* (2010) 5(10):e13704. doi: 10.1371/journal.pone.0013704

34. Li Y, Jin D, Xie W, Wen L, Chen W, Xu J, et al. PPAR-γ and wnt regulate the differentiation of MSCs into adipocytes and osteoblasts respectively. *Curr Stem Cell Res Ther* (2018) 13(3):185–92. doi: 10.2174/1574888x12666171012141908

35. Baschant U, Rauner M, Balaian E, Weidner H, Roetto A, Platzbecker U, et al. Wnt5a is a key target for the pro-osteogenic effects of iron chelation on osteoblast progenitors. *Haematologica* (2016) 101(12):1499–507. doi: 10.3324/haematol.2016.144808

36. MacDonald BT, Tamai K, He X. Wnt/beta-catenin signaling: components, mechanisms, and diseases. *Dev Cell* (2009) 17(1):9–26. doi: 10.1016/j.devcel.2009.06.016

37. Cselenyi CS, Jernigan KK, Tahinci E, Thorne CA, Lee LA, Lee E. LRP6 transduces a canonical wnt signal independently of axin degradation by inhibiting GSK3's phosphorylation of beta-catenin. *Proc Natl Acad Sci United States America* (2008) 105(23):8032–7. doi: 10.1073/pnas.0803025105

38. Okamoto M, Udagawa N, Uehara S, Maeda K, Yamashita T, Nakamichi Y, et al. Noncanonical Wnt5a enhances wnt/β-catenin signaling during osteoblastogenesis. *Sci Rep* (2014) 4:4493. doi: 10.1038/srep04493

39. Xu C, Wang J, Zhu T, Shen Y, Tang X, Fang L, et al. Cross-talking between PPAR and WNT signaling and its regulation in mesenchymal stem cell differentiation. *Curr Stem Cell Res Ther* (2016) 11(3):247–54. doi: 10.2174/1574888x10666150723145707

40. Bilkovski R, Schulte DM, Oberhauser F, Gomolka M, Udelhoven M, Hettich MM, et al. Role of WNT-5a in the determination of human mesenchymal stem cells into preadipocytes. *J Biol Chem* (2010) 285(9):6170–8. doi: 10.1074/jbc.M109.054338

41. Maeda K, Kobayashi Y, Udagawa N, Uehara S, Ishihara A, Mizoguchi T, et al. Wnt5a-Ror2 signaling between osteoblast-lineage cells and osteoclast precursors enhances osteoclastogenesis. *Nat Med* (2012) 18(3):405–12. doi: 10.1038/nm.2653

42. Parekh PI, Petro AE, Tiller JM, Feinglos MN, Surwit RS. Reversal of diet-induced obesity and diabetes in C57BL/6J mice. *Metabol: Clin Exp* (1998) 47(9):1089–96. doi: 10.1016/s0026-0495(98)90283-9

43. Olefsky J, Reaven GM, Farquhar JW. Effects of weight reduction on obesity. studies of lipid and carbohydrate metabolism in normal and hyperlipoproteinemic subjects. *J Clin Invest* (1974) 53(1):64–76. doi: 10.1172/jci107560

44. Mendes IKS, Matsuura C, Aguila MB, Daleprane JB, Martins MA, Mury WV, et al. Weight loss enhances hepatic antioxidant status in a NAFLD model induced by high-fat diet. *Appl physiol nutrition Metab = Physiologie appliquee Nutr metabolisme* (2018) 43(1):23–9. doi: 10.1139/apnm-2017-0317

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Association of selenium intake with bone mineral density and osteoporosis: the national health and nutrition examination survey

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Background: Osteoporosis (OP) is a systemic metabolic skeletal disorder characterized by a decrease in bone mineral density (BMD) and an increase in the risk of fracture. The level of selenium (Se) in serum is associated with BMD. However, the relationship between dietary and total selenium intake and parameters such as osteoporosis and BMD is unclear. By conducting National Health and Nutritional Examination Surveys (NHANES), in this study, we assessed the association of Se intake with BMD and the risk of OP among general middle-aged and elderly people.

Methods: The data were collected from three cycles of NHANES [2009–2010, 2013–2014, and 2017–2020]. Information on the dietary and supplementary Se intake was obtained from 24-h dietary recall interviews. Additionally, dual-energy X-ray absorptiometry (DXA) was performed to measure BMD, which was later transformed into T-scores; OP was diagnosed when the T-score was ≤ -2.5. We constructed a logistic regression model for the association between selenium intake and the risk of OP based on the estimated odds ratios (ORs) and the 95% confidence intervals (CIs). We also constructed a multivariable linear regression model to analyze the relationship between selenium intake and BMD.

Results: In this study, 3,250 individuals (average age: 60.01 ± 10.09 years; 51.88% females) participated. The incidence of OP was 9.35% (3.30% for males and 17.75% for females). In the logistic regression model adjusted for every interested covariate, a higher quartile of dietary Se intake (OR for quartile 4 *vs*. quartile 1: 0.63; 95% CI: 0.41–0.96; P for trend = 0.027) was related to a lower risk of OP relative to the lowest quartile. The total selenium intake also exhibited a consistent trend (OR for quartile 4 *vs*. quartile 1: 0.67; 95% CI: 0.44–1.01; P for trend = 0.049). The results of the adjusted multivariate linear regression model showed that the participants with the highest quartile of dietary Se intake (Q4) had higher BMD in the total femur (β = 0.069, P = 0.001; P for trend = 0.001), femoral neck (β = 0.064, P = 0.001; P for trend = 0.001), and total spine (β = 0.030, P = 0.136; P for trend = 0.064) compared to those in quintile 1 (Q1). A similar trend of associations was observed for the total selenium intake with BMD, which was more prominent among females, as determined by the subgroup analysis.

Conclusion: In this study, the dietary intake and total intake of selenium were positively associated with BMD, whereas they were negatively associated with the risk of OP among adults in the US. Further studies are required to verify our results and elucidate the associated biological mechanism.

KEYWORDS

selenium intake, bone mineral density (BMD), osteoporosis, NHANES, cross-sectional survey

Introduction

Osteoporosis (OP) is a commonly occurring skeletal disorder and is characterized by a decrease in bone mass, low bone mineral density (BMD), and the deterioration of bone microstructure (1, 2). A study found that in 2010, OP affected 10.2 million adults in the US who were ≥ 50 years old. The number of affected individuals might reach 13.5 million in 2030 (3). OP significantly increases the burden on the social healthcare system because of the high morbidity, mortality, and therapeutic expenses associated with it (4, 5). Various risk factors are associated with the occurrence of OP and a decrease in BMD; these factors include genetic, environmental, and dietary factors (6, 7). Dietary factors are considered to be closely associated with musculoskeletal diseases (8). Some studies have shown that macronutrients (carbohydrates, proteins, and lipids), flavonoid polyphenols, and micronutrients (phosphorus, calcium, magnesium, and vitamins D, C, and K) greatly facilitate the inhibition of osteoporosis (9). Deficiency or excess of zinc, copper, selenium, iron, cadmium, silicon, and fluorine might affect bone mineralization and lead to osteoporosis (10). Some studies have shown that trace elements can help in preventing OP (11, 12).

Selenium (Se) is an essential trace mineral element in the human body. It forms "selenoproteins" after it is incorporated into the protein polypeptide chain. Additionally, Se modulates cell processes by influencing Se-mediated antioxidant enzyme components, which scavenge reactive oxygen species (ROS) in cells. Selenium is closely associated with Keshan disease, thyroid-related diseases, and other functions and diseases (13, 14). Selenium protein P is a critical Se transporter in the bone and is very important for maintaining bone health (15). A deficiency of Se may increase the levels of ROS, and it is the most important cause of OP (16). Some studies have suggested that serum Se level is positively associated with bone outcomes, like BMD and the risk of fracture (17, 18). Only a few studies have shown a non-linear relationship (19) and an inverted U-shaped trend (20) between the dietary intake of Se and osteoporosis. Although recent studies on humans have analyzed the relationship between the dietary Se level and BMD (20), few studies have investigated the relationship of dietary and total Se with BMD and osteoporosis. Hence, the effects of selenium intake on an increase in BMD and a decrease in the risk of osteoporosis need to be investigated to develop more effective preventive measures for reducing the morbidity and mortality of OP globally.

In this study, we investigated the relationship between Se intake and changes in BMD and the risk of OP based on the cross-sectional data collected by conducting National Health and Nutritional Examination Surveys (NHANES).

Materials and methods

Participants

The NHANES was developed in 1959 as a continuous program for conducting cross-sectional examinations of the nutrition and health of people in the United States (US) annually. In this program, information on dietary, demographic, questionnaire, and laboratory data is released every two years. In this study, we included adults who were ≥50 years old, with sufficient selenium intake, and with BMD data recorded in the NHANES that was conducted in 2009–2010, 2013–2014, and 2017–2020 (Figure 1). All participants provided informed consent for this survey, as it was required by the Institutional Review Board of the National Center for Health Statistics.

Selenium intake

The information on the dietary and supplementary selenium intake was collected from two 24-h dietary recall interviews in three NHANES two-year cycles (2009–2010, 2013–2014, and 2017–2020). It was a respondent-driven approach to collect precise and comprehensive data on the food and beverage types and amounts (every water type was included) taken within 24-h immediately before the interview (midnight to midnight). In this survey, two 24-h dietary recalls were conducted; the first one was conducted in person during the mobile examination, whereas the second one was conducted after 3–10 days through a telephone interview. Information on the dietary selenium intake and supplement intake was obtained in each interview; thus, the total selenium intake for each interview was the sum of dietary selenium and supplements. The average selenium intake recorded in two consecutive interviews was considered to be the selenium intake

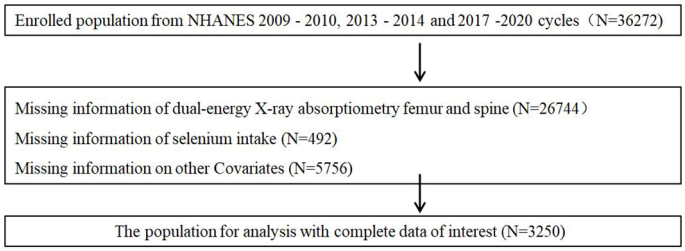

FIGURE 1
Flow diagram of inclusion criteria and exclusion criteria.

of the participants. A nutrient residual model was used to adjust the selenium intake for total energy intake (21). The approach helped adjust the total energy intake-induced confounding when analyzing the relationship between selenium and OP. Dietary Reference Intakes: Basic Guidelines for Nutritional Requirements (2006) recommended dietary reference intakes (DRIs) of total selenium intakes as follows: an estimated average intake (EAR) of 45μg/day, a recommended dietary allowance (RDA) of 55μg/day, and a tolerable maximum intake (UL) of 400μg/day, without gender differences.

Determination of BMD and the diagnosis of OP

The BMD levels were examined at different regions (total femur, femur neck, and lumbar spine) in the NHANES. Additionally, through dual-energy X-ray absorptiometry (DXA), Hologic QDR-4500A fan-beam densitometers (Hologic, Inc., Bedford, Massachusetts) were used for scanning. Generally, the left hip was examined, only if the subject reported a fracture, a pin, or a replacement of the left hip, the right hip was examined. The BMD of the lumbar spine was determined by the average level of the first to fourth lumbar vertebrae. Pregnant patients, those with a radiographic contrast material history, those with bilateral hip fractures/replacement/pins, and those who were >450 lbs were eliminated from the DXA examination. The diagnostic criteria for osteoporosis were defined using the mean of the peak BMD of the femoral neck, total hip, and lumbar spine from the NHANES III database in the United States and its corresponding standard deviation (SD) in white women aged 20 to 29 years. Then, an established method was used to transform the BMD levels in the femoral neck, total hip, and lumbar spine to T-scores (22, 23), with a T-score≤ -2.5, -2.5 < T-score ≤ -1, and > −1 indicating OP, osteopenia, and normal, respectively. Patients with osteopenia and normal individuals were considered to be non-OP.

Covariate assessment

Based on the statements on the NHANES website, trained personnel collected the data at every study site following standard procedures. In this study, the covariates were age, gender, ethnicity, education level, marital status, history of smoking, hypertension, diabetes, osteoporosis in parents, previous fractures, and body mass index (BMI, which was determined by dividing the body weight (kg) by the body height squared (m^2). Participants were weighed in kilograms using a digital weight scale. Standing height was measured using a stadiometer with a fixed vertical backboard and an adjustable head piece.

Statistical analysis

The estimates of the people in the US were generated by data weighting and analyzed by layering and clustering. For descriptive analysis, normally-distributed continuous data were represented by the mean ± standard deviation (SD), whereas non-normally distributed continuous data were expressed as the median and the quartile range. Additionally, categorical data were represented by the frequency and frequency percentage. We conducted the Student's t-test, non-parametric tests, and the Chi-squared test to analyze the statistical differences between the two groups of continuous variables that followed a normal distribution, continuous variables that did not follow a non-normal distribution, and categorical data, respectively. The dietary and total selenium intake levels were classified according to quartiles (quartiles 1–4 indicated <25^{th}, 25^{th}–50^{th}, 50^{th}–75^{th}, and >75^{th} percentile). Odds ratios (ORs) and 95% confidence intervals (CIs) were determined through logistic regression to analyze the relationship between selenium intake and the risk of OP. We used dietary and total selenium intake for reference. In Model 1, sex, body mass index, marital status, and race were adjusted. In Model 2, a history of hypertension, diabetes, osteoporosis in parents, and previous fractures were also adjusted. In Model 3, every interested covariate was adjusted. Additionally, multivariate linear regression models were constructed to determine the relationship between selenium intake and BMD in the total spine and total femur. The relationships between selenium intake and BMD, as well as the risk of OP were also analyzed through a gender-stratified subgroup analysis. The R (version 3.5.3) and SPSS (version: 24.0; SPSS, Chicago, IL) software programs were used for conducting statistical analyses. All differences among and between groups were considered to be statistically significant at $P < 0.05$ (two-sided).

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Results

Characteristics of the study population

The unqualified patients were excluded (Figure 1), and 3,250 participants were included in this study. The characteristics of the participants with and without OP are presented in Table 1. Compared to the participants without OP, those with OP were thinner and older, mainly women, with lower levels of education, widowed, divorced, or separated, with hypertension, a history of osteoporosis in parents, previous fractures, and less likely to be non-Hispanic Black. In contrast, participants without OP had a greater amount of dietary Se and total Se.

Relationship between selenium intake and osteoporosis

The relationship between selenium intake and the risk of OP was analyzed by conducting logistic regression (Table 2). In Model 1, based on the ORs and 95% CIs for the relationship between the dietary intake of Se with the risk of OP, after being adjusted for sex, body mass index, marital status, and race, a higher quartile of dietary selenium intake (0.69 (95% CI: 0.49, 0.98); 0.49 (95% CI: 0.33, 0.74); P for trend = 0.001) indicated a lower risk of OP. Additionally, dietary selenium intake showed consistent results with marginal significance (0.53 (95% CI: 0.35, 0.81); P for trend = 0.002; 0.63 (95% CI: 0.41, 0.96); P for trend = 0.027) after further

TABLE 1 Basic characteristics of the OP and non-OP population of NHANES.

Variables	No osteoporosis	Osteoporosis	P
Number, n(%)	2946(90.65%)	304(9.35%)	
Age (years)	52.00(59.00,66.00)	66.00(59.00,75.00)	<0.001
<60 ,n(%)	1519(51.6)	78(25.7)	<0.001
≥60 ,n(%)	1427(48.4)	226(74.3)	
BMI (kg/m^2), n(%)			
<25	699(23.7)	165(54.3)	<0.001
25-30	1143(38.8)	93(30.6)	
>30	1104(37.5)	46(15.1)	
Sex, n (%)			
Male	1514(51.4)	50(16.4)	<0.001
Female	1432(48.6)	254(83.6)	
Race, n (%)			
Mexican American	420(14.3)	40(13.2)	<0.001
Other Hispanic	315(10.7)	43(14.1)	
Non-Hispanic White	1243(42.2)	147(48.4)	
Non-Hispanic Black	675(22.9)	28(9.2)	
Other race	293(9.9)	46 (15.1)	
Education, n (%)			
Less than 9th grade	260(8.8)	44(14.5)	0.002
9–11th grade	330(11.2)	40(13.2)	
High school	673(22.8)	79(26.0)	
Some college	910(30.9)	75(24.7)	
College graduate	773(26.2)	66(21.7)	
Marital status, n (%)			
Married or living with partner	1922(65.2)	164(53.9)	0.012
Widowed, divorced or separated	791(26.8)	122(40.1)	
Never married	233(7.9)	18(5.9)	

(Continued)

TABLE 1 Continued

Variables	No osteoporosis	Osteoporosis	P
History of smoking			
No	1627(55.2)	186(61.2)	0.052
Yes	1319(44.8)	118(38.8)	
History of hypertension, n (%)			
No	2232(75.8)	184(60.5)	<0.001
Yes	714(24.2)	120(39.5)	
History of diabetes, n (%)			
No	2461(83.5)	264(86.8)	0.136
Yes	485(16.5)	40(13.2)	
History of osteoporosis in parents, n (%)			
No	2556(86.8)	243(79.9)	0.001
Yes	390(13.2)	61(20.1)	
History of previous fractures, n (%)			
No	2661(90.3)	253(83.2)	<0.001
Yes	285(9.7)	51(16.8)	
Intake of selenium			
Total selenium (μg/day)	111.73(80.34,149. 41)	93.03(70.26,127.00)	<0.001
Dietary selenium (μg/day)	100.53(74.49,132.60)	86.30(62.84,107.18)	<0.001
Bone mineral density			
Total femur (gm/cm^2)	0.96(0.14)	0.70(0.10)	<0.001
Femoral neck (gm/cm^2)	0.80(0.13)	0.57(0.08)	<0.001
Total spine (gm/cm^2)	1.04(0.15)	0.75(0.10)	<0.001

Categorical variables are presented as frequencies (%); continuous variables with normal distribution are shown as means (SDs); continuous variables with a skewed distribution are shown as medians (inter-quartile ranges).

adjustment for history of hypertension, diabetes, OP in parents, previous fractures (Model 2), and every interested covariate (Model 3). For the relationship between the risk of OP and the total selenium intake, consistent findings were obtained from Models 1, 2, and 3, i.e., 0.58 (95% CI: 0.39, 0.86) (P for trend = 0.005) after being adjusted for sex, BMI, marital status and race (Model 1); 0.60 (95% CI: 0.40, 0.90) (P for trend = 0.012) after further adjustment for history of hypertension, diabetes, OP in parents, and previous

TABLE 2 Odds Ratio of osteoporosis across quartiles of selenium intakes.

Intake of selenium (μg/day)	Range of selenium intake (μg/day)	Model 1[a]		Model 2[b]		Model 3[c]	
		OR (95% CI)	P-value	OR (95% CI)	P-value	OR (95% CI)	P-value
Dietary selenium (μg/day)							
Q1	≤73.15	Reference	–	Reference	–	Reference	–
Q2	73.16-98.55	0.83(0.60,1.14)	0.248	0.83(0.60,1.15)	0.269	0.88(0.63,1.22)	0.434
Q3	98.56-130.05	0.69(0.49,0.98)	0.036	0.72(0.51,1.03)	0.069	0.80(0.56,1.15)	0.230
Q4	≥130.06	0.49(0.33,0.74)	0.001	0.53(0.35,0.81)	0.003	0.63(0.41,0.96)	0.031
P for trend		0.001		0.002		0.027	

(Continued)

TABLE 2 Continued

Intake of selenium (μg/day)	Range of selenium intake (μg/day)	Model 1[a]		Model 2[b]		Model 3[c]	
		OR (95% CI)	P-value	OR (95% CI)	P-value	OR (95% CI)	P-value
Total selenium (μg/day)							
Q1	≤79.35	Reference	–	Reference	–	Reference	–
Q2	79.36-110.05	0.91(0.66,1.23)	0.572	0.91(0.66,1.27)	0.582	0.98(0.70,1.37)	0.899
Q3	110.06-147.75	0.79(0.56,1.12)	0.184	0.83(0.58,1.17)	0.285	0.88(0.62,1.23)	0.500
Q4	≥147.76	0.58(0.39,0.86)	0.007	0.60(0.40,0.90)	0.013	0.67(0.44,1.01)	0.057
P for trend		0.005		0.012		0.049	

[a]Model 1 is shown as the odds ratio (95% confidence interval); adjusted for sex, body mass index, marital status and race.
[b]Model 2 is shown as an odds ratio (95% confidence interval); further adjusted for history of hypertension, history of diabetes, history of osteoporosis in parents and history of previous fractures.
[c]Model 3 is shown as odds ratio (95% confidence interval); further adjusted for education ,age and history of smoking .

fractures (Model 2); 0.67 (95% CI: 0.44, 1.01) after being adjusted for every covariate (Model 3). Relative to the lowest quartile, the dietary and total Se intake did not show a significant inverse relationship in quartile 2.

Association of selenium intake with BMD

The results of the multivariate linear regression analysis of selenium intake with BMD are presented in Table 3. The associations between selenium intake and BMD at various bone sites were analyzed (Table 3). In the crude model, relative to the lowest quartile (Q1) of dietary selenium intake, individuals with the highest quartile (Q4) had higher BMD levels in the total femur (β = 0.106, P = 0.001, P for trend = 0.001), femur neck (β = 0.106, P = 0.001, P for trend = 0.001), and total spine (β = 0.053, P = 0.009, P for trend = 0.003). When every interested covariate was adjusted, similar significant positive relationships were also found between the BMD in the total femur and femur neck and selenium intake. A similar association was found between total selenium intake and BMD.

Relation between selenium intake and osteoporosis or BMD in the subgroup analysis by sex

To further demonstrate the relationship between Se intake levels and the risk of OP, we performed a gender-stratified subgroup analysis (Tables 4, 5). The results showed a statistically

TABLE 3 Linear regression coefficients for selenium intakes and BMD.

Intake of selenium (μg/day)	Range of selenium Intake (μg/day)	BMD (gm/cm2)					
		Total femur β (p)		Femoral neck β (p)		Total spine β (p)	
		Model 1[a]	Model 2[b]	Model 1[a]	Model 2[b]	Model 1[a]	Model 2[b]
Dietary selenium (μg/day)							
Q1	≤73.15	Reference	Reference	Reference	Reference	Reference	Reference
Q2	73.16-98.55	0.042(0.020)	0.029(0.101)	0.043(0.024)	0.029(0.114)	0.009(0.640)	0.001(0.991)
Q3	98.56-130.05	0.073(0.001)	0.051(0.004)	0.082(0.001)	0.059(0.002)	0.050(0.010)	0.035(0.072)
Q4	≥130.06	0.106(0.001)	0.069(0.001)	0.106(0.001)	0.064(0.001)	0.053(0.009)	0.030(0.136)
P for trend		0.001	0.001	0.001	0.001	0.003	0.064
Total selenium (μg/day)							
Q1	≤79.35	Reference	Reference	Reference	Reference	Reference	Reference
Q2	79.36-110.05	0.037(0.040)	0.025(0.157)	0.042(0.027)	0.029(0.117)	0.003(0.876)	-0.006(0.739)
Q3	110.06-147.75	0.060(0.001)	0.047(0.008)	0.067(0.001)	0.054(0.004)	0.029(0.142)	0.018(0.353)
Q4	≥147.76	0.087(0.001)	0.060(0.001)	0.086(0.001)	0.058(0.003)	0.047(0.019)	0.030(0.140)
P for trend		0.001	0.001	0.001	0.001	0.008	0.070

[a]Linear regression adjusted for sex, body mass index, marital status and race.
[b]Linear regression further adjusted for history of hypertension, history of diabetes, history of osteoporosis in parents , history of previous fractures, education ,age and history of smoking.

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TABLE 4 Association between selenium intake levels and BMD by sex.

Variable	Range of selenium intake (μg/day)	Male		Female	
		Model1[a]	Model 2[b]	Model1[a]	Model2[b]
		β (p)	β (p)	β (p)	β (p)
Total femur BMD(gm/cm2)					
Dietary selenium (μg/day)					
Continuous (Per 1-SD increase)		0.036(0.132)	0.039(0.101)	0.089(0.001)	0.081(0.001)
Q1	≤73.15	Reference		Reference	
Q2	73.16-98.55	0.017(0.590)	0.018(0.578)	0.036(0.131)	0.033(0.165)
Q3	98.56-130.05	0.018(0.600)	0.019(0.571)	0.074(0.001)	0.066(0.004)
Q4	≥130.06	0.072(0.040)	0.078(0.025)	0.083(0.001)	0.078(0.001)
P for trend		0.021	0.011	0.001	0.001
Total selenium (μg/day)					
Continuous (Per 1-SD increase)		0.037(0.117)	0.039(0.104)	0.074(0.001)	0.063(0.003)
Q1	≤79.35	Reference		Reference	
Q2	79.36-110.05	0.026(0.410)	0.031(0.328)	0.030(0.210)	0.024(0.307)
Q3	110.06-147.75	0.048(0.143)	0.051(0.116)	0.057(0.016)	0.048(0.045)
Q4	≥147.76	0.068(0.047)	0.074(0.032)	0.084(0.001)	0.074(0.001)
P for trend		0.044	0.034	0.001	0.001
Femoral neck BMD(gm/cm2)					
Dietary selenium (μg/day)					
Continuous (Per 1-SD increase)		0.023(0.325)	0.024(0.300)	0.082(0.001)	0.078(0.001)
Q1	≤73.15	Reference		Reference	
Q2	73.16-98.55	0.015(0.647)	0.016(0.610)	0.035(0.142)	0.035(0.140)
Q3	98.56-130.05	0.030(0.364)	0.031(0.343)	0.068(0.003)	0.063(0.001)
Q4	≥130.06	0.056(0.103)	0.061(0.081)	0.082(0.001)	0.080(0.007)
P for trend		0.072	0.055	0.001	0.001
Total selenium (μg/day)					
Continuous (Per 1-SD increase)		0.024(0.311)	0.024(0.312)	0.069(0.001)	0.063(0.003)
Q1	≤79.35	Reference		Reference	
Q2	79.36-110.05	0.018(0.557)	0.021(0.503)	0.037(0.127)	0.035(0.143)
Q3	110.06-147.75	0.051(0.115)	0.053(0.100)	0.058(0.015)	0.052(0.031)
Q4	≥147.76	0.045(0.184)	0.047(0.165)	0.084(0.001)	0.079(0.001)
P for trend		0.192	0.189	0.001	0.001
Total spine BMD(gm/cm2)					
Dietary selenium (μg/day)					
Continuous (Per 1-SD increase)		0.021(0.398)	0.020(0.423)	0.055(0.013)	0.046(0.035)
Q1	≤73.15	Reference		Reference	

(Continued)

TABLE 4 Continued

Variable	Range of selenium intake (μg/day)	Male		Female	
		Model1[a]	Model 2[b]	Model1[a]	Model2[b]
		β (p)	β (p)	β (p)	β (p)
Q2	73.16-98.55	-0.006(0.860)	-0.008(0.814)	0.001(0.982)	-0.006(0.812)
Q3	98.56-130.05	0.002(0.026)	-0.002(0.964)	0.042(0.081)	0.034(0.160)
Q4	≥130.06	0.033(0.360)	0.031(0.389)	0.066(0.008)	0.059(0.017)
P for trend		0.203	0.215	0.014	0.034
Total selenium (μg/day)					
Continuous (Per 1-SD increase)		0.030(0.217)	0.028(0.260)	0.041(0.066)	0.030(0.169)
Q1	≤79.35	Reference		Reference	
Q2	79.36-110.05	-0.004(0.892)	-0.007(0.832)	-0.002(0.937)	-0.008(0.741)
Q3	110.06-147.75	0.015(0.665)	0.011(0.745)	0.030(0.229)	0.022(0.391)
Q4	≥147.76	0.043(0.228)	0.039(0.273)	0.052(0.032)	0.043(0.075)
P for trend		0.108	0.130	0.018	0.051

[a]Model 1 is shown as the odds ratio (95% confidence interval); adjusted for sex, body mass index, marital status and race.
[b]Model 2 is shown as an odds ratio (95% confidence interval); further adjusted for history of hypertension, history of diabetes, history of osteoporosis in parents and history of previous fractures.
[c]Model 3 is shown as odds ratio (95% confidence interval); further adjusted for education ,age and history of smoking.

significant increase in total femur BMD in men and women. However, this relationship was not significantly different between the femoral neck BMD and total spine BMD in men. In women, compared to the lowest quartile (Q1) of Se intake, high Se intake was associated with higher BMD in the femoral neck (all P trend = 0.001). Similar results were obtained when selenium intake was considered to be a continuous variable (per 1-SD increase). Additionally, this positive relationship was significant between the

TABLE 5 Association between selenium intake levels and osteoporosis by sex.

Variable	Range of selenium intake (μg /day)	Male				Female			
		Model 1[a]		Model 2[b]		Model 1[a]		Model 2[b]	
		OR (95% CI)	P-value	OR (95% CI)	P-value	OR (95% CI)	P-value	OR (95% CI)	P-value
Dietary selenium (μg/day)									
Continuous (Per 1-SD increase)		1.000 (0.994,1.006)	0.980	1.002 (0.996,1.007)	0.585	0.995 (0.991,0.999)	0.009	0.996 (0.992,1.000)	0.030
Q1	≤73.15	Reference		Reference		Reference		Reference	
Q2	73.16-98.55	0.899 (0.370,2.185)	0.814	1.035 (0.143,2.591)	0.942	0.849 (0.594,1.212)	0.367	0.867 (0.603,1.247)	0.442
Q3	98.56-130.05	1.033 (0.448,2.381)	0.939	1.185 (0.497,2.827)	0.702	0.708 (0.474.1.058)	0.092	0.729 (0.485,1.097)	0.129
Q4	≥130.06	0.567 (0.224,1.435)	0.231	0.741 (0.281,1.955)	0.544	0.560 (0.344,0.911)	0.020	0.617 (0.376,0.961)	0.045
P for trend		0.229		0.534		0.002		0.031	
Total selenium (μg/day)									
Continuous (Per 1-SD increase)		0.997 (0.992,1.003)	0.330	0.999 (0.994,1.004)	0.697	0.997 (0.994,1.000)	0.036	0.997 (0.994,1.001)	0.110

(Continued)

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TABLE 5 Continued

Variable	Range of selenium intake (μg /day)	Male				Female			
		Model 1[a]		Model 2[b]		Model 1[a]		Model 2[b]	
		OR (95% CI)	P-value	OR (95% CI)	P-value	OR (95% CI)	P-value	OR (95% CI)	P-value
Q1	≤79.35	Reference		Reference		Reference		Reference	
Q2	79.36-110.05	1.238 (0.491,3.122)	0.651	1.435 (0.546,3.771)	0.463	0.971 (0.692,1.362)	0.865	0.962 (0.666,1.390)	0.838
Q3	110.06-147.75	1.608 (0.696,3.715)	0.267	1.976 (0.818,4.770)	0.130	0.676 (0.462,0.988)	0.043	0.716 (0.475,1.080)	0.111
Q4	≥147.76	0.541 (0.203,1.439)	0.218	0.720 (0.256,2.023)	0.533	0.803 (0.521,1.236)	0.318	0.709 (0.442,1.139)	0.155
P for trend		0.139		0.412		0.027		0.074	

[a]Model 1 is shown as the odds ratio (95% confidence interval); adjusted for body mass index, age, race, marital status, history of osteoporosis in parents and history of previous fractures.
[b]Model 2 is shown as an odds ratio (95% confidence interval); further adjusted for history of hypertension, history of diabetes, education and history of smoking.

highest and lowest selenium intake quartiles of total spine BMD. According to the logistic regression, the ORs were 0.56 (0.34, 0.91) and 0.62 (0.38, 0.96) in Model 1 and Model 2 for females regarding dietary selenium with OP across quartile 4 relative to quartile 1; however, this association was not significant relative to quartile 1 for men. The highest quartiles of total selenium intake were not significantly related to the risk of osteoporosis in men.

Discussion

Few studies have investigated whether diet and total selenium intake are associated with bone health. Based on US adult samples from NHANES (2009–2010, 2013–2014, and 2017–2020) in this study, we found that selenium intake was independently related to a lower risk of OP, while a higher selenium intake was related to a higher total femur/femoral neck BMD. Additionally, higher Se intake among females was related to a higher total femur/femoral neck BMD in women, but not in men.

This is the first study to determine the correlation between selenium intake and factors such as BMD and the risk of osteoporosis in adults in the US based on large data. Although some studies have investigated the effects of dietary selenium intake on bone health, and those that have are limited by small sample sizes (19, 20, 24). In our study, we analyzed the relationship between selenium intake and bone health from 3 cycles of NHANES (2009-2010, 2013-2014, and 2017-2020) in middle-aged and older adults aged 50 years or older, overcoming the small amount of data described in previous studies. In addition, studies have investigated the relationship between selenium intake and BMD of adults in the US and other countries (19, 20, 24–26), but the results of their studies were different. For example, Wolf et al. suggested in their cross-sectional study that Se intake from the diet did not have any association with BMD in the US adults (24); Xue et al. showed that an increase in the dietary Se level can predict higher BMD levels in the femur, femur neck, intertrochanter, trochanter, and that dietary selenium intake had an inverted U-shaped relationship with bone mineral density in the US adults (20); Walsh et al. conducted a randomized double-blinded controlled study and found that Se intake at 200 μg/day did not significantly alter the musculoskeletal health of postmenopausal women in the UK (25); A cross-sectional study conducted with 6,267 participants found that a higher dietary intake of Se increased the BMD of individuals in a middle-aged and elderly Chinese population (19); Recently, Xie et al. showed that selenium was positively associated with BMD and inversely associated with OP by a meta-analysis of data from different countries (26); Thus, it is necessary to further clarify the relationship between dietary selenium and bone mineral density in the US adults. Furthermore, previous study (20) have only analyzed the relationship between dietary selenium intake and bone mineral density, and did not consider selenium intake from dietary supplements. In our study, in addition to analyzing the relationship between dietary selenium intake and bone mineral density, we also analyzed the relationship between total selenium intake (dietary selenium intake and supplement intake from dietary supplements) and bone mineral density, so our study is more comprehensive and detailed than previous studies. We results showed that selenium intake was positively associated with total femur/femoral neck BMD, which partially matched the findings of previous study, supporting the finding that selenium intake is positively related to BMD. Meanwhile, very few articles have investigated the relationship between the status of selenium and osteoporosis in the US population. Although Zhang et al. suggested that Se intake was negatively associated with the risk of osteoporotic hip fracture, those researchers focused on smokers (27). Xue et al. showed an inverted U-shaped relationship between dietary selenium intake and bone mineral density, but they did not assess the relationship between dietary selenium intake and osteoporosis risk due to limitations of the original data (26). This is the first analysis between the dietary and total intake of selenium and osteoporosis among US adults. Our results indicate that dietary and total selenium intake was negative with the risk of osteoporosis in US adults.

Selenium is an essential trace mineral element in the human body. It can regulate cellular processes, such as the Se-driven

antioxidant enzyme component, which can scavenge ROS in cells (28–30). The mechanism by which selenium plays a role in the development of osteoporosis is not clear, but we hypothesized several mechanisms that link selenium and osteoporosis. First, ROS has a critical effect on the development of OP (16). Apoptosis of osteoblasts and osteocytes induced by ROS may promote the production of osteoclasts and inhibit osteogenesis and mineralization. However, excessive osteocyte apoptosis can result in oxidative stress, which can disrupt the generation of osteoclasts, increase bone loss, and result in remodeling (31, 32). Selenoproteins are transporters of Se in bones, and they are found in osteoclasts and osteoblasts. They have antioxidant activities and can scavenge the generated ROS (15, 16, 33). Second, selenium can also influence anti-inflammatory and immune processes. Since cytokines such as interleukin-6 (IL-6) are important for the pathogenic mechanism of OP. Selenium exerts an anti-inflammatory effect, partially regulated via the inhibition of cytokine activities; thus, Se regulates bone turnover to protect against OP (34, 35). Third, Se-mediated iodothyronine deiodinases help regulate thyroid hormone turnover, while Se-mediated glutathione peroxidases help in protecting the thyroid gland. Thus, Se might also affect bone health via the relationship between Se-mediated glutathione peroxidase activity and thyroid protection. A deficiency of Se might increase the blood thyroid hormone level, thus accelerating bone loss and osteoporosis (36, 37). Therefore, determining the relationship between selenium intake and OP is extremely important.

This study had some strengths. First, this study was conducted based on a nationwide survey, where BMD was determined using established approaches by expert scientists. Second, this was the first study to analyze the relationship between Se intake and parameters such as BMD and OP among adults in the US. Our results showed a potential role of appropriate dietary Se intake in preventing the development of OP. Additionally, after confounding factors were adjusted, a gender-stratified subgroup analysis was conducted to provide a reference to determine the relationship between selenium intake and changes in BMD and risk of osteoporosis. However, our study had certain limitations. First, this was a cross-sectional study, and residual confounding due to additional unmeasured factors was not eliminated, although certain covariates were adjusted. Second, we acquired dietary intake information from the NHANES based on 24-h dietary recall interviews, which might be associated with recall bias. Hence, our results should be confirmed by conducting larger, prospective studies.

To summarize, the level of dietary and total Se intake was positively associated with BMD and negatively associated with the risk of OP among adults in the US. However, larger prospective studies are needed to confirm our findings.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding authors.

Ethics statement

The studies involving humans were approved by the Institutional Review Board of the National Center for Health Statistics. The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

Author contributions

SP analyzed the data, and wrote the manuscript. GZ and DW designed the study and revised the manuscript. SP is the first author. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

References

1. Hendrickx G, Boudin E, Van Hul W. A look behind the scenes: the risk and pathogenesis of primary osteoporosis. *Nat Rev Rheumatol* (2015) 11(8):462–74. doi: 10.1038/nrrheum.2015.48

2. Kanis JA, McCloskey EV, Johansson H, Oden A, Melton LJ 3rd, Khaltaev N. A reference standard for the description of osteoporosis. *Bone* (2008) 42(3):467–75. doi: 10.1016/j.bone.2007.11.001

3. Duan W, Meng X, Sun Y, Jia C. Association between polycyclic aromatic hydrocarbons and osteoporosis: data from NHANES, 2005-2014. *Arch Osteoporos* (2018) 13(1):112. doi: 10.1007/s11657-018-0527-4

4. Cai S, Fan J, Zhu L, Ye J, Rao X, Fan C, et al. Bone mineral density and osteoporosis in relation to all-cause and cause-specific mortality in NHANES: A population-based cohort study. *Bone* (2020) 141:115597. doi: 10.1016/j.bone.2020.115597

5. Lorentzon M, Nilsson AG, Johansson H, Kanis JA, Mellstrom D, Sundh D. Extensive undertreatment of osteoporosis in older Swedish women. *Osteoporos Int* (2019) 30(6):1297–305. doi: 10.1007/s00198-019-04872-4

6. Lunde A, Tell GS, Pedersen AB, Scheike TH, Apalset EM, Ehrenstein V, et al. The role of comorbidity in mortality after hip fracture: A nationwide norwegian study of 38,126 women with hip fracture matched to a general-population comparison cohort. *Am J Epidemiol* (2019) 188(2):398–407. doi: 10.1093/aje/kwy251

7. Rizzoli R, Biver E, Brennan-Speranza TC. Nutritional intake and bone health. *Lancet Diabetes Endocrinol* (2021) 9(9):606–21. doi: 10.1016/S2213-8587(21)00119-4

8. Aaseth J, Boivin G, Andersen O. Osteoporosis and trace elements–an overview. *J Trace Elem Med Biol* (2012) 26(2-3):149–52. doi: 10.1016/j.jtemb.2012.03.017

9. Martiniakova M, Babikova M, Mondockova V, Blahova J, Kovacova V, Omelka R. The role of macronutrients, micronutrients and flavonoid polyphenols in the prevention and treatment of osteoporosis. *Nutrients* (2022) 14(3):523. doi: 10.3390/nu14030523

10. Ratajczak AE, Rychter AM, Zawada A, Dobrowolska A, Krela-Kazmierczak I. Do only calcium and vitamin D matter? Micronutrients in the diet of inflammatory bowel diseases patients and the risk of osteoporosis. *Nutrients* (2021) 13(2):525. doi: 10.3390/nu13020525

11. Zhang H, Wang A, Shen G, Wang X, Liu G, Yang F, et al. Hepcidin-induced reduction in iron content and PGC-1beta expression negatively regulates osteoclast differentiation to play a protective role in postmenopausal osteoporosis. *Aging (Albany NY)* (2021) 13(8):11296–314. doi: 10.18632/aging.202817

12. Ceylan MN, Akdas S, Yazihan N. Is zinc an important trace element on bone-related diseases and complications? A meta-analysis and systematic review from serum level, dietary intake, and supplementation aspects. *Biol Trace Elem Res* (2021) 199(2):535–49. doi: 10.1007/s12011-020-02193-w

13. Zhou H, Wang T, Li Q, Li D. Prevention of keshan disease by selenium supplementation: a systematic review and meta-analysis. *Biol Trace Elem Res* (2018) 186(1):98–105. doi: 10.1007/s12011-018-1302-5

14. Winther KH, Rayman MP, Bonnema SJ, Hegedus L. Selenium in thyroid disorders - essential knowledge for clinicians. *Nat Rev Endocrinol* (2020) 16(3):165–76. doi: 10.1038/s41574-019-0311-6

15. Pietschmann N, Rijntjes E, Hoeg A, Stoedter M, Schweizer U, Seemann P, et al. Selenoprotein P is the essential selenium transporter for bones. *Metallomics* (2014) 6(5):1043–9. doi: 10.1039/C4MT00003J

16. Manolagas SC. From estrogen-centric to aging and oxidative stress: a revised perspective of the pathogenesis of osteoporosis. *Endocr Rev* (2010) 31(3):266–300. doi: 10.1210/er.2009-0024

17. Hoeg A, Gogakos A, Murphy E, Mueller S, Kohrle J, Reid DM, et al. Bone turnover and bone mineral density are independently related to selenium status in healthy euthyroid postmenopausal women. *J Clin Endocrinol Metab* (2012) 97(11):4061–70. doi: 10.1210/jc.2012-2121

18. Galvez-Fernandez M, Grau-Perez M, Garcia-Barrera T, Ramirez-Acosta S, Gomez-Ariza JL, Perez-Gomez B, et al. Arsenic, cadmium, and selenium exposures and bone mineral density-related endpoints: The HORTEGA study. *Free Radic Biol Med* (2021) 162:392–400. doi: 10.1016/j.freeradbiomed.2020.10.318

19. Wang Y, Xie D, Li J, Long H, Wu J, Wu Z, et al. Association between dietary selenium intake and the prevalence of osteoporosis: a cross-sectional study. *BMC Musculoskelet Disord* (2019) 20(1):585. doi: 10.1186/s12891-019-2958-5

20. Xue G, Liu R. Association between dietary selenium intake and bone mineral density in the US general population. *Ann Transl Med* (2022) 10(16):869. doi: 10.21037/atm-22-3441

21. Willett WC, Howe GR, Kushi LH. Adjustment for total energy intake in epidemiologic studies. *Am J Clin Nutr* (1997) 65(4 Suppl):1220S–8S; discussion 9S-31S. doi: 10.1093/ajcn/65.4.1220S

22. Looker AC, Wahner HW, Dunn WL, Calvo MS, Harris TB, Heyse SP, et al. Updated data on proximal femur bone mineral levels of US adults. *Osteoporos Int* (1998) 8(5):468–89. doi: 10.1007/s001980050093

23. Bass MA, Sharma A, Nahar VK, Chelf S, Zeller B, Pham L, et al. Bone mineral density among men and women aged 35 to 50 years. *J Am Osteopath Assoc* (2019) 119(6):357–63. doi: 10.7556/jaoa.2019.064

24. Wolf RL, Cauley JA, Pettinger M, Jackson R, Lacroix A, Leboff MS, et al. Lack of a relation between vitamin and mineral antioxidants and bone mineral density: results from the Women's Health Initiative. *Am J Clin Nutr* (2005) 82(3):581–8. doi: 10.1093/ajcn/82.3.581

25. Walsh JS, Jacques RM, Schomburg L, Hill TR, Mathers JC, Williams GR, et al. Effect of selenium supplementation on musculoskeletal health in older women: a randomised, double-blind, placebo-controlled trial. *Lancet Healthy Longev* (2021) 2(4):e212–e21. doi: 10.1016/S2666-7568(21)00051-9

26. Xie H, Wang N, He H, Yang Z, Wu J, Yang T, et al. The association between selenium and bone health: a meta-analysis. *Bone Joint Res* (2023) 12(7):423–32. doi: 10.1302/2046-3758.127.BJR-2022-0420.R1

27. Zhang J, Munger RG, West NA, Cutler DR, Wengreen HJ, Corcoran CD. Antioxidant intake and risk of osteoporotic hip fracture in Utah: an effect modified by smoking status. *Am J Epidemiol* (2006) 163(1):9–17. doi: 10.1093/aje/kwj005

28. Allan CB, Lacourciere GM, Stadtman TC. Responsiveness of selenoproteins to dietary selenium. *Annu Rev Nutr* (1999) 19:1–16. doi: 10.1146/annurev.nutr.19.1.1

29. Lei XG, Cheng WH, McClung JP. Metabolic regulation and function of glutathione peroxidase-1. *Annu Rev Nutr* (2007) 27:41–61. doi: 10.1146/annurev.nutr.27.061406.093716

30. Sunde RA. Molecular biology of selenoproteins. *Annu Rev Nutr* (1990) 10:451–74. doi: 10.1146/annurev.nu.10.070190.002315

31. Domazetovic V, Marcucci G, Iantomasi T, Brandi ML, Vincenzini MT. Oxidative stress in bone remodeling: role of antioxidants. *Clin cases Miner Bone Metab* (2017) 14(2):209–16. doi: 10.11138/ccmbm/2017.14.1.209

32. Robling AG, Bonewald LF. The osteocyte: new insights. *Annu Rev Physiol* (2020) 82:485–506. doi: 10.1146/annurev-physiol-021119-034332

33. Jakob F, Becker K, Paar E, Ebert-Duemig R, Schutze N. Expression and regulation of thioredoxin reductases and other selenoproteins in bone. *Methods Enzymol* (2002) 347:168–79. doi: 10.1016/S0076-6879(02)47015-2

34. Heaney RP. Calcium, dairy products and osteoporosis. *J Am Coll Nutr* (2000) 19(2 Suppl):83S–99S. doi: 10.1080/07315724.2000.10718088

35. Duntas LH. Selenium and inflammation: underlying anti-inflammatory mechanisms. *Horm Metab Res* (2009) 41(6):443–7. doi: 10.1055/s-0029-1220724

36. Schomburg L, Kohrle J. On the importance of selenium and iodine metabolism for thyroid hormone biosynthesis and human health. *Mol Nutr Food Res* (2008) 52(11):1235–46. doi: 10.1002/mnfr.200700465

37. Williams GR, Bassett JHD. Thyroid diseases and bone health. *J Endocrinol Invest* (2018) 41(1):99–109. doi: 10.1007/s40618-017-0753-4

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Strong association of lumbar disk herniation with diabetes mellitus: a 12-year nationwide retrospective cohort study

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Background: Despite reports on the association between diabetes mellitus (DM) and lumbar disk herniation (LDH), large-scale, nationwide studies exploring this relationship are lacking. We aimed to examine the profiles of DM in individuals with LDH and explore the potential mechanisms underlying the development of these disorders.

Methods: This retrospective, population-based study was conducted between 2008 and 2019 using data from the National Health Insurance (NHI) research database in Taiwan. The primary outcome was the date of initial LDH diagnosis, death, withdrawal from the NHI program, or end of the study period.

Results: In total, 2,662,930 individuals with and 16,922,546 individuals without DM were included in this study; 719,068 matched pairs were established following propensity score matching (1:1 ratio) for sex, age, comorbidities, smoking, alcohol consumption, antihyperglycemic medications, and index year. The adjusted risk for developing LDH was 2.33-fold (95% confidence interval: 2.29–2.37; $P<0.001$), age-stratified analysis revealed a significantly greater risk of LDH in every age group, and both males and females were approximately twice as likely to develop LDH in the DM compared with non-DM cohort. Individuals with DM and comorbidities had a significantly higher risk of developing LDH than those without, and the serial models yielded consistent results. Treatment with metformin, sulfonylureas, meglitinides, thiazolidinediones, dipeptidyl peptidase-4 inhibitors, or alpha-glucosidase inhibitors was associated with a more than 4-fold increased risk of LDH in the DM cohort. DM was strongly associated with the long-term development of

LDH; over the 12-year follow-up period, the cumulative risk of LDH was significantly higher in patients with than without DM (log-rank $P<0.001$).

Conclusion: DM is associated with an increased risk of LDH, and advanced DM may indicate a higher risk of LDH.

KEYWORDS

diabetes mellitus, lumbar disk herniation, intervertebral degenerative disk disease, anti-diabetic medications, chronic back pain

Introduction

Diabetes mellitus (DM) is a chronic disease characterized by elevated blood glucose levels, and is associated with various comorbidities. Types 1 and 2 are the two primary forms of DM, with type 2 representing approximately 90% of DM cases. Type 1 DM, also known as autoimmune DM, is a chronic disease characterized by insulin deficiency and hyperglycemia caused by the elimination of pancreatic β-cells (1). DM can affect various organ systems, resulting in severe complications over time. Individuals with type 2 DM are at risk of both microvascular and macrovascular complications, including retinopathy, nephropathy, neuropathy, and cardiovascular comorbidities. Insulin resistance and impaired insulin secretion are the primary defects of type 2 DM (2), and several antihyperglycemic medications (AHMs) with various mechanisms of action for reducing blood sugar have been developed. Commonly used oral AHMs include metformin (biguanide class), sulfonylureas (Sus), meglitinides, thiazolidinediones (TZDs), alpha-glucosidase inhibitors (Agis), dipeptidyl peptidase-4 inhibitors (DPP4is), and sodium-glucose cotransporter-2 inhibitors (SGLT2is). Injective AHMs include GLP-1 receptor agonists (GLP1Ras) and insulin.

Lumbar disk herniation (LDH) is a common cause of lower back and unilateral leg pain that commonly occurs during the fourth and fifth decades of life, affecting a significant portion of the population, with a lifetime prevalence of 10%. The occurrence of Modic changes in the lumbar region exhibited a significant increase in both the 40s and 60s (3). Similarly, the prevalence of severe intervertebral disc degeneration in the lumbar region demonstrated a significant increase in individuals aged 20s, 30s, 50s, and 70s (3). Approximately 5–20 cases of LDH per 1,000 adults occur annually, with around 95% of herniations occurring at L4-L5 or L5-S1 (4). Degeneration of intervertebral disks is a leading cause of back pain; disk degeneration, disk herniation, and radicular pain result from an imbalance between catabolic and anabolic responses (5), and disk degeneration is typically associated with herniations. Male sex, taller height, intensive work, obesity, and smoking were reported to predict LDH recurrence (6, 7), and although the relationship between DM and lumbar disk degeneration has been the subject of research, the findings remain inconsistent. Some studies have reported cases wherein DM is a risk factor in patients with multiple disk herniation. Notably, patients who underwent surgery for lumbar disk disease had a significantly higher incidence of DM than those who underwent surgery for other reasons (8). Park et al. (9) revealed that type 2 DM is significantly associated with lumbar spine disorders and frequent spinal procedures, while another study revealed a positive relationship between DM and lumbar disk diseases, including LDH (10). Additionally, a longer duration and poor control of hyperglycemia was reported to aggravate disk degeneration (11). Based on magnetic resonance imaging findings, another study found no conclusive evidence suggesting that insulin-dependent DM has a significant impact on bone density or disk degeneration (12). Therefore, whether DM is a risk factor for lumbar disk disease remains to be clarified.

Large-scale cohort studies of this topic are lacking; therefore, we aimed to delineate the association between DM and LDH by conducting a nationwide study to determine whether any difference in the risk of LDH exists between individuals with and without DM.

Methods

Study population

This study utilized data from the National Health Insurance Research Database (NHIRD), which is maintained by the National Health Research Institute. The NHIRD contains information from the Taiwan National Health Insurance (NHI) program, which has provided healthcare coverage to nearly all residents since its inception in 1995. By the end of 2010, the NHI program had enrolled more than 27 million people, representing approximately 99% of Taiwan's total population. The NHIRD encrypts the identification information of each patient in the database to protect patient privacy; therefore, our investigation did not

Abbreviations: AGis, alpha-glucosidase inhibitors; AS, ankylosing spondylitis; aHR, adjusted hazard ratio; cHR, crude hazard ratio; CBP, chronic back pain; CKD, chronic kidney disease; CLD, chronic liver disease; DM, diabetes mellitus; DPP4is, dipeptidyl peptidase 4 inhibitors; GLP1RAs, glucagon-like peptide 1 receptor agonists; ICD, International Classification of Diseases; LDH, lumbar disk herniation; DM, diabetes mellitus; SGLT2is, sodium–glucose cotransporter 2 inhibitors; SUs, sulfonylureas; TZDs, thiazolidinediones; 95% CI, 95% confidence interval.

include any personal, institutional, or other data links between two or more databases. Using the International Classification of Diseases, Ninth and Tenth Revisions, Clinical Modifications (ICD-9-CM and ICD-10-CM) codes, inpatient and outpatient diagnoses were determined. This study was conducted in accordance with the principles of the Declaration of Helsinki and approved by the Institutional Review Board of China Medical University Hospital (approval number CMUH110-REC3-133(CR-1)). A waiver of informed consent was granted by the Institutional Review Board owing to the use of deidentified data in the present study. The access date to the NHIRD was on May 31, 2023.

Study design

Patients diagnosed with either type 1 or 2 DM between 2008 and 2018 were identified in our institution's clinical database using ICD codes (ICD-9 250; ICD-10 E08–E13). The study included Taiwanese individuals aged ≥20 years, with a study period from January 1, 2008, to December 31, 2018; patients were followed up until December 31, 2019. Accurate coding of diagnoses was ensured by requiring at least two outpatient visits or one hospitalization for inclusion. The exclusion criteria were: (1) an index date before 2008 or after 2018; (2) prior LDH diagnosis; (3) a history of kyphosis (ICD-9-CM codes 737.0, 737.1; ICD-10-CM codes M40.0–M40.3), lordosis (ICD-9-CM code 737.2; ICD-10-CM codes M40.4, M40.5), scoliosis (ICD-9-CM 737.3; ICD-10-CM M41), or spine fracture (ICD-9-CM codes 733.82, 805, 806, 808, 905, V54.8; ICD-10-CM code S32); (4) an age <20 years or >100 years; or (5) missing information regarding sex or age. The follow-up period was defined as the time between the index date (date of initial DM diagnosis) and end date (date of LDH diagnosis, or December 31, 2019, whichever occurred first). The non-DM control group was matched to individuals with DM based on enrollment criteria. This study was conducted in accordance with the Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) guidelines for reporting observational studies (Supplementary Table 1).

Main outcome and covariates

The main outcome of this study was the development of LDH. We censored patients on the date of the respective outcome, death, or withdrawal from the NHIRD, or at the end of follow-up on December 31, 2019, whichever came first. During the follow-up period, the incidence rates of LDH were compared between the case and control groups, with LDH defined by ICD-9-CM codes (722.10, 722.11) and ICD-10-CM codes (M51.25, M51.26, and M51.27), and DM defined by ICD-9-CM (250) and ICD-10-CM (E08–E13) codes.

To identify potential confounders that could affect LDH development, we considered sex, age, income, common comorbidities, and AHMs. The comorbidities accounted for were hypertension (ICD-9-CM codes 401–405; ICD-10-CM codes I10–I15), dyslipidemia (ICD-9-CM code 272; ICD-10-CM codes E75 and E78), chronic liver disease (CLD) (ICD-9-CM code 571; ICD-10-CM code K70, K73, and K74), chronic kidney disease (CKD) (ICD-9-CM codes 585, 586; ICD-10-CM codes N18, N19), neoplasm (ICD-9-CM code 140–239; ICD-10-CM codes C00–D49), and obesity (ICD-9-CM codes 278, 783.1; ICD-10-CM codes E66–E68 and R63.5).

Several diseases involve the bone, thereby altering the bone density of the skeletal infrastructure. Therefore, we included the following variables: bone metastasis (ICD-9-CM code 198.5; ICD-10-CM codes C79.5 and C7B.03), ankylosing spondylitis (ICD-9-CM code 720; ICD-10-CM codes M08.1, M45, M46, M48.8, and M49), and multiple myeloma (ICD-9-CM code 203; ICD-10-CM codes C88, C90, and Z51). Social behaviors, such as smoking (ICD-9-CM codes 305.1, V15.82; ICD-10-CM codes F17 and Z87.891) and alcohol consumption (ICD-9-CM code 305.0; ICD-10-CM code F10), were also included. For patients with DM, AHMs were prescribed to control blood glucose, including metformin (Anatomical Therapeutic Chemical [ATC] code A10BA02), SUs (ATC code A10BB), meglitinides (ATC code A10BX02), TZDs (ATC code A10BG), DPP4is (ATC code A10BH), SGLT2is (ATC code A10BK), AGis (ATC code A10BF01), GLP1RAs (ATC code A10BJ), and insulin (ATC code A10A).

Statistical analysis

We used propensity score matching to reduce selection bias and improve the comparability of variables — such as sex, age, income, comorbidities, AHMs, smoking, alcohol consumption, and index year — between the DM and non-DM groups. The closest propensity score was computed, and matched pairs were created using the nearest-neighbor method, with a significance level of standardized mean difference of <0.1 indicating a significant difference between the cohorts. We used the Cox proportional hazards model to compare outcomes between the two groups, with crude and multivariate-adjusted hazard ratios (HRs) adjusted for sex, age, comorbidities, AHMs, and index year. Patients were censored if they developed LDH, died, or reached the end of the follow-up period on December 31, 2019, whichever occurred first. We performed Kaplan-Meier analysis and log-rank tests to compare the cumulative incidence of LDH between the DM and non-DM groups. Statistical analyses were performed using SAS (version 9.5; SAS Institute, Cary, NC, USA), and a two-tailed *P*-value <0.05 was considered statistically significant.

Results

Patient characteristics

Among the data obtained between January 1, 2008, and December 31, 2019, we identified 31,488,321 individuals from the database. After excluding ineligible patients, we included 2,662,930 and 16,992,546 individuals in the DM and non-DM cohorts, respectively. Figure 1 presents a flowchart of the study. We performed 1:1 propensity score matching based on the variables mentioned in the Methods section, resulting in 719,068 matched

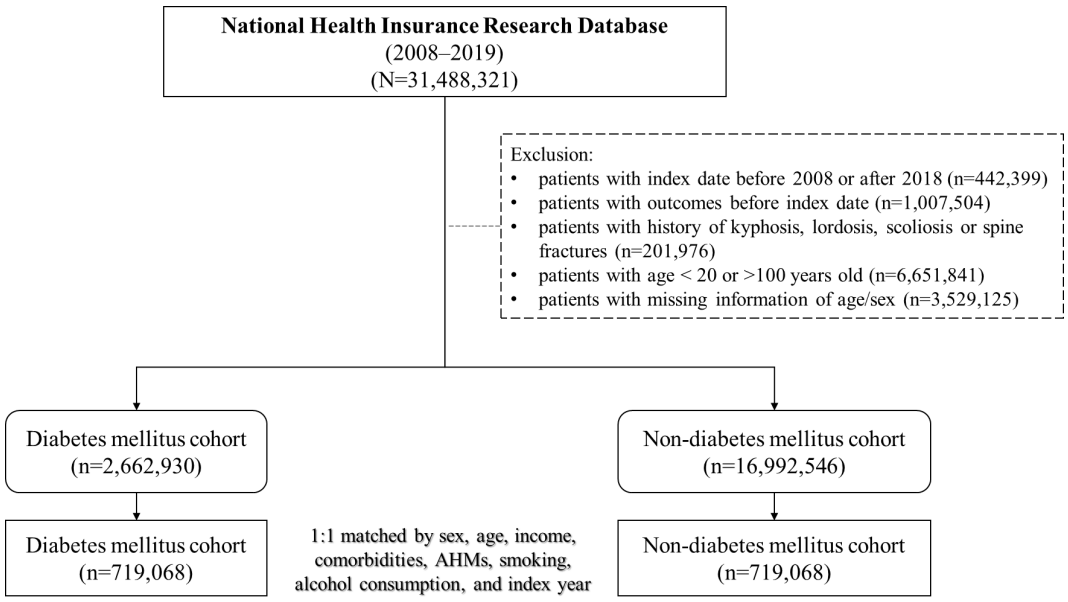

FIGURE 1
Flow chart for patients with diabetes mellitus and comparison cohort.

pairs of patients with and without DM. In the matched cohorts, the mean age of the DM cohort was 59.59 ± 15.32 years, and 50.27% were female; the mean follow-up duration was 5.7 ± 3.48 years. Table 1 presents the baseline demographics of the included participants; the two cohorts showed similar baseline characteristics.

Multivariate analyses

Figure 2 shows a forest plot of the risk factors for LDH in individuals with DM; Supplementary Table 2 summarizes these risk factors. In the multivariable Cox regression analysis, 20,729 (2.88%) patients with LDH did not have a previous diagnosis of DM, and 45,243 (6.29%) patients with LDH were diagnosed with DM before the occurrence of LDH (incidence rate: 4.6 *vs.* 11.0 per 1,000 person-years). The crude HR (cHR) of DM was 2.38 (95% confidence interval [CI], 2.34–2.42, $P<0.001$) in patients with LDH. Individuals with DM had a higher risk of developing LDH than those without DM after adjusting for sex, age, comorbidities and AHMs (adjusted HR [aHR], 2.33; 95% CI, 2.29–2.37; $P<0.001$). The risk of LDH was found to increase in individuals aged 40–59 years and 60–79 years when compared with individuals aged 20–39 years, with aHRs of 1.33 and 1.39, respectively. However, this risk decreased in individuals aged >80 years. Males had a significantly lower risk of LDH (aHR, 0.87; 95% CI, 0.86–0.88; $P<0.001$) than females, and income level was not associated with LDH. Patients with comorbidities — such as hypertension, dyslipidemia, CLD, CKD, obesity, smoking, and alcohol consumption — had a significantly higher risk of LDH, whereas those with cancer had a significantly lower risk of LDH. Notably, patients with AS had a significantly elevated risk of LDH (aHR, 1.56; 95% CI, 1.46–1.66; $P<0.001$). Participants using 1 or >2 AHMs had a prominent risk of LDH (aHR, 1.27 and 1.32, respectively).

Figure 3 presents a forest plot of the risk factors for LDH in individuals with and without DM; Supplementary Table 3 summarizes these findings. Individuals with DM had a significantly higher risk of developing LDH, regardless of sex, age, income level, or the coexistence of any comorbidity. Patients using AHMs have a more prominent risk of LDH than those who do not; notably, individuals receiving GLP1RAs had 10.46-fold higher risk of developing LDH than those did not (95% CI, 1.01–108.29; $P=0.0489$). The use of metformin, SUs, meglitinides, TZDs, DPP4is, or AGis was associated with a more than 4-fold increased risk of LDH in patients with than without DM, and the number of AHMs used was positively associated with the risk of LDH development.

Stratified analyses

To investigate the effect of covariates, four models were used to determine the risk of LDH in patients both with and without DM. Table 2 presents the HRs and 95% CIs for the two cohorts, as well as each model. In Model 1, the cHR was examined. In models 2–4, the aHRs were obtained based on adjustments to different variables.

Duration analysis

Table 3 presents the risk of LDH in both cohorts according to the duration of LDH diagnosis. Regarding follow-up periods <4 years, the aHR of LDH development in the DM cohort was 2.6 (95% Cl, 2.54–2.66; $P<0.001$). After >10 years of follow-up, the risk of LDH in the DM cohort remained significantly higher than in the non-DM cohort (aHR, 1.51; 95% CI, 1.37–1.68; $P<0.001$).

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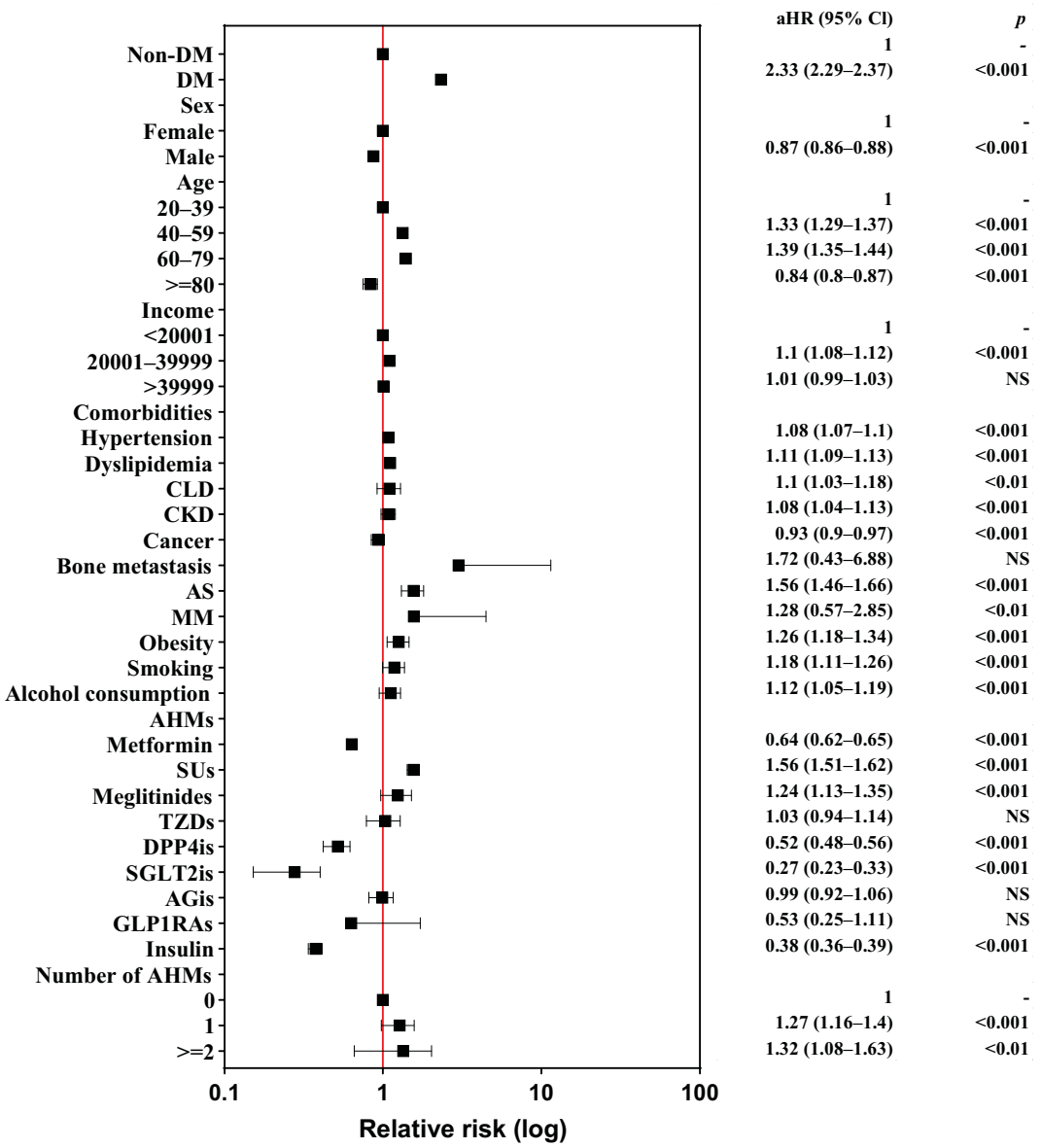

FIGURE 2
Forest plot of risk factors for lumbar disk herniation among individuals. AHMs, anti-hyperglycemic medications; aHR, adjusted hazard ratio; AGis, alpha-glucosidase inhibitors; AS, ankylosing spondylitis; CLD, chronic liver disease; CKD, chronic kidney disease; DM, diabetes mellitus; DPP4is, dipeptidyl peptidase-4 inhibitors; GLP1RAs, glucagon-like peptide-1 receptor agonists; MM, multiple myeloma; NS, nonsignificant; SGLT2is, sodium-glucose cotransporter 2 inhibitors; SUs, sulfonylureas; TZDs, thiazolidinediones; 95% CI, 95% confidence interval.

Cumulative incidence of LDH

Figure 4 illustrates the Kaplan-Meier cumulative incidence of LDH, which was significantly higher in the DM than non-DM cohort (log-rank $P<0.001$).

Discussion

In recent years, there has been a notable increase in the occurrence of both type 1 and type 2 DM, suggesting that a large proportion of the population faces challenges and complications associated with this chronic condition. A study conducted in this context revealed that individuals with DM displayed elevated levels of LDH. These results suggest that inadequate long-term management of DM may contribute to the development of LDH, and potentially increase the chances of requiring surgical intervention.

Degenerative disk disease poses a significant healthcare issue, leading to persistent and often intense back pain that has a detrimental impact on the patient's wellbeing, and contributes to rising healthcare expenses. Understanding the risk factors associated with lumbar disk degeneration is crucial to implementing strategies that prevent or slow disease development and progression. Recent studies indicate a higher vulnerability to intervertebral disk disease in females compared with males; still, the specific impact of DM on intervertebral disk degeneration based on

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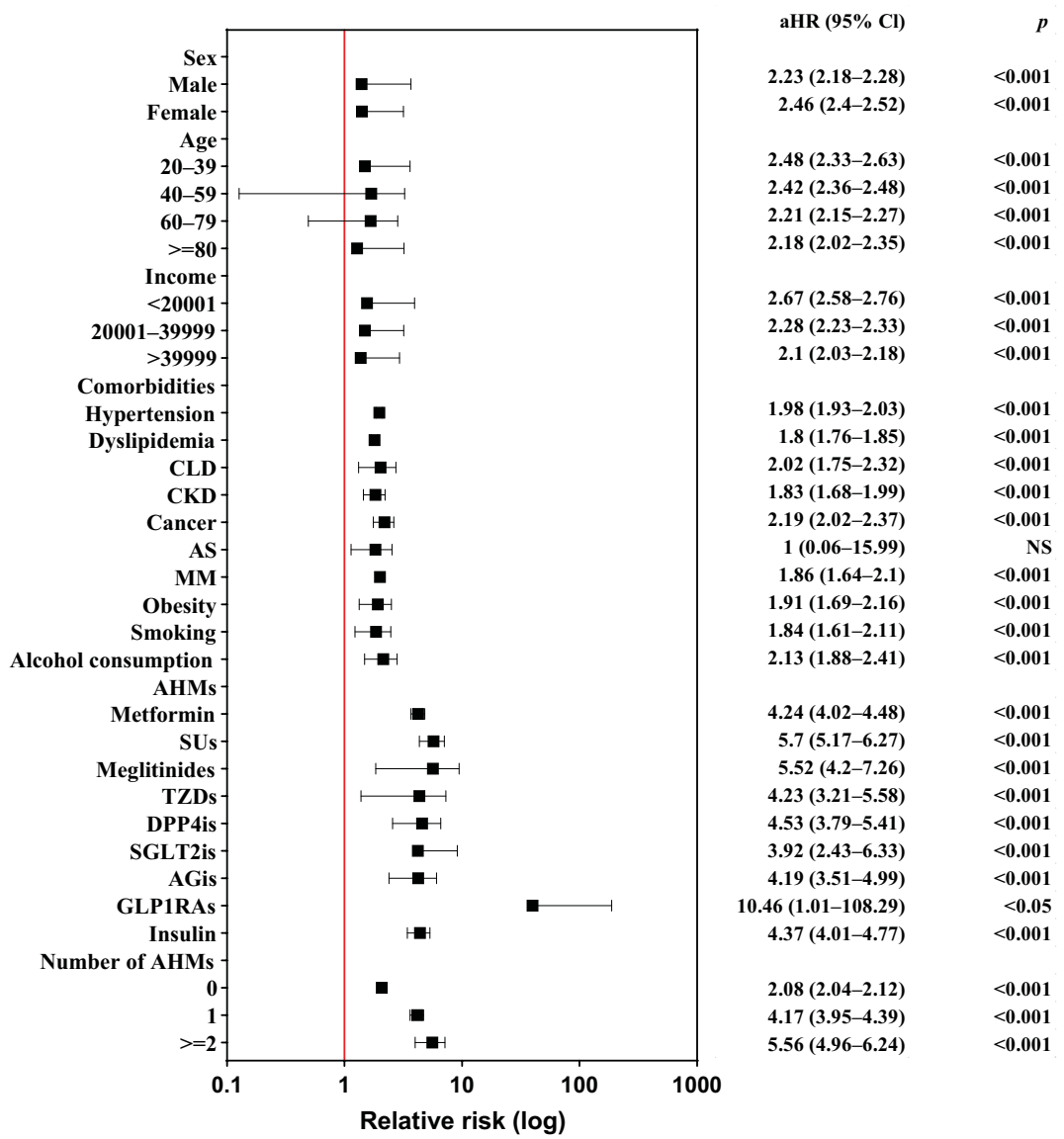

FIGURE 3
Forest plot of risk factors for lumbar disk herniation among individuals with and without diabetes mellitus. AHMs, anti-hyperglycemic medications; aHR, adjusted hazard ratio; AGis, alpha-glucosidase inhibitors; AS, ankylosing spondylitis; CLD, chronic liver disease; CKD, chronic kidney disease; DPP4is, dipeptidyl peptidase-4 inhibitors; GLP1RAs, glucagon-like peptide-1 receptor agonists; MM, multiple myeloma; NS, nonsignificant; SGLT2is, sodium–glucose cotransporter 2 inhibitors; SUs, sulfonylureas; TZDs, thiazolidinediones; 95% CI, 95% confidence interval.

differences in sex remains unclear (13). Our study reported consistent findings that males bear a lower risk of LDH than females (aHR, 0.87; 95% CI, 0.86–0.88). Several clinical studies have demonstrated that the incidence of intervertebral disk disease is higher in individuals with obesity and DM (14); notably, growing evidence indicates a correlation between a high body mass index (BMI), obesity, or overweight, and an increased risk of intervertebral disk degeneration (15). Özcan-Ekş et al. (16) found that severe intervertebral disc disease was significantly more prevalent in obese individuals compared to non-obese individuals, with a prevalence rate of 73.5% in obese patients compared to 50.4% in non-obese patients. In addition, there was a higher likelihood of obese patients exhibiting Modic changes at any lumbar level, particularly in women. This result corroborates our research outcomes. In the present study, obesity was found to increase the risk of LDH (aHR, 1.12; 95% CI, 1.05–1.19).

Associations were also found between dyslipidemia and LDH levels (aHR, 1.11; 95% CI, 1.09–1.13); however, the relationship between serum lipid levels and back pain remains under debate. Some theories propose that advanced atherosclerosis may play a role in microvessel disease and spinal disk degeneration. Abnormal lipid levels have also been suggested as a potential mechanism that leads to atherosclerosis in the blood vessels of the lumbar region, which in turn can cause low back pain. Additionally, individuals with high TG levels were more likely to experience disk herniation (odds ratio, 2.974; 95% CI, 1.488–5.945) (17). The age-adjusted

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TABLE 1 Characteristics for individuals with and without diabetes mellitus.

	Non-DM (N=719,068)		DM (N=719,068)		
Variables	n	%	n	%	SMD
Sex					
Female	364088	50.63	361509	50.27	0.007
Male	354980	49.37	357559	49.73	0.007
Age					
20–39	68300	9.50	73128	10.17	0.023
40–59	285965	39.77	292912	40.74	0.02
60–79	281903	39.20	271858	37.81	0.029
≥80	82900	11.53	81170	11.29	0.008
mean, (SD)	60.09	15.17	59.59	15.32	0.033
Income					
<20001	199799	27.79	203113	28.25	0.01
20001–39999	360788	50.17	357082	49.66	0.01
>39999	158481	22.04	158873	22.09	0.001
Comorbidities					
Hypertension	367936	51.17	343790	47.81	0.067
Dyslipidemia	272958	37.96	254643	35.41	0.053
CLD	15237	2.12	11611	1.61	0.037
CKD	40519	5.63	33905	4.72	0.042
Cancer	44068	6.13	42315	5.88	0.01
Bone metastasis	26	0.00	30	0.00	0.001
AS	9044	1.26	7814	1.09	0.016
MM	138	0.02	142	0.02	<0.001
Obesity	12024	1.67	10009	1.39	0.023
Spine fractures surgery	1921	0.27	4011	0.56	0.03
Smoking	11905	1.66	10707	1.49	0.013
Alcohol consumption	15644	2.18	12682	1.76	0.03
Anti-hyperglycemic medications					
Metformin	121995	16.97	133879	18.62	0.043
SUs	31771	4.42	41406	5.76	0.061
Meglitinides	6194	0.86	8247	1.15	0.029
TZDs	4715	0.66	6662	0.93	0.031
DPP4is	22698	3.16	30507	4.24	0.058
SGLT2is	6421	0.89	8597	1.20	0.03
AGis	9338	1.30	11382	1.58	0.024
GLP1RAs	237	0.03	414	0.06	0.012
Insulin	112035	15.58	91586	12.74	0.082
Number of anti-hyperglycemic medications					
0	571979	79.54	538570	74.90	0.111
1	105712	14.70	135817	18.89	0.112
≥2	41377	5.75	44681	6.21	0.019
Follow-up years of LDH, mean (SD)	6.26	3.31	5.7	3.48	0.163

AGis, alpha-glucosidase inhibitors; AS, ankylosing spondylitis; N, number of events; CLD, chronic liver disease; CKD, chronic kidney disease; DM, diabetes mellitus; DPP4is, dipeptidyl peptidase 4 inhibitors; GLP1RAs, glucagon-like peptide 1 receptor agonists; LDH, lumbar disk herniation; MM, Multiple myeloma; SD, standard deviation; SGLT2is, sodium–glucose cotransporter 2 inhibitors; SMD, standardized mean difference; SUs, sulfonylureas; TZDs, thiazolidinediones.

prevalence of low back pain was inversely associated with HDL cholesterol levels, and positively associated with triglyceride; however, after accounting for age, the total cholesterol levels were not significantly associated with low back pain in either gender (18). Cholesterol levels are also associated with CBP in patients with DM; elevated LDL cholesterol levels were associated with CBP, whereas elevated HDL cholesterol levels were negatively associated.

Recently, smoking was shown to negatively influence LDH levels, likely due to microangiopathy. In the present study, we found that smokers had a greater probability of suffering from LDH (aHR, 1.18; 95% Cl, 1.11–1.26) compared with nonsmokers. Two potential mechanisms for disk degeneration caused by smoking have been postulated: (1) downregulation of glycosaminoglycan biosynthesis and cell proliferation mediated by nicotine, and (2) decreased supply of nutrients to the intervertebral disk. The results of our study align with those of previous studies that established a correlation between DM and degenerative disk diseases (9, 19).

TABLE 2 Hazard ratios and 95% confidence intervals for lumbar disk herniation in different models.

	Non-DM	DM
LDH		
Number of events	20,729	45,243
PY	4,499,516	4,102,043
IR	4.61	11.03
Model1. cHR (95% CI)	(Reference)	2.38 (2.34, 2.42)***
Model2. aHR (95% CI)	(Reference)	2.37 (2.33, 2.41)***
Model3. aHR (95% CI)	(Reference)	2.38 (2.34, 2.42)***
Model4. aHR (95% CI)	(Reference)	2.33 (2.29, 2.37)***

Model2: adjusted to sex and age.
Model3: adjusted to Model2 (sex, age), and comorbidities.
Model4: adjusted to Model3 (sex, age, comorbidities), and anti-hyperglycemic medications.
aHR, adjusted hazard ratio; cHR, crude hazard ratio; DM, diabetes mellitus; IR, incidence rate per 1,000 person-years; LDH, lumbar herniation; PY, person-years. ***P <0.001.

TABLE 3 The risks of lumbar disk herniation in the diabetes mellitus cohort relative to the non-diabetes mellitus cohort in terms of different follow-up period.

	Non-DM			DM						
Follow-up years	n	PY	IR	n	PY	IR	cHR	(95% CI)	aHR	(95% CI)
<4	11501	2008268	5.73	29605	1897823	15.60	2.69	(2.63, 2.74)***	2.6	(2.54, 2.66)***
4–7	5664	1401677	4.04	10223	1253535	8.16	2.02	(1.95, 2.08)***	1.96	(1.89, 2.02)***
8–10	2919	822924	3.55	4524	714300	6.33	1.8	(1.72, 1.88)***	1.76	(1.68, 1.84)***
>10	645	266647	2.42	891	236384	3.77	1.52	(1.37, 1.68)***	1.51	(1.37, 1.68)***

aHR, adjusted hazard ratio; cHR, crude hazard ratio; DM, diabetes mellitus; IR, incidence rate per 1,000 person-years; PY, person-years.
†: adjusted by sex, age, comorbidities, and anti-hyperglycemic medications. ***P <0.001.

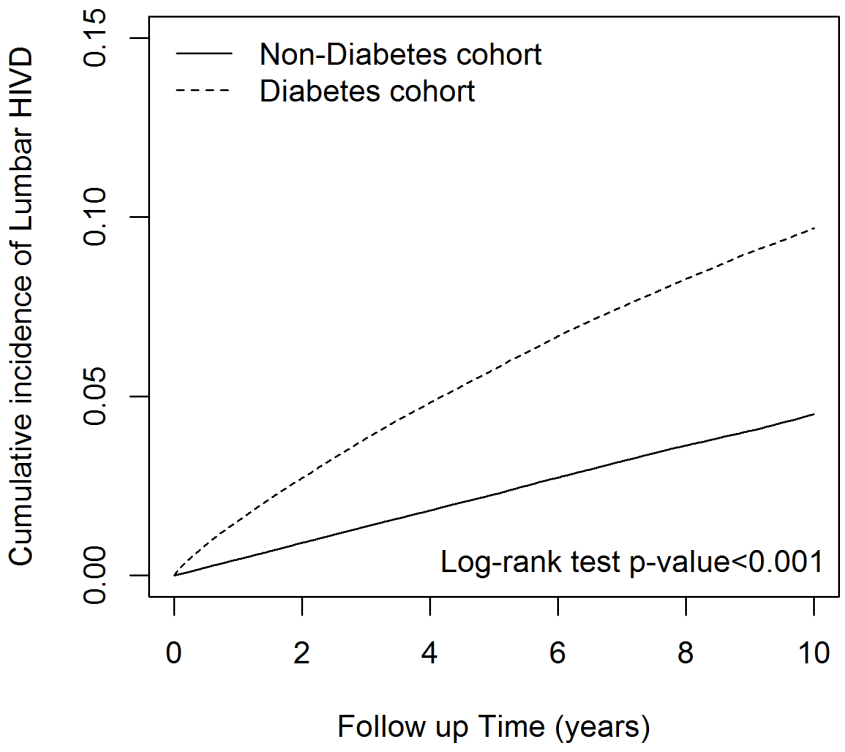

FIGURE 4
The cumulative incidence of lumbar disk herniation in diabetes mellitus cohort and control cohort.

Furthermore, previous investigations demonstrated that individuals with DM tend to experience worse outcomes after lumbar discectomy than nondiabetic controls, including higher rates of reoperation and longer hospital stays (20).

Elevated preoperative HbA1c levels and long-term DM are risk factors for unfavorable outcomes following cervical laminoplasty in patients with DM and cervical spondylotic myelopathy (21). In a review of patients who underwent discectomy for LDH, Mobbs et al. (20) reported higher rates of LDH recurrence and reoperation in patients with DM (28%) than in controls (3.5%). However, Vogt et al. (22) did not find a correlation between a history of DM and the prevalence of L4–L5 degenerative spondylolisthesis. A duration >10 years and poor control of type 2 DM are risk factors for lumbar disk degeneration, with a longer duration associated with more severe disk degeneration (23). The duration of DM is also associated with the need for spinal surgery, suggesting that the cumulative effects of DM over time may contribute to degenerative changes requiring surgical intervention. Lumbar degenerative disk disease is associated with male sex, HbA1c levels, and venous glucose (24). This study also reported a potential link between DM and lumbar spinal stenosis. A Mendelian randomization analysis revealed a causal effect of type 2 DM on degenerative disk disease that persisted even when adjusted to BMI (25). Magnetic resonance imaging revealed a strong correlation between the severity and duration of DM and the presence of Modic changes (26). DM is also associated with poor outcomes following lumbar discectomy and

cervical laminoplasty; a meta-analysis demonstrated that DM increased the risk of postoperative mortality, surgical site infection, deep venous thrombosis, and prolonged hospitalization after spinal surgery (27).

A positive correlation has been identified between DM and degenerative lumbar disk disease; high preoperative HbA1c levels and long-term DM are risk factors for poor cervical laminoplasty outcomes in patients with DM and cervical spondylotic myelopathy (21). Several studies have also suggested that hyperglycemia promotes the formation of advanced glycation end products in the nucleus pulposus, which contributes to the progression of disk degeneration. Recent animal studies have examined the association between hyperglycemia and intervertebral disk degeneration. An animal study indicated that DM accelerates disk degeneration through microangiopathy (28); additionally, microvascular disease — a characteristic of DM — may impair disk nutrition and contribute to degeneration. In a study using a rat model, hyperglycemia stimulated disk autophagy — a process of cellular self-degradation — and accelerated stress-induced senescence in nucleus pulposus cells. Autophagy in nucleus pulposus and annulus fibrosus cells also appears to play a significant role in lumbar degenerative diseases. Two studies have demonstrated that high glucose-induced oxidative stress accelerates premature stress-induced senescence in young rat annulus fibrosis cells (29, 30).

The evaluation of proteoglycans in the intervertebral disks of individuals with DM has revealed a reduction in sulfate incorporation into glycosaminoglycan molecules, and lower rates of glycosylation. These findings align with those of a previous study conducted by Robinson et al. (31), who observed a lower presence of proteoglycans in the intervertebral disks of patients with than without DM. These variations may contribute to elevated vulnerability to recurrent herniation in individuals with DM, as sulfation and proteoglycans are recognized for their role in reinforcing the collagen matrix of the disk. Nevertheless, despite the histological evidence, clinical studies have not established a conclusive association between DM and the rate of recurrent LDH.

Notably, the administration of AHMs, such as SUs or meglitinides, was found to be significantly associated with an increased risk of LDH; additionally, the simultaneous use of multiple AHMs, which suggests inadequate blood sugar control, was significantly associated with an increased risk of LDH. Conversely, the use of metformin, DPP4is, SGLT2is, or insulin was significantly associated with a lower risk of LDH. No significant association with LDH was observed for TZDs, AGis, or GLP1RAs. In multivariate analyses, patients with DM using any AHM exhibited a higher risk of LDH than those without DM. This suggests that worsening hyperglycemia, which requires medication, is associated with an increased risk of LDH.

This study demonstrated that participants who used AHMs were at a higher risk of LDH than those who did not. Furthermore, patients who were coadministered >2 AHMs were at a significantly higher risk of developing LDH than those who were coadministered <2 AHMs. These findings suggest that patients with poorly controlled DM tend to exhibit more severe disk degeneration than those with adequate control, as well as that DM is a risk factor for LDH, with an effect dependent on the duration and level of disease control. Additionally, the study observed that the use of medications like metformin, DPP4is, SGLT2is, or exogenous insulin was associated with a lower incidence of LDH.

DM is a complex condition that likely contributes to LDH via various mechanisms; furthermore, the correlation between AHMs and LDH can vary depending on specific clinical circumstances. Therefore, maintaining strict blood glucose control is crucial for preventing or delaying lumbar degenerative diseases in older patients with DM (30). This study acknowledges the importance of further investigation to understand the mechanisms underlying the association between DM and LDH, as well as the disease burden of DM in spinal pathologies; thus, further prospective comparative studies with longer follow-up periods are required to confirm our results.

This study has some limitations, including its retrospective cohort design; however, observational studies cannot provide insight into the causal relationship between DM and interverbal degenerative disk disease, even when based on larger sample sizes. Second, the NHIRD lacks relevant clinical and laboratory information, such as BMI, lipid profiles, and HbA1c levels. Third, although we adjusted for various confounding factors, the residual confounding factors may have biased our results; cohort studies are usually associated with bias due to uncovered and unobserved confounding factors. Last, our findings only be related to the Taiwanese population; thus, similar studies should be performed in different countries to determine whether our observations apply to other populations. Despite the notable limitations mentioned above, the primary objective of this study was to evaluate the overall correlation between DM burden and LDH levels. However, to delve into more precise inquiries, future investigations should consider conducting smaller and more targeted studies.

Conclusions

In recent years, the prevalence of both type 1 and type 2 DM has increased in children and adolescents, indicating that a growing population is at risk of complications associated with this chronic disease. This study aimed to explore the relationship between DM and LDH. These findings revealed that higher LDH burden metrics were identified in patients with than without DM, suggesting that advanced DM contributes to the development of LDH. Elevated blood sugar levels, modified proteoglycan composition, microvascular diseases, and cholesterol levels are potential factors involved in the mechanisms underlying LDH in individuals with DM. In conclusion, early and strict blood glucose control is important to prevent the development of lumbar degenerative diseases in patients with DM.

IRB approval status

This study was reviewed and approved by the Institutional Review Board of China Medical University Hospital (ID number CMUH110-REC3-133(CR-1)).

Impact statement

Diabetes mellitus contributes to the development of lumbar disk herniation; thus, early and strict blood glucose control is important to prevent the development of lumbar degenerative diseases in these patients.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

Ethics statement

The studies involving humans were approved by Institutional Review Board of China Medical University Hospital. The studies were conducted in accordance with the local legislation and intitutional requirements. Written informed consent for participation was not required from the participants or the participants' legal guardians because only deidentified data was obtained and used in the present study.

Author contributions

J-XL: Conceptualization, Investigation, Methodology, Visualization, Writing – original draft, Writing – review & editing, Software. T-JH: Data curation, Formal Analysis, Methodology, Software, Writing – review & editing. S-BH: Data curation, Project administration, Software, Validation, Writing – review & editing. Y-HL: Project administration, Supervision, Validation, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2023.1260566/full#supplementary-material

References

1. Katsarou A, Gudbjornsdottir S, Rawshani A, Dabelea D, Bonifacio E, Anderson BJ, et al. Type 1 diabetes mellitus. *Nat Rev Dis Primers* (2017) 3:17016. doi: 10.1038/nrdp.2017.16

2. DeFronzo RA, Ferrannini E, Groop L, Henry RR, Herman WH, Holst JJ, et al. Type 2 diabetes mellitus. *Nat Rev Dis Primers* (2015) 1:15019. doi: 10.1038/nrdp.2015.19

3. Eksi MS, Orhun O, Yasar AH, Dursun AT, Berikol G, Borekci A, et al. At what speed does spinal degeneration gear up?: Aging Paradigm in patients with Low Back Pain. *Clin Neurol Neurosurg* (2022) 215:107187. doi: 10.1016/j.clineuro.2022.107187

4. Amin RM, Andrade NS, Neuman BJ. Lumbar disc herniation. *Curr Rev Musculoskelet Med* (2017) 10(4):507–16. doi: 10.1007/s12178-017-9441-4

5. Risbud MV, Shapiro IM. Role of cytokines in intervertebral disc degeneration: pain and disc content. *Nat Rev Rheumatol* (2014) 10(1):44–56. doi: 10.1038/nrrheum.2013.160

6. Shimia M, Babaei-Ghazani A, Sadat BE, Habibi B, Habibzadeh A. Risk factors of recurrent lumbar disk herniation. *Asian J Neurosurg* (2013) 8(2):93–6. doi: 10.4103/1793-5482.116384

7. Shepard N, Cho W. Recurrent lumbar disc herniation: A review. *Global Spine J* (2019) 9(2):202–9. doi: 10.1177/2192568217745063

8. Sakellaridis N. The influence of diabetes mellitus on lumbar intervertebral disk herniation. *Surg Neurol* (2006) 66(2):152–4. doi: 10.1016/j.surneu.2006.01.019

9. Park CH, Min KB, Min JY, Kim DH, Seo KM, Kim DK. Strong association of type 2 diabetes with degenerative lumbar spine disorders. *Sci Rep* (2021) 11(1):16472. doi: 10.1038/s41598-021-95626-y

10. Hassoon A, Bydon M, Kerezoudis P, Maloney PR, Rinaldo L, Yeh HC. Chronic low-back pain in adult with diabetes: NHANES 2009-2010. *J Diabetes Complications* (2017) 31(1):38–42. doi: 10.1016/j.jdiacomp.2016.10.025

11. Kakadiya G, Gohil K, Gandbhir V, Shakya A, Soni Y. Hyperglycemia and its influence on development of lumbar degenerative disc disease. *N Am Spine Soc J* (2020) 2:100015. doi: 10.1016/j.xnsj.2020.100015

12. Videman T, Battie MC, Gibbons LE, Kaprio J, Koskenvuo M, Kannus P, et al. Disc degeneration and bone density in monozygotic twins discordant for insulin-dependent diabetes mellitus. *J Orthop Res* (2000) 18(5):768–72. doi: 10.1002/jor.1100180514

13. Best MJ, Buller LT, Falakassa J, Vecchione D. Risk factors for nonroutine discharge in patients undergoing spinal fusion for intervertebral disc disorders. *Iowa Orthop J* (2015) 35:147–55.

14. Samartzis D, Karppinen J, Chan D, Luk KD, Cheung KM. The association of lumbar intervertebral disc degeneration on magnetic resonance imaging with body mass index in overweight and obese adults: a population-based study. *Arthritis Rheum* (2012) 64(5):1488–96. doi: 10.1002/art.33462

15. Zhou J, Mi J, Peng Y, Han H, Liu Z. Causal associations of obesity with the intervertebral degeneration, low back pain, and sciatica: A two-sample mendelian randomization study. *Front Endocrinol (Lausanne)* (2021) 12:740200. doi: 10.3389/fendo.2021.740200

16. Ozcan-Eksi EE, Turgut VU, Kucuksuleymanoglu D, Eksi MS. Obesity could be associated with poor paraspinal muscle quality at upper lumbar levels and degenerated

spine at lower lumbar levels: Is this a domino effect? *J Clin Neurosci* (2021) 94:120–7. doi: 10.1016/j.jocn.2021.10.005

17. Zhang Y, Zhao Y, Wang M, Si M, Li J, Hou Y, et al. Serum lipid levels are positively correlated with lumbar disc herniation–a retrospective study of 790 Chinese patients. *Lipids Health Dis* (2016) 15:80. doi: 10.1186/s12944-016-0248-x

18. Heuch I, Heuch I, Hagen K, Zwart JA. Associations between serum lipid levels and chronic low back pain. *Epidemiology* (2010) 21(6):837–41. doi: 10.1097/EDE.0b013e3181f20808

19. Frymoyer JW. Degenerative spondylolisthesis: diagnosis and treatment. *J Am Acad Orthop Surg* (1994) 2(1):9–15. doi: 10.5435/00124635-199401000-00002

20. Mobbs RJ, Newcombe RL, Chandran KN. Lumbar discectomy and the diabetic patient: incidence and outcome. *J Clin Neurosci* (2001) 8(1):10–3. doi: 10.1054/jocn.2000.0682

21. Machino M, Yukawa Y, Ito K, Inoue T, Kobayakawa A, Matsumoto T, et al. Risk factors for poor outcome of cervical laminoplasty for cervical spondylotic myelopathy in patients with diabetes. *J Bone Joint Surg Am* (2014) 96(24):2049–55. doi: 10.2106/JBJS.N.00064

22. Vogt MT, Rubin D, Valentin RS, Palermo L, Donaldson WF 3rd, Nevitt M, et al. Lumbar olisthesis and lower back symptoms in elderly white women. *Study Osteoporotic Fractures. Spine (Phila Pa 1976)* (1998) 23(23):2640–7. doi: 10.1097/00007632-199812010-00020

23. Liu X, Pan F, Ba Z, Wang S, Wu D. The potential effect of type 2 diabetes mellitus on lumbar disc degeneration: a retrospective single-center study. *J Orthop Surg Res* (2018) 13(1):52. doi: 10.1186/s13018-018-0755-8

24. Chen R, Liang X, Huang T, Zhong W, Luo X. Effects of type 1 diabetes mellitus on lumbar disc degeneration: a retrospective study of 118 patients. *J Orthop Surg Res* (2020) 15(1):280. doi: 10.1186/s13018-020-01784-6

25. Jin P, Xing Y, Xiao B, Wei Y, Yan K, Zhao J, et al. Diabetes and intervertebral disc degeneration: A Mendelian randomization study. *Front Endocrinol (Lausanne)* (2023) 14:1100874. doi: 10.3389/fendo.2023.1100874

26. Eksi MS, Kara M, Ozcan-Eksi EE, Aytar MH, Gungor A, Ozgen S, et al. Is diabetes mellitus a risk factor for modic changes?: A novel model to understand the association between intervertebral disc degeneration and end-plate changes. *J Orthop Sci* (2020) 25(4):571–5. doi: 10.1016/j.jos.2019.09.005

27. Luo W, Sun RX, Jiang H, Ma XL. The effect of diabetes on perioperative complications following spinal surgery: a meta-analysis. *Ther Clin Risk Manag* (2018) 14:2415–23. doi: 10.2147/TCRM.S185221

28. Chen S, Liao M, Li J, Peng H, Xiong M. The correlation zbetween microvessel pathological changes of the endplate and degeneration of the intervertebral disc in diabetic rats. *Exp Ther Med* (2013) 5(3):711–7. doi: 10.3892/etm.2012.868

29. Park JS, Park JB, Park IJ, Park EY. Accelerated premature stress-induced senescence of young annulus fibrosus cells of rats by high glucose-induced oxidative stress. *Int Orthop* (2014) 38(6):1311–20. doi: 10.1007/s00264-014-2296-z

30. Kong JG, Park JB, Lee D. Effect of high glucose on stress-induced senescence of nucleus pulposus cells of adult rats. *Asian Spine J* (2015) 9(2):155–61. doi: 10.4184/asj.2015.9.2.155

31. Robinson D, Mirovsky Y, Halperin N, Evron Z, Nevo Z. Changes in proteoglycans of intervertebral disc in diabetic patients. A possible cause of increased back pain. *Spine (Phila Pa 1976)* (1998) 23(8):849–55. doi: 10.1097/00007632-199804150-00001

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Association between serum polyunsaturated fatty acids and bone mineral density in US adults: NHANES 2011-2014

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Objective: The purpose of this study was to investigate the association between serum polyunsaturated fatty acids (PUFAs) and bone mineral density (BMD).

Methods: We performed a cross-sectional study based on data from the National Health and Nutrition Examination Survey (NHANES) 2011-2014. The weighted multiple linear regression model was utilized to determine the association between serum PUFAs and BMD. Further smoothed curve fitting and threshold effect analysis were conducted. Finally, we performed a subgroup analysis.

Results: In total, 1979 participants aged 20-59 years were enrolled. After adjusting for all covariates, we found that serum docosapentaenoic acid (DPA) was positively associated with head BMD (β = 0.0015, 95% CI: 0.0004, 0.0026, P = 0.008296) and lumbar spine BMD (β = 0.0005, 95% CI: 0.0000, 0.0010, P = 0.036093), and serum eicosadienoic acid (EDA) was negatively associated with thoracic spine BMD (β = -0.0008, 95% CI: -0.0016, -0.0000, P = 0.045355). Smoothed curve fitting revealed a nonlinear positive association between serum DPA and lumbar spine BMD. Threshold effect analysis indicated that the threshold of serum DPA was 81.4 μmol/L. Subgroup analysis revealed a positive correlation between serum DPA and head BMD in the subgroup aged 50-59 years (β = 0.0025, 95% CI: 0.0002, 0.0049, P = 0.035249) and females (β = 0.0026, 95% CI: 0.0008, 0.0044, P = 0.005005). There was a positive relationship between serum DPA and lumbar spine BMD in females (β = 0.0008, 95% CI: 0.0001, 0.0015, P = 0.017900) and a negative association between serum EDA and thoracic spine BMD in the subgroup aged 30-39 years (β = -0.0016, 95% CI: -0.0031, -0.0001, P = 0.041331), males (β = -0.0012, 95% CI: -0.0023, -0.0001, P = 0.039364) and other races (β = -0.0021, 95% CI: -0.0037, -0.0006, P = 0.008059).

Conclusion: This study demonstrated a linear positive relationship between serum DPA and head BMD, a nonlinear positive association between serum DPA and lumbar spine BMD, and a linear negative correlation between serum EDA and thoracic spine BMD in US adults.

KEYWORDS

polyunsaturated fatty acids, docosapentaenoic acid (DPA), eicosadienoic acid (EDA), bone mineral density, NHANES

Introduction

As a global public health issue, osteoporosis is defined as a degenerative skeletal disorder that manifests as the disruption of bone microstructure and reduced bone mass, resulting in decreased bone strength and higher fracture risk (1, 2). According to a report by the Surgeon General (US), approximately 10 million (M) Americans over the age of 50 years are affected by osteoporosis (3). In Europe, approximately 22M women and 5.5M men have been diagnosed with osteoporosis (4). Most importantly, osteoporosis-related fragility fractures can lead to poor quality of life, severe economic burden, and significantly elevated mortality, especially hip fractures (5, 6). Therefore, the importance of exploring factors associated with osteoporosis should be emphasized.

Both genetic and nongenetic factors strongly correlate with the development of osteoporosis (7). Diet and nutrients, as nongenetic factors, have attracted more attention due to their impact on osteoporosis (8, 9). Polyunsaturated fatty acids (PUFAs) consist of two subtypes, n-3 and n-6, and are essential fatty acids acquired mainly through fish and vegetable oils. After consumption, PUFAs can be transformed into a sequence of long-chain derivatives in the human body. Alpha-linolenic acid (ALA) can be metabolized into eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), all of which are crucial components of n-3 PUFAs. Linoleic acid (LA) can be converted into arachidonic acid (AA), both of which are fundamental constituents of n-6 PUFAs (10, 11). The role of n-6 PUFAs and their metabolites including prostaglandins, leukotrienes, and thromboxanes has been linked to various physiological processes such as inflammation generation, and platelet activation, while the n-3 PUFAs have been proven to trigger opposing physiological effects (12, 13). Previous studies have shown that PUFAs are associated with various chronic diseases, including cardiovascular events (14), diabetes (15), depressive disorders (16), osteoarthritis (17, 18), and osteoporosis (19, 20).

Bone mineral density (BMD) scores are widely utilized to evaluate bone mass and diagnose osteoporosis. However, the research evidence for the relationship between dietary PUFAs and BMD remains equivocal. One cohort study found a negative association between dietary PUFA intake and femoral neck BMD in premenopausal women (21). A cross-sectional study demonstrated a positive correlation between dietary intake of PUFAs and total BMD among adults aged 20–59 years (22). While a randomized clinical trial found no correlation between the supplementation with n-3 PUFAs and BMD was observed in kidney transplant recipients (23). These contradictory findings warrant additional investigation. In addition to dietary information, biological samples, such as serum, plasma, or red blood cells (RBCs), can be utilized for PUFA assessment. Currently, only limited research has explored the connection between PUFAs derived from biological samples and BMD (24, 25). Therefore, this research aims to investigate the correlation between serum PUFAs and BMD among adults aged 20-59 years using the National Health and Nutrition Examination Survey (NHANES) 2011-2014.

Methods

Study population

NHANES is a health project that aims to investigate the health and nutrition status of the US population. The data of 19931 participants from the 2011-2014 cycle was utilized to evaluate the correlation between serum PUFAs and BMD, and to explore differential action of N-3 and N-6 PUFAs on bone. First, we eliminated subjects with ages less than 20 years (n=8602). Second, participants without complete information on serum PUFAs, head BMD, lumbar spine BMD, thoracic spine BMD, trunk BMD, and total BMD were excluded (n=9267). Third, we excluded participants missing information on sample weights (n=39) and covariates (n=44). Finally, we extracted 1979 participants in the study for final analysis. The inclusion and exclusion details of the study participants were shown in Figure 1. The NHANES study received approval from the NCHS Ethics Review Board and all participants provided written informed consent.

Serum PUFAs measurements

Serum specimens were processed, stored, and shipped to the Division of Laboratory Sciences, National Center for Environmental Health, Centers for Disease Control and Prevention, Atlanta, GA for analysis. Fatty acids are detected using electron capture negative-ion mass spectrometry within 34 minutes. Finally, we selected 11 PUFAs for further analysis, including alpha-Linolenic acid (ALA, 18:3n-3), stearidonic acid (SDA, C18:4n-3), eicosapentaenoic acid (EPA, 20:5n-3), docosapentaenoic acid (DPA, 22:5n-3), docosahexaenoic acid

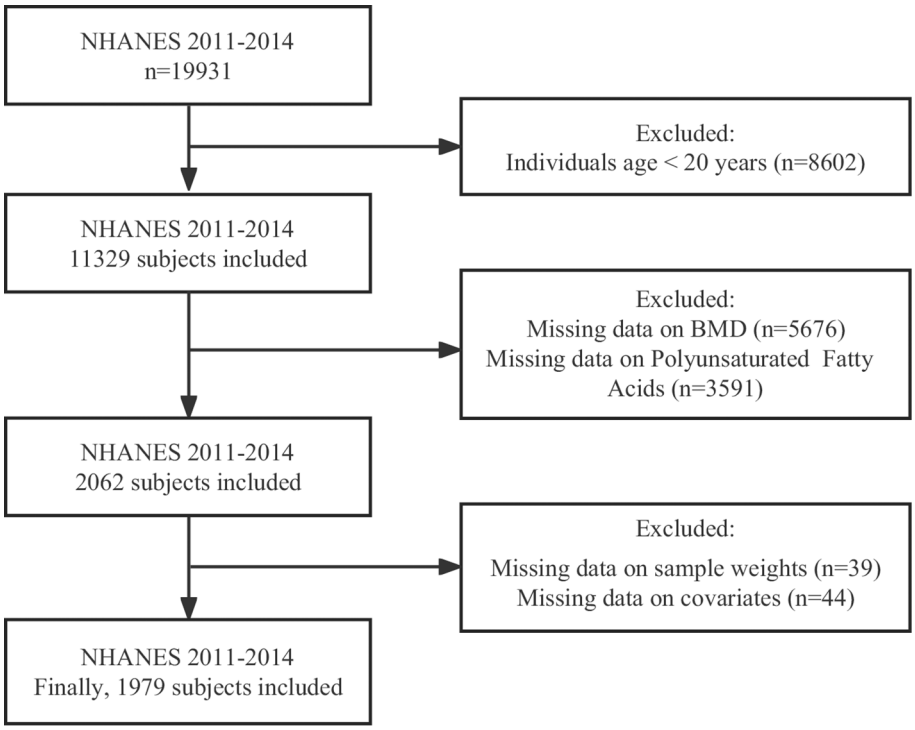

FIGURE 1
The selection flowchart of the participants.

(DHA, 22:6n-3), linoleic acid (LA, 18:2n-6), gamma-linolenic acid (GLA, 18:3n-6), eicosadienoic acid (EDA, 20:2n-6), homo-gamma-linolenic acid (HGLA, 20:3n-6), arachidonic acid (AA, 20:4n-6), docosapentaenoic acid (DPA, 22:5n-6).

BMD measurements

Dual-energy X-ray absorptiometry (DXA) is a widely used technology for evaluating BMD owing to its rapidity, simplicity, and minimal radiation exposure (26). The DXA examinations based on QDR 4500A fan-beam densitometers (Hologic Inc) were administered by trained and certified radiology technologists and the whole body DXA scans provided the BMD data of the head, lumbar spine, thoracic spine, trunk and total body. Trunk BMD was defined as BMD measurements for trunk bone, including thoracic and lumbar spine, left and right ribs, and pelvis.

Covariates

Confounding factors potentially associated with BMD were enrolled in this analysis. The demographic data included age, sex, race, and educational level. Body mass index (BMI) was defined as weight(kg) divided by the square of height(m2). Moderate recreational activities were obtained from the questionnaire (in a typical week do you do any moderate-intensity sports, fitness, or recreational activities that cause a small increase in breathing or heart rate such as brisk walking, bicycling, swimming, or volleyball for at least 10 minutes continuously) and we also included smoking data (smoked at least 100 cigarettes in life). Laboratory data included alkaline phosphatase, serum phosphorus, serum calcium, serum bilirubin, uric acid, total cholesterol, triglyceride, glycohemoglobin, and urine albumin creatinine ratio was collected from the fasting blood samples.

Statistical analysis

The study analysis was performed by using EmpowerStats (http://www.empowerstats.com and R (4.2.3 version) software. Continuous variables were expressed as the mean ± standard deviation and categorical variables were expressed as numbers(n) and percentages (%). The weighted multiple linear regression model was utilized to determine the relationship between serum PUFAs and BMD. No covariates were adjusted in Model 1. Age, gender, and race were adjusted in Model 2. Age, gender, race, educational level, BMI, moderate recreational activities, smoked at least 100 cigarettes in life, alkaline phosphatase, serum phosphorus, serum calcium, serum bilirubin, uric acid, total cholesterol, triglyceride, glycohemoglobin, urine albumin creatinine ratio were adjusted in Model 3. Then, we conducted subgroup analysis by age, gender, and race. $P < 0.05$ was considered statistically significant. Further, we explored the association between serum PUFAs and BMD by using smoothed curve fitting and a weighted generalized additive model (GAM). The threshold effect of serum DPA on lumbar spine BMD was calculated using two-piece linear regression models.

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Results

Baseline characteristics of participants

Of the 1979 study subjects, 1014(52%) were males and 965(48%) were females. Respectively, the average age of males and females was 38.85 ± 11.47 and 39.39 ± 11.69 years old. In terms of educational level, smoked at least 100 cigarettes in life, serum phosphorus, serum calcium, serum bilirubin, uric acid, triglyceride, urine albumin creatinine ratio, GLA, SDA, DPA, DHA, head BMD, thoracic Spine BMD, total BMD, we observed a significantly statistical difference between the two groups. There were no statistical differences in lumbar spine BMD and trunk BMD between the two groups. The weighted, detailed baseline information of the subjects was shown in Table 1.

TABLE 1 Baseline characteristics of participants.

Characteristic	Males, n = 1014 (52%)[1]	Females, n = 965 (48%)[1]	P Value[2]
Age (years)	38.85±11.47	39.39±11.69	0.361
Race/ethnicity, n (%)			0.181
Mexican American	130.00 (9.71%)	123.00 (9.38%)	
Other Hispanic	104.00 (7.30%)	104.00 (7.12%)	
Non-Hispanic White	424.00 (65.57%)	372.00 (62.78%)	
Non-Hispanic Black	164.00 (9.86%)	209.00 (12.28%)	
Other Race	192.00 (7.55%)	157.00 (8.44%)	
Educational level, n (%)			**0.004**
Less than 9th grade	56.00 (3.61%)	37.00 (2.53%)	
9-11th grade	144.00 (12.08%)	121.00 (10.90%)	
High school graduate/GED or equivalent	224.00 (23.02%)	171.00 (15.95%)	
Some college or AA degree	298.00 (30.08%)	335.00 (35.09%)	
College graduate or above	292.00 (31.21%)	301.00 (35.53%)	
Body mass index (kg/m2)	28.48±5.91	28.84±7.31	0.613
Smoked at least 100 cigarettes			**<0.001**
Yes	482.00 (47.40%)	307.00 (34.31%)	
No	532.00 (52.60%)	658.00 (65.69%)	
Moderate recreational activities			0.074
Yes	428.00 (42.76%)	453.00 (48.41%)	
No	586.00 (57.24%)	512.00 (51.59%)	
Alkaline phosphatase (IU/L)	63.38±17.35	62.74±20.67	0.151
Serum phosphorus (mmol/L)	1.17±0.17	1.23±0.17	**<0.001**
Serum calcium (mmol/L)	2.36±0.08	2.33±0.08	**<0.001**
Serum bilirubin (umol/L)	13.67±5.76	11.14±4.54	**<0.001**
Uric acid (umol/L)	364.07±69.69	276.70±63.06	**<0.001**
Total Cholesterol (mmol/L)	4.91±1.03	4.93±1.02	0.901
Triglyceride (mmol/L)	1.55±1.25	1.16±0.74	**<0.001**
Glycohemoglobin (%)	5.51±0.92	5.47±0.85	0.544
Urine albumin creatinine ratio (mg/g)	24.08±194.13	17.93±64.22	**<0.001**
Total n-6 PUFAs (umol/L)	4,791.80±1,210.66	4,677.43±1,033.95	0.257

(Continued)

Associations between serum PUFAS and BMD

We performed a weighted multiple linear regression model to investigate the relationship between serum PUFAs and BMD. After all the covariates were adjusted (model 3), we found that serum DPA was positively associated with head BMD (β = 0.0015, 95% Cl: 0.0004,0.0026, P = 0.008296) and lumbar spine BMD (β = 0.0005, 95% Cl: 0.0000,0.0010, P = 0.036093), serum EDA was negatively associated with thoracic spine BMD (β = -0.0008, 95% Cl: -0.0016,-0.0000, P = 0.045355). No relationship was observed between serum PUFAs and trunk BMD and total BMD, result details are shown in Table 2.

Smoothed curve fitting and threshold effect analysis

Smoothed curve fitting revealed the linear association between serum DPA and head BMD (Figure 2), serum EDA, and thoracic spine BMD (Figure 3). A nonlinear association was found between serum DPA and lumbar BMD (Figure 4). Threshold effect analysis by using a two-piecewise linear regression model indicated the turning point of serum DPA was 81.4umol/L (Table 3). Serum DPA was positively associated with lumbar spine BMD (β = 0.0007, 95% Cl: 0.0001, 0.0012, P = 0.0208) when serum DPA <81.4umol/L. When serum DPA > 81.4umol/L, the relationship was not significant (β = 0.0001, 95% Cl: -0.0008, 0.0010, P = 0.8507).

Subgroup analysis

Furthermore, we conducted a subgroup analysis by age, gender, and race. In terms of serum DPA and head BMD, when stratified by age, a positive association between serum DPA and head BMD was observed in the subgroup aged 50-59 Years (β = 0.0025, 95% Cl: 0.0002,0.0049, P = 0.035249). When stratified by gender, we found that serum DPA was positively associated with head BMD in females (β = 0.0026, 95% Cl: 0.0008,0.0044, P = 0.005005), the

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TABLE 1 Continued

Characteristic	Males, n = 1014 (52%)[1]	Females, n = 965 (48%)[1]	P Value[2]
LA (umol/L)	3,665.25±1,006.08	3,572.93±820.37	0.447
GLA (umol/L)	64.06±36.34	56.00±31.50	**<0.001**
EDA (umol/L)	23.16±9.41	22.41±7.89	0.413
HGLA (umol/L)	164.05±61.32	166.07±62.49	0.627
AA (umol/L)	854.74±241.84	838.85±260.28	0.133
DPA (umol/L)	20.53±8.39	21.17±9.59	0.655
Total n-3 PUFAs (umol/L)	352.25±159.99	348.24±141.49	0.516
ALA (umol/L)	91.99±59.71	83.87±41.69	0.139
SDA (umol/L)	4.14±3.60	3.57±2.77	**0.004**
EPA (umol/L)	61.32±44.31	58.75±45.14	0.262
DPA (umol/L)	54.88±21.66	47.75±18.09	**<0.001**
DHA (umol/L)	139.91±67.64	154.30±71.57	**<0.001**
n-6/n-3 (umol/L)	14.91±3.90	14.54±3.68	0.057
Head BMD (g/cm2)	2.12±0.32	2.29±0.38	**<0.001**
Lumbar Spine BMD (g/cm2)	1.03±0.15	1.03±0.15	0.387
Thoracic Spine BMD (g/cm2)	0.84±0.11	0.79±0.11	**<0.001**
Trunk BMD (g/cm2)	0.92±0.11	0.86±0.09	<0.001
Total BMD (g/cm2)	1.15±0.11	1.08±0.10	**<0.001**

[1]Mean±SD for continuous; n (unweighted) (%) for categorical.
[2]Wilcoxon rank-sum test for complex survey samples; chi-squared test with Rao & Scott's second-order correction.
Bold values represent statistical significance.

result was shown in Table 4. In terms of serum DPA and lumbar spine BMD, we found a positive relationship in females (β = 0.0008, 95% Cl: 0.0001, 0.0015, P = 0.017900) when stratified by gender, the result was shown in Table 5. In terms of serum EDA and thoracic spine BMD, a negative association was found in the subgroup aged 30-39 Years (β = -0.0016, 95% Cl: -0.0031, -0.0001, P = 0.041331), males (β = -0.0012, 95% Cl: -0.0023, -0.0001, P = 0.039364) and other race (β = -0.0021, 95% Cl: -0.0037, -0.0006, P = 0.008059) when stratified by age, gender and race respectively, the result was shown in Table 6.

Discussion

For the first time, we utilized the NHANES database to evaluate the association between serum PUFAs and BMD. This study demonstrated a nonlinear positive association between serum DPA and lumbar spine BMD, a linear positive relationship between serum DPA and head BMD, and a linear negative correlation between serum EDA and thoracic spine BMD in US adults. Therefore, we speculated that serum n-3 PUFAs were beneficial for BMD, while n-6 PUFAs had the opposite effect.

Recent years have seen accumulating evidence suggesting potential associations between PUFAs and various human diseases (14–16), including those related to bone health (19, 20). Numerous clinical studies have delved into the relationship between dietary PUFAs and bone health. For instance, a cross-sectional study based on NHANES database demonstrated a positive correlation between total dietary intake of PUFAs and total BMD among adults aged 20–59 years (22). A positive association was also discerned between the consumption of PUFAs intake and both total BMD and lumbar spine BMD (27). However, it should be noted that this association appeared to be limited to the specific demographic of older women without hormone therapy

TABLE 2 Relationship between serum PUFAs and BMD.

	Head BMD		Lumbar spine BMD		Thoracic spine BMD		Trunk BMD		Total BMD	
	β(95%Cl)	P-value	β(95%Cl)	P-value	β(95%Cl)	P-value	β(95%Cl)	P-value	β(95%Cl)	P-value
LA	0.0000 (-0.0000,0.0000)	0.643407	-0.0000 (-0.0000,0.0000)	0.917583	0.0000 (-0.0000,0.0000)	0.217497	0.0000 (-0.0000,0.0000)	0.532287	0.0000 (-0.0000,0.0000)	0.401307
GLA	-0.0000 (-0.0006,0.0005)	0.916053	0.0000 (-0.0002,0.0003)	0.748661	-0.0000 (-0.0002,0.0001)	0.623080	-0.0000 (-0.0002,0.0002)	0.968531	-0.0000 (-0.0002,0.0002)	0.973614
EDA	-0.0006 (-0.0032,0.0020)	0.661861	-0.0003 (-0.0014,0.0008)	0.625047	**-0.0008(-0.0016,-0.0000)**	**0.045355**	-0.0006 (-0.0013,0.0002)	0.128612	-0.0003 (-0.0010,0.0005)	0.453765
HGLA	0.0002 (-0.0002,0.0005)	0.320140	0.0001 (-0.0001,0.0002)	0.250818	0.0000 (-0.0001,0.0001)	0.951152	0.0000 (-0.0001,0.0001)	0.418553	0.0001 (-0.0000,0.0002)	0.077997
AA	0.0000 (-0.0001,0.0001)	0.709653	0.0000 (-0.0000,0.0001)	0.286111	-0.0000 (-0.0000,0.0000)	0.337952	-0.0000 (-O0.0000,0.0000)	0.614450	-0.0000 (-0.0000,0.0000)	0.296193
DPA	-0.0004 (-0.0024,0.0016)	0.715433	0.0004 (-0.0005,0.0012)	0.405885	-0.0003 (-0.0009,0.0003)	0.371307	-0.0001 (-0.0007,0.0005)	0.783197	-0.0002 (-0.0007,0.0004)	0.510352
Total n-6 PUFAs	0.0000 (-0.0000,0.0000)	0.549169	0.0000 (-0.0000,0.0000)	0.735193	0.0000 (-0.0000,0.0000)	0.430938	0.0000 (-0.0000,0.0000)	0.648891	0.0000 (-0.0000,0.0000)	0.578619
ALA	-0.0002 (-0.0006,0.0003)	0.501371	-0.0001 (-0.0003,0.0001)	0.364921	0.0000 (-0.0001,0.0002)	0.802385	-0.0000 (-0.0002,0.0001)	0.572231	-0.0000 (-0.0001,0.0001)	0.962142
SDA	-0.0036 (-0.0096,0.0025)	0.253160	-0.0022 (-0.0048,0.0004)	0.097405	-0.0010 (-0.0029,0.0008)	0.259009	-0.0010 (-0.0028,0.0008)	0.268148	-0.0003 (-0.0020,0.0014)	0.710131

(Continued)

TABLE 2 Continued

	Head BMD		Lumbar spine BMD		Thoracic spine BMD		Trunk BMD		Total BMD	
	β(95%CI)	P-value	β(95%CI)	P-value	β(95%CI)	P-value	β(95%CI)	P-value	β(95%CI)	P-value
EPA	0.0001 (-0.0003,0.0004)	0.767557	-0.0000 (-0.0002,0.0002)	0.975971	-0.0001 (-0.0002,0.0000)	0.260300	-0.0000 (-0.0001,0.0001)	0.589247	0.0000 (-0.0001,0.0001)	0.778855
DPA	**0.0015 (0.0004,0.0026)**	**0.008296**	**0.0005 (0.0000,0.0010)**	**0.036093**	-0.0001 (-0.0005,0.0002)	0.411804	-0.0000 (-0.0004,0.0003)	0.860290	0.0002 (-0.0001,0.0005)	0.303189
DHA	-0.0001 (-0.0004,0.0001)	0.334231	-0.0000 (-0.0002,0.0001)	0.448581	-0.0000 (-0.0001,0.0000)	0.412422	-0.0001 (-0.0001,0.0000)	0.164544	-0.0001 (-0.0001,0.0000)	0.123513
Total n-3 PUFAs	-0.0000 (-0.0002,0.0001)	0.751520	-0.0000 (-0.0001,0.0000)	0.636003	-0.0000 (-0.0001,0.0000)	0.364455	-0.0000 (-0.0001,0.0000)	0.241699	-0.0000 (-0.0001,0.0000)	0.529926
n-6/n-3	0.0006 (-0.0038,0.0051)	0.783726	0.0003 (-0.0015,0.0022)	0.727518	0.0007 (-0.0006,0.0020)	0.303120	0.0006 (-0.0006,0.0019)	0.327898	0.0004 (-0.0009,0.0016)	0.546783

Age, gender, race, educational level, BMI, moderate recreational activities, smoked at least 100 cigarettes in life, alkaline phosphatase, serum phosphorus, serum calcium, serum bilirubin, uric acid, total cholesterol, triglyceride, glycohemoglobin, urine albumin creatinine ratio were adjusted in the weighted multiple linear regression model.
Bold values represent statistical significance.

which limited the applicability of this conclusion to other populations. required verification. On the contrary, a longitudinal study demonstrated that an increase in dietary intake of PUFAs and monounsaturated fatty acids (MUFAs) was correlated with decreased femoral neck BMD in women aged 45–55 years of age (21). In addition, total dietary PUFAs intake increased the fracture risk in the older age group >65 years, according to Martínez-Ramírez et al. (28). However, a cohort research demonstrated no association between total dietary PUFAs consumption and hip fracture risk (29). These studies presented varied results regarding the effect of total dietary intake of PUFAs on BMD or fracture risk. The inconsistencies could potentially be attributed to specific factors such as age, gender, and the site of BMD measurement in the study population, which highlighted the need for further and comprehensive investigation. In addition, differences in the impact of dietary PUFAs subgroups on bone health should also be given due consideration.

In fact, the mechanisms by which n-3 PUFAs and n-6 PUFAs function within various body tissues, including bones, have been revealed to differ (12, 30). A pivotal factor is the direction of mesenchymal stem cell (MSC) differentiation, which steers either osteogenesis or adipogenesis. The peroxisome proliferator-activated receptor γ (PPARγ) has a crucial role in driving the differentiation of MSC into adipocytes, subsequently inhibiting osteogenesis (31). Existing studies have shown that n-6 PUFAs impede osteogenesis through the upregulation of PPARγ expression and downregulation of Runx2 expression (32), whereas n-3 PUFAs manifest converse effects (33). N-6 PUFAs are also known to trigger RANKL–RANK signaling, leading to osteoclastogenesis and bone loss (32, 34), a process which is suppressed by n-3 PUFAs (35, 36). Furthermore, n-6 PUFAs are found to elevate pro-inflammatory cytokine levels, promoting bone resorption (37, 38). The outcomes of these experimental findings propose that n-3 PUFAs exert beneficial

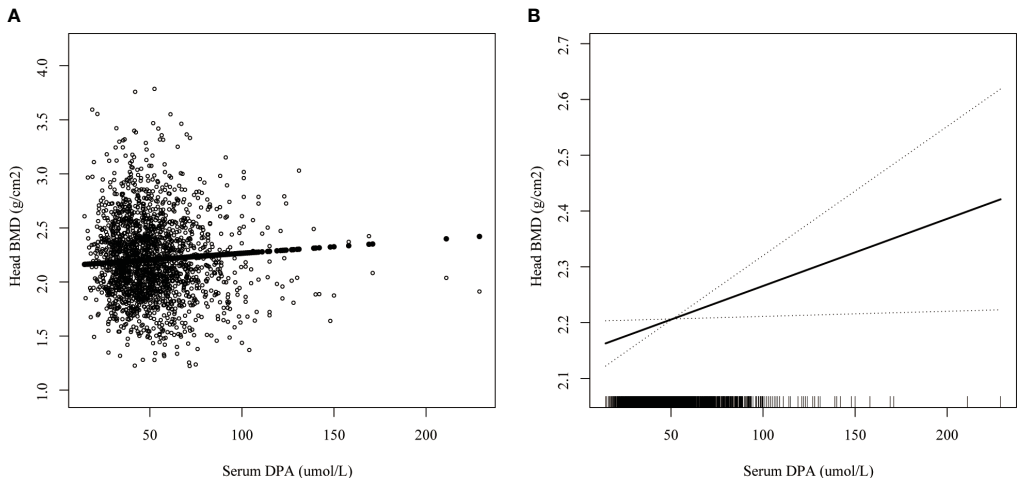

FIGURE 2
The association between serum DPA and head BMD. **(A)** each black point represents a sample. **(B)** the solid black line represents the smooth curve fit between variables. Dotted line bands represent the 95% confidence interval of the fit. Age, gender, race, educational level, BMI, moderate recreational activities, smoked at least 100 cigarettes in life, alkaline phosphatase, serum phosphorus, serum calcium, serum bilirubin, uric acid, total cholesterol, triglyceride, glycohemoglobin, urine albumin creatinine ratio were adjusted.

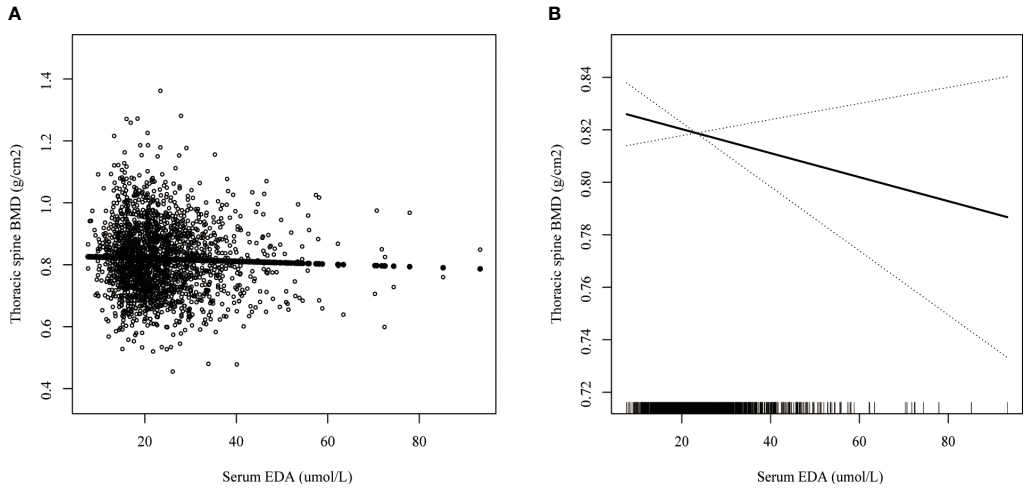

FIGURE 3
The association between serum EDA and thoracic spine BMD. **(A)** each black point represents a sample. **(B)** the solid black line represents the smooth curve fit between variables. Dotted line bands represent the 95% confidence interval of the fit. Age, gender, race, educational level, BMI, moderate recreational activities, smoked at least 100 cigarettes in life, alkaline phosphatase, serum phosphorus, serum calcium, serum bilirubin, uric acid, total cholesterol, triglyceride, glycohemoglobin, urine albumin creatinine ratio were adjusted.

influences on bone health, standing in contrast to the detrimental effects of n-6 PUFAs. Therefore, further assessment of the effects of different PUFA subclasses on human bone health is required.

A cross-sectional study utilizing the NHANES database showed that dietary supplementation with n-3 PUFAs (EPA, DHA, and SDA) was positively associated with lumbar spine BMD among adults >60 years of age (39). Correspondingly, another cross-sectional study reported a beneficial impact of dietary n-3 PUFAs consumption on lumbar spine BMD in postmenopausal women (40). Beyond its impact on lumbar spine BMD, dietary intake of n-3 PUFAs was also found to enhance hip BMD in the female population aged 19-25 years (41). In addition, dietary n-3 PUFAs intake reduced the levels of biological markers of bone resorption, suggesting that n-3 PUFAs reduced bone loss potentially by inhibiting osteoclast activity (42). While the majority of studies, encompassing the aforementioned ones, endorsed the beneficial effects of dietary n-3 PUFAs on bone health, aligning with prior experimental results, there still existed certain studies that reached inconsistent conclusions. Within the context of a randomized clinical trial, no correlation between n-3 PUFAs supplementation and BMD was observed amongst kidney transplant recipients (23). Furthermore, a comprehensive meta-analysis also suggested that supplementation with dietary n-3 PUFAs had no positive impact on BMD (43), a conclusion that deviated from the results of

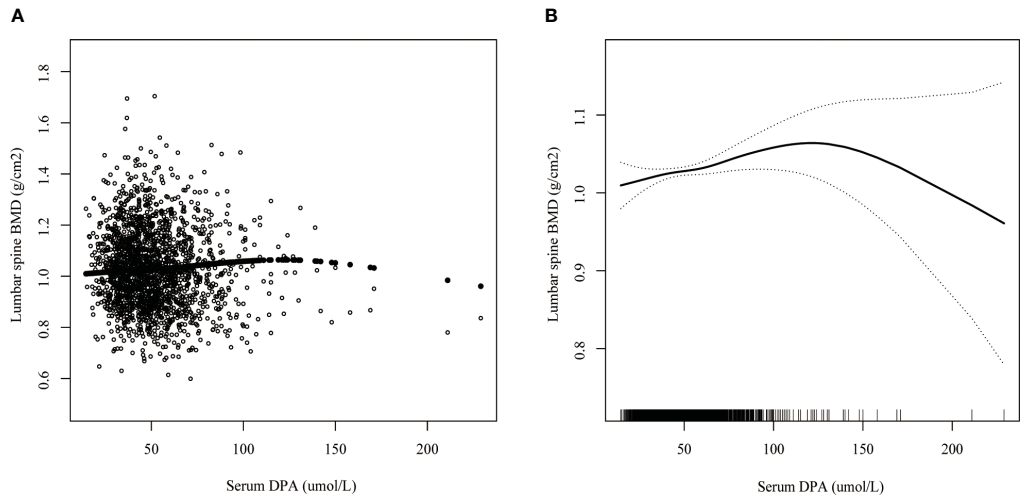

FIGURE 4
The association between serum DPA and lumbar spine BMD. **(A)** each black point represents a sample. **(B)** the solid black line represents the smooth curve fit between variables. Dotted line bands represent the 95% confidence interval of the fit. Age, gender, race, educational level, BMI, moderate recreational activities, smoked at least 100 cigarettes in life, alkaline phosphatase, serum phosphorus, serum calcium, serum bilirubin, uric acid, total cholesterol, triglyceride, glycohemoglobin, urine albumin creatinine ratio were adjusted.

TABLE 3 Threshold effect analysis of serum DPA (umol/L) and lumbar spine BMD(g/cm2) using two-piecewise linear regression model.

Lumbar spine BMD(g/cm2)	
Fitting by linear regression model	**0.0005 (0.0000, 0.0010) 0.0361**
Fitting by two-piecewise linear regression model	
Turn point of serum DPA (umol/L)	81.4
<81.4, effect 1	**0.0007 (0.0001, 0.0012) 0.0208**
>81.4,, effect 2	0.0001 (-0.0008, 0.0010) 0.8507
Log likelihood ratio test	0.305

Age, gender, race, educational level, BMI, moderate recreational activities, smoked at least 100 cigarettes in life, alkaline phosphatase, serum phosphorus, serum calcium, serum bilirubin, uric acid, total cholesterol, triglyceride, glycohemoglobin, urine albumin creatinine ratio were adjusted.
Bold values represent statistical significance.

experimental findings. We proposed that two principal factors contributed to this issue. First, the majority of studies have concentrated on establishing an association between dietary PUFAs consumption and BMD or fracture risk. Nonetheless, data on dietary PUFAs was predominantly obtained from food-frequency questionnaires, which may result in an inaccurate assessment of PUFAs intake. Second, dietary intake of PUFAs did not fully align with bioavailable PUFAs, a factor that could be influenced by the digestion and absorption process. Our hypothesis has garnered support from various studies. For instance, one study highlighted that dietary PUFAs intake was not correlated with serum levels of PUFAs (44). Another study demonstrated that the dietary intake of ALA did not impact its plasma levels, with the connection between dietary consumption and plasma concentration only proving significant for LA, AA, EPA, and DHA (45). Therefore, we advocate the use of biological specimens such as serum, plasma, or red blood cells (RBCs) for a more accurate assessment of PUFAs as opposed to relying solely on dietary information.

However, only a limited number of studies have investigated the association between PUFA levels in biological samples and BMD. A cross-sectional study involving 301 Spanish postmenopausal women demonstrated a positive correlation between the plasma concentration of n-3 PUFAs (inclusive of ALA, EPA, and DHA) and BMD in the spine and neck of the femur (24). A significantly positive correlation between the serum concentration of n-3 PUFAs and femur BMD was observed exclusively within the group having a low n-6:n-3 ratio in another study (25), suggesting that high serum concentrations of n-6 PUFAs potentially impeded the bone health-promoting effects of n-3 PUFAs. This study also showed that the serum concentration of ALA, a class of n-3 PUFAs, was negatively correlated with creatinine-corrected urinary deoxypyridinoline, suggesting that n-3 PUFAs may promote BMD by inhibiting bone resorption. Moreover, PUFA levels from the biological samples were also associated with fracture risk. In a cohort study

TABLE 4 Subgroup analysis of the relationship between serum DPA and head BMD.

	Model 1		Model 2		Model 3	
	β(95%CI)	P-value	β(95%CI)	P-value	β(95%CI)	P-value
Serum DPA	-0.0005(-0.0013,0.0003)	0.210125	0.0002(-0.0006,0.0010)	0.649524	**0.0015(0.0004,0.0026**	**0.008296**
Stratified by age						
20-29 Years	-0.0016(-0.0034,0.0002)	0.087833	-0.0001(-0.0019,0.0018)	0.955135	0.0000(-0.0025,0.0025)	0.977817
30-39 Years	-0.0014(-0.0030.0.0002)	0.092035	0.0002(-0.0013.0.0018)	0.773166	0.0007(-0.0017,0.0031)	0.577333
40-49 Years	-0.0013(-0.0028,0.0002)	0.094098	0.0001(-0.0013,0.0016)	0.848408	0.0013(-0.0009,0.0035)	0.249357
50-59 Years	-0.0004(-0.0021,0.0012)	0.600252	-0.0001(-0.0017,0.0016)	0.948671	**0.0025(0.0002,0.0049)**	**0.035249**
Stratified by gender						
Male	0.0002(-0.0007,0.0011)	0.662386	0.0001(-0.0008,0.0011)	0.818739	0.0008(-0.0006,0.0023)	0.243991
Female	0.0002(-0.0011,0.0015)	0.754838	0.0002(-0.0012,0.0016)	0.784873	**0.0026(0.0008,0.0044)**	**0.005005**
Stratified by race						
Mexican American	-0.0004(-0.0024,0.0016)	0.703844	0.0010(-0.0012,0.0031)	0.366396	0.0016(-0.0014,0.0046)	0.285512
Other Hispanic	-0.0002(-0.0027,0.0024)	0.902879	0.0007(-0.0019,0.0034)	0.586048	0.0037(-0.0002,0.0075)	0.061983
Non-Hispanic white	0.0001(-0.0011,0.0012)	0.921793	0.0003(-0.0009,0.0015)	0.624622	0.0014(-0.0004,0.0032)	0.128920
Non-Hispanic black	-0.0023(-0.0050,0.0004)	0.093270	-0.0017(-0.0044,0.0010)	0.217913	0.0017(-0.0020,0.0054)	0.362165
Other race	-0.0005(-0.0020,0.0011)	0.557601	-0.0009(-0.0024,0.0007)	0.269804	0.0014(-0.0007,0.0036)	0.190573

Model 1: no covariates were adjusted.
Model 2: age, gender, and race were adjusted.
Model 3: age, gender, race, educational level, BMI, moderate recreational activities, smoked at least 100 cigarettes in life, alkaline phosphatase, serum phosphorus, serum calcium, serum bilirubin, uric acid, total cholesterol, triglyceride, glycohemoglobin, urine albumin creatinine ratio were adjusted.
Bold values represent statistical significance.

TABLE 5 Subgroup analysis of the relationship between serum DPA and lumbar spine BMD.

	Model 1		Model 2		Model 3	
	β(95%CI)	P-value	β(95%CI)	P-value	β(95%CI)	P-value
Serum DPA	-0.0004 (-0.0008, -0.0001)	0.007858	-0.0002 (-0.0005, 0.0002)	0.379742	**0.0005 (0.0000, 0.0010)**	**0.036093**
Stratified by age						
20-29 Years	0.0001 (-0.0007, 0.0010)	0.759573	0.0003 (-0.0006, 0.0012)	0.490057	-0.0001 (-0.0013, 0.0010)	0.834775
30-39 Years	-0.0003 (-0.0009, 0.0004)	0.441149	-0.0001 (-0.0007, 0.0006)	0.866197	0.0006 (-0.0004, 0.0017)	0.222370
40-49 Years	-0.0009 (-0.0015, -0.0002)	0.008880	-0.0005 (-0.0012, 0.0001)	0.125960	0.0003 (-0.0007, 0.0012)	0.580044
50-59 Years	-0.0000 (-0.0006, 0.0006)	0.922797	-0.0001 (-0.0007, 0.0005)	0.796055	0.0007 (-0.0002, 0.0016)	0.119122
Stratified by gender						
Male	-0.0005 (-0.0009, -0.0000)	0.036530	-0.0003 (-0.0007, 0.0001)	0.197322	0.0003 (-0.0003, 0.0010)	0.324876
Female	-0.0004 (-0.0009, 0.0001)	0.160154	0.0001 (-0.0004, 0.0007)	0.663739	**0.0008 (0.0001, 0.0015)**	**0.017900**
Stratified by race						
Mexican American	-0.0003 (-0.0011, 0.0005)	0.437910	-0.0001 (-0.0009, 0.0007)	0.853077	0.0001 (-0.0011, 0.0012)	0.882147
Other Hispanic	0.0003 (-0.0007, 0.0014)	0.527626	0.0010 (-0.0002, 0.0021)	0.094548	0.0010 (-0.0006, 0.0027)	0.212801
Non-Hispanic white	-0.0003 (-0.0008, 0.0002)	0.203654	-0.0002 (-0.0007, 0.0004)	0.549060	0.0005 (-0.0003, 0.0013)	0.188309
Non-Hispanic black	-0.0011 (-0.0022, 0.0000)	0.058988	-0.0009 (-0.0021, 0.0003)	0.144914	0.0006 (-0.0010, 0.0022)	0.493524
Other race	-0.0006 (-0.0012, 0.0001)	0.090649	-0.0004 (-0.0011, 0.0003)	0.255681	0.0008 (-0.0001, 0.0017)	0.084806

Bold values represent statistical significance.

including 1438 participants, there was a decrease in fracture risk with increased plasma levels of n-3 PUFAs and EPA, while n-6 PUFAs and AA manifested the contrary effects (46). A nested case –control study found that elevated levels of total n-3 PUFAs, ALA, and EPA derived from RBCs, along with a high n-6:n-3 ratio, were associated with a decreased risk of hip fracture (47). These studies indicated that high level of n-3 PUFAs from biological samples were beneficial for promoting BMD or mitigated fracture risk, whereas n-

TABLE 6 Subgroup analysis of the relationship between serum EDA and thoracic spine BMD.

	Model 1		Model 2		Model 3	
	β(95%CI)	P-value	β(95%CI)	P-value	β(95%CI)	P-value
Serum EDA	-0.0005 (-0.0010, 0.0001)	0.110023	-0.0005 (-0.0010, 0.0001)	0.082590	**-0.0008 (-0.0016, -0.0000)**	**0.045355**
Stratified by age						
20-29 Years	0.0005 (-0.0005, 0.0016)	0.326502	0.0010 (-0.0000, 0.0021)	0.055493	0.0004 (-0.0011, 0.0018)	0.619783
30-39 Years	-0.0004 (-0.0014, 0.0007)	0.516746	-0.0003 (-0.0014, 0.0007)	0.546388	**-0.0016 (-0.0031, -0.0001)**	**0.041331**
40-49 Years	-0.0003 (-0.0013, 0.0008)	0.582345	-0.0003 (-0.0013, 0.0007)	0.580130	-0.0003 (-0.0018, 0.0011)	0.663472
50-59 Years	-0.0020 (-0.0033, -0.0007)	0.003020	-0.0023 (-0.0035, -0.0010)	0.000356	-0.0019 (-0.0039, 0.0001)	0.058075
Stratified by gender						
Male	-0.0003 (-0.0010, 0.0004)	0.453229	-0.0004 (-0.0011, 0.0003)	0.263614	**-0.0012 (-0.0023, -0.0001)**	**0.039364**
Female	-0.0011 (-0.0019, -0.0002)	0.016808	-0.0008 (-0.0016, 0.0001)	0.078244	-0.0007 (-0.0018, 0.0004)	0.215719
Stratified by race						
Mexican American	0.0010 (-0.0001, 0.0020)	0.077203	0.0007 (-0.0004, 0.0017)	0.206954	-0.0002 (-0.0019, 0.0015)	0.782629
Other Hispanic	-0.0001 (-0.0016, 0.0014)	0.884382	-0.0003 (-0.0018, 0.0013)	0.730043	-0.0015 (-0.0038, 0.0007)	0.187988
Non-Hispanic white	-0.0002 (-0.0011, 0.0007)	0.674408	-0.0004 (-0.0013, 0.0005)	0.418354	-0.0007 (-0.0020, 0.0006)	0.292860
Non-Hispanic black	-0.0031 (-0.0052, -0.0010)	0.003744	-0.0030 (-0.0051, -0.0009)	0.005217	-0.0002 (-0.0030, 0.0026)	0.882039
Other race	-0.0015 (-0.0027, -0.0003)	0.012820	-0.0022 (-0.0034, -0.0010)	0.000402	**-0.0021 (-0.0037, -0.0006)**	**0.008059**

Bold values represent statistical significance.

6 PUFAs exerted an opposing effect. Although the conclusions drawn from these studies aligned well with the outcomes of prior experimental results, certain limitations, such as the sample size, the diversity in the types of PUFAs assessed, and the limited number of sites for BMD measurement, needed to be duly acknowledged.

It was worth noting that BMD in various regions had varying clinical significance. Low BMD in the spine might be linked to fragility fractures. Moreover, low head BMD could be connected to hearing impairments (48) and malocclusion in adolescents (49). Therefore, to improve the comprehensiveness and reliability of the study, we included 11 serum PUFAs and 5 sites of BMD to investigate their associations using the NHANES large sample data (n=1979). First, our study revealed a nonlinear positive association between serum DPA and lumbar spine BMD, a relationship that remained consistent solely within the female subgroup but was absent in the male subgroup. Moreover, threshold effect analysis indicated that when serum DPA > 81.4umol/L, the positive relationship was no more significant. Regarding the threshold and nonlinear relationship, previous research proposed that excess dietary intake of n-3 PUFAs beyond the threshold level conferred no additional benefits to bone health (23). In a related animal study, increased consumption of DHA, a type of n-3 PUFA, was shown to benefit BMD, bone mineral content, and peak bone mass. However, no additional benefits were not observed in the group with higher intake of DHA (50). Therefore, we postulated that a threshold might exist for the promotion of lumbar spine BMD by some of the serum N-3 PUFAs, such as DPA. However, this hypothesis warranted further validation through additional clinical studies. Second, our investigation discerned a linear and positive relationship between serum DPA and head BMD, a trend that was consistent within the female subgroup, though not apparent in the male counterpart. A meta-analysis demonstrated that n-3 PUFA supplementation had a better favorable impact on BMD in females (43). An animal study reported that female offspring of mice supplemented with n-3 PUFA had better bone health than male offspring (51). These studies suggested that there was a potential sex differences in the promotion of BMD by n-3 PUFAs, which explained why the positive association of DPA with head BMD and lumbar spine BMD was only significant in the female subgroup in this study. In addition, the positive trend between serum DPA and head BMD was significant in the subgroup aged 50-59 years. BMD decreased with age, potentially highlighting the importance of DPA in promoting BMD among participants aged 50-59 years. Regarding the subtle differences in the effects of serum DPA on head BMD and lumbar spine BMD, we speculated that they are related to the different morphology and activity of osteoclasts, osteogenic capacity of bone marrow stromal cells at the different sites (52–54).Third, our research demonstrated that serum EDA levels had a linear and negative correlation with thoracic spine BMD, which remained consistent within the subgroup aged 30-39 years, males and other race. However, we needed further evidence from large clinical studies to support this subgroup association.

This study had some advantages. First, the research data were extracted from the NHANES database, which ensured the accuracy and representativeness of the data. Second, covariates potentially associated with BMD were adjusted to improve the reliability of this study. However, limitations cannot be ignored. First, it is important to note that this study was a cross-sectional study, which precluded drawing causal inferences regarding serum PUFAs and BMD. Second, this study focused on participants 20-59 years of age, and the conclusions cannot be directly extrapolated to older populations or adolescents. Third, the levels of PUFAs from biological samples were more difficult to obtain compared to food frequency questionnaires and had higher economic costs. Fourth, the relationship between serum PUFAs and BMD was weak. Finally, there is a lack of experimental validation in this study.

In the future, we will conduct further studies focusing on the following aspects. First, we will design clinical randomized controlled trials to investigate the causal association between serum PUFAs and BMD. Second, we aim to expand the study to include elderly individuals who are at high risk for osteoporosis and adolescents. Third, we will investigate the relationship between dietary PUFA intake and serum PUFA levels and ascertain the factors that have an impact on this relationship. Fourth, we will experimentally validate the effects of PUFAs on osteogenesis markers, adipogenesis markers, and inflammatory markers (TNF-α, IL-1β, IL-6, and COX-2).

Conclusions

In conclusion, this study demonstrated a linear positive relationship between serum DPA and head BMD, a nonlinear positive association between serum DPA and lumbar spine BMD, and a linear negative correlation between serum EDA and thoracic spine BMD in US adults aged 20-59 years.

Data availability statement

Publicly available datasets were analyzed in this study. This data can be found here: https://www.cdc.gov/nchs/nhanes/index.htm.

Ethics statement

The studies involving humans were approved by NCHS Research Ethics Review Board (ERB). The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

Author contributions

HL: Formal Analysis, Methodology, Writing – original draft, Writing – review & editing. CX: Formal Analysis, Methodology, Writing – original draft, Writing – review & editing. YL: Methodology, Software, Writing – review & editing. JZ: Methodology, Software, Supervision, Writing – review & editing. YH: Methodology, Software, Writing – review & editing. RZ: Methodology, Software, Writing – review & editing. NZ: Supervision, Writing – review & editing. ZZ: Supervision, Writing – review & editing. XL: Funding acquisition, Supervision, Validation, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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References

1. Compston JE, McClung MR, Leslie WD. Osteoporosis. *Lancet* (2019) 393 (10169):364–76. doi: 10.1016/S0140-6736(18)32112-3

2. Consensus development conference: diagnosis, prophylaxis, and treatment of osteoporosis. *Am J Med* (1993) 94(6):646–50. doi: 10.1016/0002-9343(93)90218-E

3. Office of the Surgeon General (US). *Bone health and osteoporosis: A report of the surgeon general*. Rockville (MD): office of the surgeon general (US) (2004). Available at: http://www.ncbi.nlm.nih.gov/books/NBK45513/.

4. Hernlund E, Svedbom A, Ivergård M, Compston J, Cooper C, Stenmark J, et al. Osteoporosis in the European Union: medical management, epidemiology and economic burden. A report prepared in collaboration with the International Osteoporosis Foundation (IOF) and the European Federation of Pharmaceutical Industry Associations (EFPIA). *Arch Osteoporos* (2013) 8(1):136. doi: 10.1007/s11657-013-0136-1

5. Zheng XQ, Xu L, Huang J, Zhang CG, Yuan WQ, Sun CG, et al. Incidence and cost of vertebral fracture in urban China: a 5-year population-based cohort study. *Int J Surg* (2023) 109(7):1910–8. doi: 10.1097/JS9.0000000000000411

6. Clynes MA, Harvey NC, Curtis EM, Fuggle NR, Dennison EM, Cooper C. The epidemiology of osteoporosis. *Br Med Bull* (2020) 133(1):105–17. doi: 10.1093/bmb/ldaa005

7. Hendrickx G, Boudin E, Van Hul W. A look behind the scenes: the risk and pathogenesis of primary osteoporosis. *Nat Rev Rheumatol* (2015) 11(8):462–74. doi: 10.1038/nrrheum.2015.48

8. Guo D, Zhao M, Xu W, He H, Li B, Hou T. Dietary interventions for better management of osteoporosis: An overview. *Crit Rev Food Sci Nutr* (2023) 63(1):125–44. doi: 10.1080/10408398.2021.1944975

9. Muñoz-Garach A, García-Fontana B, Muñoz-Torres M. Nutrients and dietary patterns related to osteoporosis. *Nutrients* (2020) 12(7):1986. doi: 10.3390/nu12071986

10. Djuricic I, Calder PC. Beneficial outcomes of omega-6 and omega-3 polyunsaturated fatty acids on human health: an update for 2021. *Nutrients* (2021) 13(7):2421. doi: 10.3390/nu13072421

11. Kapoor B, Kapoor D, Gautam S, Singh R, Bhardwaj S. Dietary polyunsaturated fatty acids (PUFAs): uses and potential health benefits. *Curr Nutr Rep* (2021) 10 (3):232–42. doi: 10.1007/s13668-021-00363-3

12. Schmitz G, Ecker J. The opposing effects of n-3 and n-6 fatty acids. *Prog Lipid Res* (2008) 47(2):147–55. doi: 10.1016/j.plipres.2007.12.004

13. Harwood JL. Polyunsaturated fatty acids: conversion to lipid mediators, roles in inflammatory diseases and dietary sources. *Int J Mol Sci* (2023) 24(10):8838. doi: 10.3390/ijms24108838

14. Marklund M, Wu JHY, Imamura F, Del Gobbo LC, Fretts A, de Goede J, et al. Biomarkers of dietary omega-6 fatty acids and incident cardiovascular disease and mortality. *Circulation* (2019) 139(21):2422–36. doi: 10.1161/CIRCULATIONAHA.118.038908

15. Forouhi NG, Imamura F, Sharp SJ, Koulman A, Schulze MB, Zheng J, et al. Association of Plasma Phospholipid n-3 and n-6 Polyunsaturated Fatty Acids with Type 2 Diabetes: The EPIC-InterAct Case-Cohort Study. *PloS Med* (2016) 13(7): e1002094. doi: 10.1371/journal.pmed.1002094

16. Chang JPC, Chang SS, Yang HT, Chen HT, Chien YC, Yang B, et al. Omega-3 polyunsaturated fatty acids in cardiovascular diseases comorbid major depressive disorder - Results from a randomized controlled trial. *Brain Behav Immun* (2020) 85:14–20. doi: 10.1016/j.bbi.2019.03.012

17. Loef M, Ioan-Facsinay A, Mook-Kanamori DO, Willems van Dijk K, de Mutsert R, Kloppenburg M, et al. The association of plasma fatty acids with hand and knee osteoarthritis: the NEO study. *Osteoarthritis Cartilage* (2020) 28(2):223–30. doi: 10.1016/j.joca.2019.10.002

18. Stonehouse W, Benassi-Evans B, Bednarz J, Vincent AD, Hall S, Hill CL. Krill oil improved osteoarthritic knee pain in adults with mild to moderate knee osteoarthritis: a 6-month multicenter, randomized, double-blind, placebo-controlled trial. *Am J Clin Nutr* (2022) 116(3):672–85. doi: 10.1093/ajcn/nqac125

19. Longo AB, Ward WE. PUFAs, bone mineral density, and fragility fracture: findings from human studies. *Adv Nutr* (2016) 7(2):299–312. doi: 10.3945/an.115.009472

20. El-Sayed E, Ibrahim K. Effect of the types of dietary fats and non-dietary oils on bone metabolism. *Crit Rev Food Sci Nutr* (2017) 57(4):653–8. doi: 10.1080/10408398.2014.914889

21. Macdonald HM, New SA, Golden MHN, Campbell MK, Reid DM. Nutritional associations with bone loss during the menopausal transition: evidence of a beneficial effect of calcium, alcohol, and fruit and vegetable nutrients and of a detrimental effect of fatty acids. *Am J Clin Nutr* (2004) 79(1):155–65. doi: 10.1093/ajcn/79.1.155

22. Fang ZB, Wang GX, Cai GZ, Zhang PX, Liu DL, Chu SF, et al. Association between fatty acids intake and bone mineral density in adults aged 20-59: NHANES 2011-2018. *Front Nutr* (2023) 10:1033195. doi: 10.3389/fnut.2023.1033195

23. Jørgensen HS, Eide IA, Jenssen T, Åsberg A, Bollerslev J, Godang K, et al. Marine n-3 polyunsaturated fatty acids and bone mineral density in kidney transplant recipients: A randomized, placebo-controlled trial. *Nutrients* (2021) 13(7):2361. doi: 10.3390/nu13072361

24. Roncero-Martín R, Aliaga I, Moran JM, Puerto-Parejo LM, Rey-Sánchez P, de la Luz Canal-Macías M, et al. Plasma fatty acids and quantitative ultrasound, DXA and pQCT derived parameters in postmenopausal spanish women. *Nutrients* (2021) 13 (5):1454. doi: 10.3390/nu13051454

25. Feehan O, Magee PJ, Pourshahidi LK, Armstrong DJ, Slevin MM, Allsopp PJ, et al. Associations of long chain polyunsaturated fatty acids with bone mineral density and bone turnover in postmenopausal women. *Eur J Nutr* (2023) 62(1):95–104. doi: 10.1007/s00394-022-02933-9

26. Baran DT, Faulkner KG, Genant HK, Miller PD, Pacifici R. Diagnosis and management of osteoporosis: guidelines for the utilization of bone densitometry. *Calcif Tissue Int* (1997) 61(6):433–40. doi: 10.1007/s002239900362

27. Järvinen R, Tuppurainen M, Erkkilä AT, Penttinen P, Kärkkäinen M, Salovaara K, et al. Associations of dietary polyunsaturated fatty acids with bone mineral density in elderly women. *Eur J Clin Nutr* (2012) 66(4):496–503. doi: 10.1038/ejcn.2011.188

28. Martínez-Ramírez MJ, Palma S, Martínez-González MA, Delgado-Martínez AD, de la Fuente C, Delgado-Rodríguez M. Dietary fat intake and the risk of osteoporotic fractures in the elderly. *Eur J Clin Nutr* (2007) 61(9):1114–20. doi: 10.1038/sj.ejcn.1602624

29. Virtanen JK, Mozaffarian D, Willett WC, Feskanich D. Dietary intake of polyunsaturated fatty acids and risk of hip fracture in men and women. *Osteoporos Int* (2012) 23(11):2615–24. doi: 10.1007/s00198-012-1903-3

30. Saini RK, Keum YS. Omega-3 and omega-6 polyunsaturated fatty acids: Dietary sources, metabolism, and significance - A review. *Life Sci* (2018) 203:255–67. doi: 10.1016/j.lfs.2018.04.049

31. Takada I, Kouzmenko AP, Kato S. Wnt and PPARgamma signaling in osteoblastogenesis and adipogenesis. *Nat Rev Rheumatol* (2009) 5(8):442–7. doi: 10.1038/nrrheum.2009.137

32. Casado-Díaz A, Santiago-Mora R, Dorado G, Quesada-Gómez JM. The omega-6 arachidonic fatty acid, but not the omega-3 fatty acids, inhibits osteoblastogenesis and induces adipogenesis of human mesenchymal stem cells: potential implication in osteoporosis. *Osteoporos Int* (2013) 24(5):1647–61. doi: 10.1007/s00198-012-2138-z

33. Nakanishi A, Tsukamoto I. n-3 polyunsaturated fatty acids stimulate osteoclastogenesis through PPARγ-mediated enhancement of c-Fos expression, and suppress osteoclastogenesis through PPARγ-dependent inhibition of NFkB activation. *J Nutr Biochem* (2015) 26(11):1317–27. doi: 10.1016/j.jnutbio.2015.06.007

34. Wada T, Nakashima T, Hiroshi N, Penninger JM. RANKL-RANK signaling in osteoclastogenesis and bone disease. *Trends Mol Med* (2006) 12(1):17–25. doi: 10.1016/j.molmed.2005.11.007

35. Wang Z, Wu J, Li L, Wang K, Wu X, Chen H, et al. Eicosapentaenoic acid supplementation modulates the osteoblast/osteoclast balance in inflammatory environments and protects against estrogen deficiency-induced bone loss in mice. *Clin Nutr* (2023) 42(9):1715–27. doi: 10.1016/j.clnu.2023.07.022

36. Zhan Q, Tian Y, Han L, Wang K, Wang J, Xue C. The opposite effects of Antarctic krill oil and arachidonic acid-rich oil on bone resorption in ovariectomized mice. *Food Funct* (2020) 11(8):7048–60. doi: 10.1039/D0FO00884B

37. Abshirini M, Ilesanmi-Oyelere BL, Kruger MC. Potential modulatory mechanisms of action by long-chain polyunsaturated fatty acids on bone cell and chondrocyte metabolism. *Prog Lipid Res* (2021) 83:101113. doi: 10.1016/j.plipres.2021.101113

38. Rozner R, Vernikov J, Griess-Fishheimer S, Travinsky T, Penn S, Schwartz B, et al. The role of omega-3 polyunsaturated fatty acids from different sources in bone development. *Nutrients* (2020) 12(11):3494. doi: 10.3390/nu12113494

39. Mangano KM, Kerstetter JE, Kenny AM, Insogna KL, Walsh SJ. An investigation of the association between omega 3 FA and bone mineral density among older adults: results from the National Health and Nutrition Examination Survey years 2005–2008. *Osteoporos Int* (2014) 25(3):1033–41. doi: 10.1007/s00198-013-2501-8

40. Nawata K, Yamauchi M, Takaoka S, Yamaguchi T, Sugimoto T. Association of n-3 polyunsaturated fatty acid intake with bone mineral density in postmenopausal women. *Calcif Tissue Int* (2013) 93(2):147–54. doi: 10.1007/s00223-013-9743-5

41. Kuroda T, Ohta H, Onoe Y, Tsugawa N, Shiraki M. Intake of omega-3 fatty acids contributes to bone mineral density at the hip in a younger Japanese female population. *Osteoporos Int* (2017) 28(10):2887–91. doi: 10.1007/s00198-017-4128-7

42. Griel AE, Kris-Etherton PM, Hilpert KF, Zhao G, West SG, Corwin RL. An increase in dietary n-3 fatty acids decreases a marker of bone resorption in humans. *Nutr J* (2007) 6:2. doi: 10.1186/1475-2891-6-2

43. Gao J, Xie C, Yang J, Tian C, Zhang M, Lu Z, et al. The effects of n-3 PUFA supplementation on bone metabolism markers and body bone mineral density in adults: A systematic review and meta-analysis of RCTs. *Nutrients* (2023) 15(12):2806. doi: 10.3390/nu15122806

44. Lelli D, Antonelli Incalzi R, Ferrucci L, Bandinelli S, Pedone C. Association between PUFA intake and serum concentration and mortality in older adults: A cohort study. *Clin Nutr* (2020) 39(2):510–5. doi: 10.1016/j.clnu.2019.02.030

45. Astorg P, Bertrais S, Laporte F, Arnault N, Estaquio C, Galan P, et al. Plasma n-6 and n-3 polyunsaturated fatty acids as biomarkers of their dietary intakes: a cross-sectional study within a cohort of middle-aged French men and women. *Eur J Clin Nutr* (2008) 62(10):1155–61. doi: 10.1038/sj.ejcn.1602836

46. Harris TB, Song X, Reinders I, Lang TF, Garcia ME, Siggeirsdottir K, et al. Plasma phospholipid fatty acids and fish-oil consumption in relation to osteoporotic fracture risk in older adults: the Age, Gene/Environment Susceptibility Study. *Am J Clin Nutr* (2015) 101(5):947–55. doi: 10.3945/ajcn.114.087502

47. Orchard TS, Ing SW, Lu B, Belury MA, Johnson K, Wactawski-Wende J, et al. The association of red blood cell n-3 and n-6 fatty acids with bone mineral density and hip fracture risk in the women's health initiative. *J Bone Miner Res* (2013) 28(3):505–15. doi: 10.1002/jbmr.1772

48. Mendy A, Vieira ER, Albatineh AN, Nnadi AK, Lowry D, Gasana J. Low bone mineral density is associated with balance and hearing impairments. *Ann Epidemiol* (2014) 24(1):58–62. doi: 10.1016/j.annepidem.2013.10.012

49. Konstantynowicz J, Sierpinska T, Kaczmarski M, Piotrowska-Jastrzebska J, Golebiewska M. Dental malocclusion is associated with reduced systemic bone mineral density in adolescents. *J Clin Densitom* (2007) 10(2):147–52. doi: 10.1016/j.jocd.2007.02.002

50. Farahnak Z, Freundorfer MT, Lavery P, Weiler HA. Dietary docosahexaenoic acid contributes to increased bone mineral accretion and strength in young female Sprague-Dawley rats. *Prostaglandins Leukot Essent Fatty Acids* (2019) 144:32–9. doi: 10.1016/j.plefa.2019.04.005

51. Vamadeva SG, Patel K, Ravi Mangu S, Ellur G, Sukhdeo SV, Sharan K. Maternal omega-3 LC-PUFA supplementation programs an improved bone mass in the offspring with a more pronounced effect in females than males at adulthood. *J Nutr Biochem* (2023) 113:109245. doi: 10.1016/j.jnutbio.2022.109245

52. Akintoye SO, Lam T, Shi S, Brahim J, Collins MT, Robey PG. Skeletal site-specific characterization of orofacial and iliac crest human bone marrow stromal cells in same individuals. *Bone* (2006) 38(6):758–68. doi: 10.1016/j.bone.2005.10.027

53. Goldberg S, Grynpas MD, Glogauer M. Heterogeneity of osteoclast activity and bone turnover in different skeletal sites. *Arch Oral Biol* (2016) 71:134–43. doi: 10.1016/j.archoralbio.2016.06.026

54. de Souza Faloni AP, Schoenmaker T, Azari A, Katchburian E, Cerri PS, de Vries TJ, et al. Jaw and long bone marrows have a different osteoclastogenic potential. *Calcif Tissue Int* (2011) 88(1):63–74. doi: 10.1007/s00223-010-9418-4

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The effects of popular diets on bone health in the past decade: a narrative review

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Bone health encompasses not only bone mineral density but also bone architecture and mechanical properties that can impact bone strength. While specific dietary interventions have been proposed to treat various diseases such as obesity and diabetes, their effects on bone health remain unclear. The aim of this review is to examine literature published in the past decade, summarize the effects of currently popular diets on bone health, elucidate underlying mechanisms, and provide solutions to neutralize the side effects. The diets discussed in this review include a ketogenic diet (KD), a Mediterranean diet (MD), caloric restriction (CR), a high-protein diet (HP), and intermittent fasting (IF). Although detrimental effects on bone health have been noticed in the KD and CR diets, it is still controversial, while the MD and HP diets have shown protective effects, and the effects of IF diets are still uncertain. The mechanism of these effects and the attenuation methods have gained attention and have been discussed in recent years: the KD diet interrupts energy balance and calcium metabolism, which reduces bone quality. Ginsenoside-Rb2, metformin, and simvastatin have been shown to attenuate bone loss during KD. The CR diet influences energy imbalance, glucocorticoid levels, and adipose tissue, causing bone loss. Adequate vitamin D and calcium supplementation and exercise training can attenuate these effects. The olive oil in the MD may be an effective component that protects bone health. HP diets also have components that protect bone health, but their mechanism requires further investigation. In IF, animal studies have shown detrimental effects on bone health, while human studies have not. Therefore, the effects of diets on bone health vary accordingly.

KEYWORDS

ketogenic diet (KD), Mediterranean diet (MD), caloric restriction (CR), high-protein diet, intermittent fasting (IF), bone health

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1 Introduction

Diet is an indispensable component of our daily life, and its impact on the human body has been the subject of extensive research. Over the years, different dietary interventions have been considered as lifestyle interventions that can prevent or treat various diseases such as obesity, cardiovascular disease, epilepsy, and metabolic diseases (1–4). Nutrients participate in every physiological process, regulate metabolism, and play critical roles in each system of the human body, including the skeletal system (5). Various types of diets have different effects on bone health. In this review, we aim to summarize the influences and potential mechanisms of several currently popular diets on bone health, based on both animal and human studies. These diets include the ketogenic diet (KD), the Mediterranean diet (MD), caloric restriction (CR), a high-protein diet (HP), and intermittent fasting (IF). Information about these diets can be found in Table 1.

2 Method

In this review, we conducted a search of Web of Science's core database from January 2012 to November 2022, to identify published articles about the effect of different kinds of diets on bone health. The topics were utilized when searching included "ketogenic diet", "Mediterranean diet", "caloric restriction", "high-protein diet", and "intermittent fasting", combined with "bone" or "calcium". All relevant randomized controlled trials (RCTs), observational studies, and reviews were screened and integrated. Case studies, letters, and conference papers or reports were excluded. Table 2 summarizes most of the cited human studies and Table 3 summarizes most of the cited animal studies.

TABLE 1 Characteristics and examples of various types of diets.

Ketogenic diet	Characteristics	High fat intake, moderate protein consumption, and low carbohydrate intake
	Macronutrient ratio	Fat:protein:carbohydrate = 55%–60%:30%–35%:5%–10%
	Type	Classic long-chain triglyceride (LCT) ketogenic diet
		Medium-chain triglyceride (MCT) ketogenic diet
		Modified Atkins diet (MAD)
		Low glycemic index treatment
Mediterranean diet	Characteristics	Plant-focused, healthy fat emphasis
	Common food categories	Vegetable, fruit, bean, whole grain, extra virgin olive oil, nut
Caloric restriction diet	Characteristics	Reduced daily caloric intake, without malnutrition or deprivation of essential nutrients
	Calorie reduction ratio	20%
High-protein diet	Characteristics	High protein focus, elevated consumption, varied sources, balanced nutrition
	Protein intake	More than 25% of calories from protein, over 1.6 g of protein per kilo of body weight
Intermittent fasting	Characteristics	Only eat during a specific time
	Type	Complete alternate-day fasting
		Modified fasting regimens
		Time-restricted feeding
		Religious fasting
		Ramadan fasting

3 Ketogenic diets and bone health

3.1 The definition of ketogenic diets

KDs are characterized by a low intake of carbohydrates and a normal to high intake of fat, leading to increased utilization of ketones or fats in the body, similar to changes that occur during periods of starvation. Typically, these diets recommend that only 5% of calories come from carbohydrates, while 75% come from fats and 20% from protein, though the total calorie intake and ratio of energy sources can be adjusted based on individual needs.

3.2 Effects of ketogenic diets on bone health

3.2.1 Evidence from animal studies

Most studies have predominantly shown that the KD has an unfavorable effect on bone health. In mice, Wu et al. used the Micro-CT technique and a three-point bending test to assess the bone quality of 8-week-old mice fed a 4:1 KD for 12 weeks. The results indicated that both the cancellous and cortical bone architecture of long bones were compromised (6). A further study on the vertebrae also found a decrease in bone quality (54). Aikawa et al. researched the skeletal systems of aged mice that underwent exercise training and were fed a KD during the experiment, reporting that KD impaired bone mass, trabecular microstructure, and compromised the benefits regarding bone health after exercise (7). Zengin et al. found that a 4-week consumption of an "Atkin-style" KD diet or low protein KD could impair the bone quality of adult male rats. Their femur trabecular bone volume was relatively low, while this effect was not seen in female rats (8). Ding et al. reported that the bone loss was more significant in

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TABLE 2 Summary of the animal studies.

Author	Outcome considered and method for evaluating diet and/or bone health parameters	Study type	Model	Findings
Wu et al. (6)	Cancellous and cortical bone architecture	RCT	Forty female C57BL/6J mice randomly divided into four groups: SD+Sham, SD+OVX, KD +Sham, and KD+OVX; fed for 12 weeks.	KD adversely affects both cancellous and cortical bone in long bones. Combining KD and OVX may exacerbate bone loss.
Aikawa et al. (7)	Bone mass, trabecular microstructure and lumbar BMC	RCT	Male C57BL/6 mice randomly divided into four experimental groups: control diet and sedentary, control diet and exercise, LCHF diet and sedentary, and LCHF diet and exercise; fed for 12 weeks.	The LCHF diet impairs bone mass and certain trabecular microstructures in older mice, and reduces the beneficial effects of exercise on lumbar BMC.
Zengin et al. (8)	Trabecular bone volume, serum IGF-I, and the bone formation marker P1NP	RCT	Twelve-week-old male and female Wistar rats randomly divided into three experimental groups: CD, LC-HF-1, and LC-HF-2; fed for 4 weeks.	In male rats, LC-HF diets lead to a reduction in trabecular bone volume, serum IGF-I, and the bone formation marker P1NP, while no such effects are observed in females.
Ding et al. (9)	Bone density and microstructure	RCT	14 male 6-week-old Sprague–Dawley rats randomly divided into two experimental groups: control and KD group; fed for 12 weeks.	The ketogenic diet negatively impacts bone density and microstructure, primarily in appendicular bones, with minimal effects on axial bones like the L4 vertebrae.
Liu et al. (10)	Spinal fusion, microstructures and bone mass	RCT	32 Sprague–Dawley rats randomly divided into two experimental groups: KD and SD; fed for 8 weeks.	KD delayed spinal fusion and decreased bone mass in posterolateral lumbar spinal fusion in rats.
Zhou et al. (11)	BALP, TRACP, OCN, PPAR-γ, cathepsin K, TRAP, bone microstructure, biomechanical properties.	RCT	30 female (aged 8 weeks) C57BL/6J mice randomly divided into three experimental groups: sham, KD, and KD + Rb2; fed for 12 weeks.	Ginsenoside-Rb2 reduced KD-induced bone loss and improved biomechanics, increasing bone volume fraction from 2.3% to 6.0%.
Tagliaferri et al. (12)	Bone density, oxidative stress, inflammation.	RCT	Six-week-old female C57BL/6J mice randomly divided into six experimental groups: 4 OVX and 2 SH; fed for 30 days.	Virgin olive oil with vitamin D3 improved bone density and reduced oxidative stress in OVX mice.
Puel et al. (13)	BMD, spleen weight, plasma fibrinogen levels.	RCT	98 rats randomly divided into seven experimental groups: 20 SH, 26 OVX with standard diet, and 4 additional OVX groups receiving oleuropein at 2.5, 5, 10, or 15 mg/kg body weight; fed for 100 days.	Oleuropein reduced bone loss and improved inflammatory markers in OVX rats at all tested doses except 5 mg/kg BW.
Shen et al. (14)	Body composition, IGF-I, leptin, adiponectin, glutathione peroxidase, TNF-α mRNA, bone volume, BMD, bone strength.	RCT	30 Sprague–Dawley rats divided into HFD, RD, and LFD groups based on weight gain; fed various diets for up to 8 months.	Restricted diet improved body composition but weakened bone structure and strength in obese rats.
Behrendt et al. (15)	Ct.BMD, Tb.BMD, BV/TV, Tb.N.	RCT	Mice divided into CR groups and AL control; fed up to 74 weeks.	Lifelong caloric restriction (CR) worsened cortical bone in young mice but improved trabecular bone in older mice.
Colman et al. (16)	OC, CTX, NTX, PTH, 25 (OH)D	RCT	30 male rhesus monkeys fed by CR divided into C and R groups; R group reduced by 100 calories; fed for 3 months.	Long-term caloric restriction (CR) led to a decline in bone mass and density compared to control monkeys, but without pathological osteopenia.
Li et al. (17)	BMAT alterations, BMD	Observational study	BMAd-specific Cre mouse model in which we knocked out adipose triglyceride lipase (ATGL, Pnpla2 gene)	Caloric restriction induced significant increases in genes related to extracellular matrix organization and skeletal development.
Takeda et al. (18)	The BMD of tibia, femoral breaking force and energy	RCT	47 male Wistar rats (5 weeks old) divided into diet and exercise sub-groups; fed for 60 days.	Both inadequate and excessive protein intake can affect bone strength, while a protein intake of approximately 20% promotes bone mass and strength development.
Nebot et al. (19)	TV, BV, BMD	RCT	88 male Sprague–Dawley rats (6 weeks old) divided into 11 groups with SD and HFD diets; fed for 21 weeks.	Caloric restriction resulted in significant alterations in trabecular microstructure, characterized by an increase in trabecular number

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TABLE 2 Continued

Author	Outcome considered and method for evaluating diet and/or bone health parameters	Study type	Model	Findings
				and a reduction in trabecular spacing, with no changes in bone volume (BV).
Tirapegui et al. (20)	Carcass, proteoglycan synthesis, IGF-I concentration, total tissue RNA, protein concentration and protein synthesis	RCT	16 newly weaned Wistar rats divided into G12 and G26 diet groups; fed for 3 weeks.	Compared to a low-protein diet, a high-protein diet resulted in lower fat mass but showed no significant changes in protein nutritional status.
Kamel et al. (21)	Glucose, insulin, TGs, cholesterol, PTH, OPG, DPD, NTX-1, TRAP-5b, BMD, BMC	RCT	40 male rats divided into control, control+IF, DEX, and DEX+IF groups; treated for 90 days.	IF corrected GIO in rats by inhibiting osteoclastogenesis and PTH secretion and stimulating osteoblast activity.
Kamel et al. (22)	Thyroid abnormality, bone remodeling ability	RCT	8 pregnant Wistar rats divided into fasting and normally fed groups; fed for 21 days after birth.	IF imposed on embryonic rats resulted in a collapse of bone remodeling to some extent.
Shin et al. (23)	BMD	RCT	Female Sprague–Dawley rats divided into four groups: AD-AL, AD-IMF, Non-AD-AL, and Non-AD-IMF; diets for 4 weeks post β-amyloid infusion.	IF exacerbated bone density loss in Alzheimer's disease-induced estrogen-deficient rats.
Xu et al. (24)	BMD, ALP, TRAP, BMSC	RCT	30 male 6-week-old Sprague–Dawley rats divided into Control, KD, and EODKD groups; fed for 12 weeks.	Compared to KD, EODKD exhibited higher ketone levels but also inhibited the bone resorption process and early bone formation differentiation.

BMD, Bone Mineral Density; BMC, Bone Mineral Content; IGF-I, Insulin-like Growth Factor I; P1NP, Procollagen Type 1 N-Terminal Propeptide; BALP, Bone Alkaline Phosphatase; TRACP, Tartrate-Resistant Acid Phosphatase; OCN, Osteocalcin; PPAR-γ, Peroxisome Proliferator-Activated Receptor Gamma; TRAP, Tartrate-Resistant Acid Phosphatase; Ct.BMD, Cortical Bone Mineral Density; Tb.BMD, Trabecular Bone Mineral Density; BV/TV, Bone Volume per Total Volume; Tb.N, Trabecular Number; OC, Osteocalcin; CTX, C-Terminal Telopeptide; NTX, N-Terminal Telopeptide; PTH, Parathyroid Hormone; 25(OH)D, 25-Hydroxyvitamin D; BMAT, Bone Marrow Adipose Tissue; TV, Total Volume; BV, Bone Volume; ALP, Alkaline Phosphatase; BMSC, Bone Marrow Stromal Cells; TGs, Triglycerides; OPG, Osteoprotegerin; DPD, Deoxypyridinoline; NTX-1, N-Terminal Telopeptide of type I collagen; TRAP-5b, Tartrate-Resistant Acid Phosphatase 5b; SD, Standard Diet; OVX, Ovariectomized; KD, Ketogenic Diet; AL, Ad Libitum; LCHF, Low-Carbohydrate High-Fat; CD, Control Diet; IMF, Intermittent Fasting; SH, Sham-Operated; HFD, High-Fat Diet; RD, Restricted Diet; LFD, Low-Fat Diet; CR, Caloric Restriction; IF, Intermittent Fasting; DEX, Dexamethasone; ICV, Intracerebroventricular; EODKD, Every-Other-Day Ketogenic Diet; RCT, Randomized Controlled Trial; LC-HF-1, "Atkins-Style" Protein-Matched Diet; LC-HF-2, Ketogenic Low-Protein Diet; G12, Libitum Diets Containing 12% Protein; G26, Libitum Diets Containing 26% Protein.

TABLE 3 Summary of the human studies.

Author	Population	Diet	Outcome considered and method for evaluating diet and/or bone health parameters	Findings
Hahn et al. (25)	33 children	KD	Bone mass, Serum 25-OHD levels	KG patients showed vitamin D deficiency and reduced bone mass; Vitamin D supplementation increased KG bone mass by 8.1% in 12 months.
Simm et al. (26)	29 patients	KD	DXA, BMD, BMAD, osteocalcin	Patients on a KD showed a trend towards reduced LS-BMD *Z* scores
Svedlund et al. (27)	39 Children with intractable epilepsy, glucose transporter type 1 deficiency syndrome, or pyruvate dehydrogenase complex deficiency	MAD	Bone mass (total body, lumbar spine, and hip)	MAD has no significant effect on bone mass
Gomez-Arbelaez et al. (28)	20 adult obese patients	KD	BMC and BMD via DXA	KD leaves BMC and BMD statistically unchanged via DXA.
Athinarayanan et al. (29)	349 type 2 diabetes patients	KD	Spine BMD	Diabetes resolution and no adverse effect on bone health were observed in the experiment group.

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TABLE 3 Continued

Author	Population	Diet	Outcome considered and method for evaluating diet and/or bone health parameters	Findings
Bertoli et al. (30)	3 adult patients with GLUT-1 DS	KD	BMD	Long-term KD had no major negative effects on body composition or bone health in adults with GLUT-1 DS.
Vargas-Molina et al. (31)	21 adult resistance-trained women	KD	BMD	KD led to a significant reduction in systolic blood pressure and a small favorable effect on BMD.
Carter et al. (32)	30 obese patients	KD	BSAP, bone turnover ratio, and UNTx	Dieters lost more weight than controls but no significant change in bone turnover markers or ratio was observed.
Heikura et al. (33)	30 world-class race walkers	KD	CTX, OC, and P1NP	Short-term LCHF diet impaired markers of bone modeling/remodeling.
Draaisma et al. (34)	38 epileptic children	KD	Lumbar *Z*-score, BMD	Children on KDT have low normal BMD that may further decrease. Intravenous bisphosphonate therapy showed a statistically significant increase in BMD.
Nestares et al. (35)	59 children with celiac disease (CD), 40 non-celiac children	MD	BMC, bone *Z*-score, and BMD	MD adherence was associated with higher lean mass and bone health in CD children.
Seiquer et al. (36)	20 male adolescents	MD	Calcium absorption and retention	MD led to increased calcium absorption and retention, and decreased urinary calcium excretion.
Julian et al. (37)	492 Spanish adolescents	MD	BMD	Fruits, nuts, cereals, and roots were associated with higher BMC, but significance was lost when adjusted for lean mass and physical activity.
Pérez-Rey et al. (38)	442 premenopausal women	MD	Ad-SOS, BMD	Higher adherence to the MD was positively associated with better bone mass measurements in Spanish premenopausal women.
Cervo et al. (39)	794 community-dwelling men	MD	BMD and risk of incident falls	MD was associated with lower incident fall rates in older men. No association was found between MEDI-LITE scores and BMD or physical function parameters.
Feart et al. (40)	1,482 older French adults	MD	Risk of bone fractures	Higher MeDi adherence was not associated with a decreased risk of fractures in older French persons.
Villareal et al. (41)	218 non-obese, younger adults	CR	BMD, C-telopeptide, TRAP, BSAP	CR led to significant bone loss at crucial sites for osteoporotic fracture due to changes in body composition, hormones, and nutrients.
Tirosh et al. (42)	424 obese and overweight participants	CR	BMD at femoral neck and spine	Weight loss diets had sex-specific effects on BMD: men showed an increase in spine BMD, while women had a decrease in BMD at all sites.
Pop et al. (43)	38 overweight and obese men	CR	Body weight, BMD, BMC, cortical thickness, 25-OHD	CR in overweight and obese men did not decrease BMD or alter bone geometry.
Von Thun et al. (44)	42 postmenopausal women	CR	BMD	People with CR lost BMD at the FN and trochanter after 2 years, irrespective of weight regain or maintenance.
Hinton et al. (45)	40 overweight or obese women	CR	BMD	Hip and lumbar spine BMD decreased with weight loss due to CR and did not recover after weight regain, regardless of exercise.
Armamento-Villareal et al. (46)	107 obese adults	CR	Thigh muscle volume, hip BMD	In the population following CR, thigh muscle mass is related to hip BMD, and a decrease in muscle mass caused by the diet can lead to a decrease in BMD

(Continued)

TABLE 3 Continued

Author	Population	Diet	Outcome considered and method for evaluating diet and/or bone health parameters	Findings
Antonio et al. (47)	24 exercise-trained women	HP	Whole-body BMD, lumbar BMD, T-scores, lean body mass, and fat mass.	Six months of an HP diet did not affect whole body or lumbar BMD, T-scores, lean body mass, or fat mass.
Lee et al. (48)	12,812 subjects in NHANES	HP	Femoral BMD, T-scores	HP was associated with higher femoral BMD and T-scores in subjects without CKD while CKD patients did not benefit from an HP diet in terms of femoral BMD
Gao et al. (49)	4,447 subjects in NHANES	HP	T-scores, BMD	A high-protein, low-carbohydrate diet may benefit bone health with a significant positive effect on T-score and reduced the risk of low BMD.
Murphy et al. (50)	7 patients with chronic kidney disease and low energy availability	HP	Leptin, IGF-1, P1NP, CTX-I	HP did not mitigate the adverse effects of LEA on bone turnover or leptin levels.
Martens et al. (51)	64 healthy lean midlife/older adults	TRF	Lean mass, BMD	TRF appears to be a feasible and safe dietary intervention for healthy non-obese older adults without negatively impact lean mass, bone density, or nutrient intake.
Clayton et al. (52)	16 lean participants	IF with energy restriction	Serum level of CTX, PINP, PTH	IF with energy restriction does not affect bone metabolism markers like CTX, PINP, and PTH.
Papageorgiou et al. (53)	10 eumenorrheic women	CR	P1NP, CTX, IGF-1, Leptin	Low EA achieved through CR led to a decrease in bone formation but no change in bone resorption.

the appendicular rather than axial bone of rats fed a 3:1 KD (9). Meanwhile, Liu et al. found that a KD can delay the spinal fusion of rats after surgery (10) and confirmed that the microstructures and properties of cancellous bone deteriorated as a result of the interrupted balance of bone resorption and formation (55). Rats fed with KD had significantly lower alkaline phosphatase (ALP) activity and higher tartrate-resistant acid phosphatase (TRAP) activity, and the osteogenic ability of their bone marrow stromal cells was also found to be impaired (56). By measuring TRAP, collagen type I (CoLI), and osteocalcin (OCN) staining, mice fed a KD were found to have upregulated osteoclast activities. When combined with ovariectomy, the osteoblast activities were found to be downregulated (6).

Attention has been drawn to how to relieve the side effect of KD on bone health. Liu et al. found that the bone quality loss induced by KD can be relieved by ginsenoside-Rb2, which inhibits bone resorption and osteogenic differentiation. Metformin can also reduce bone loss by enhancing osteoblast proliferation and inhibiting osteoclast differentiation (11, 57). Zhou et al. demonstrated the protective effect of simvastatin on the bones of mice that were compromised by KD, and the mechanism may be the facilitation of osteoblast differentiation and inhibition of osteoclast differentiation (58). Previous studies have reported that simvastatin can induce the expression of bone morphogenetic protein (BMP)-2, which improves bone formation (59).

3.2.2 Evidence from human studies

3.2.2.1 KD in epileptic children

The alteration of bone health in children treated with KD has been studied since the 1970s. Five epileptic children treated with KD therapy were reported to have disordered mineral metabolism, and their bone mass and serum 25-OHD levels were found to be decreased compared to the normal control (25). In another observational study, researchers investigated the bone health of 29 epileptic children aged 0.5–6.5 years who persisted with a KD for at least 6 months. After measuring with dual-energy x-ray absorptiometry (DXA), they reported a decrease of 0.16 units of bone mineral density *Z* score per year relative to age-matched children (26). In terms of the Modified Atkins Diet (MAD), the intake of protein is not restricted and the KD ratio is 1:1–2:1. A recent study reported that a 24-month MAD did not significantly affect the bone mass and height of children who were diagnosed with intractable epilepsy, glucose transporter type 1 deficiency syndrome, or pyruvate dehydrogenase complex deficiency (27). Most notably, the causes of damaged bone mineral status in epileptic children can also include medication side effects, seizures, and mobile ability (60); thus, more high-level evidence is required to determine the extent of how much KD is to blame for impaired bone growth in epileptic children.

3.2.2.2 KD in adults

The recent applications of the KD in adults were mainly focused on the treatment of metabolic diseases including obesity, diabetes, and glucose transporter 1 deficiency syndrome (GLUT-1 DS). Regarding obesity, a study observed 20 adult obese patients who were treated with the KD for 4 months, and with the measurement of DXA, both their bone mineral content (BMC) and bone mineral density (BMD) were statistically unchanged (28). In terms of diabetes, a study enrolled 262 type2 diabetes patients who were

treated with a low-carbonate KD to achieve and sustain nutrition ketosis (blood BHB level of 0.5–3.0 mmol/L). It was found that their spine BMD remained stable from baseline to a 2-year follow-up (29). In regard to GTUT-1 DS, the alteration of bone mass of three adult patients who were treated with normocaloric 3:1 KD for 5 years was observed, and the BMD of all three patients decreased in the first 3 years and remained stable thereafter. At the 5-year follow-up, all patients' BMDs were in the normal range (30). In a study with healthy participants, 21 adult resistance-trained women were randomly assigned to a non-KD or low-carbohydrate KD group for 8 weeks. The results revealed that the KD group displayed a significant increase in BMD after 8 weeks, while the NKD group showed no significant change. However, no statistical significance was found between the 2 groups (31). In recent years, most studies on adults proved that a KD could improve disease conditions and reduce harm to bone health. However, detrimental effects on bone quality especially in children should be given great consideration. Additionally, because the proportion and type of fat in KD were not always recorded and controlled in current studies, and the duration of KD intervention varied among studies, more high-level studies with standardized study methods and large sample sizes are necessary to reach a final judgement.

Current research on the KD indicates that its effects on bone metabolism vary among different age groups and genders. In terms of obese patients, Carter et al. compared 15 obese patients who underwent KD treatment with another 15 matched obese patients without diet intervention for 3 months. No significant difference was found in the comparison of their bone-specific alkaline phosphatase (BSAP) and urinary cross-linked N-telopeptides of type I collagen (UNTx), indicating a negative effect on bone turnover rate in obese patients (32). As to the world-class athletes who underwent a short-term KD for 3.5 weeks, bone resorption markers (cross-linked C-terminal telopeptide of type I collagen, CTX) increased, while the bone formation marker (procollagen 1 N-terminal propeptide, P1NP) decreased (33). Since bone is the major reservoir of calcium, calcium metabolism can provide another perspective on how a KD affects bone metabolism. Current studies have revealed that a KD could decrease calcium digestibility, release calcium from bone to blood, and promote abnormal excretion of calcium. Hawkes et al. observed cases of epileptic children who were treated with KD therapy and then expanded the research into a multi-center study. In general, it was found that children developed hypercalcemia after an average of 2.1 years. Furthermore, moderately elevated urinary calcium excretion, and low levels of serum alkaline phosphatase, PTH, and 1,25-dihydroxyvitamin D were also noticed (Figure 1) (61, 62).

Since previous studies have noticed the compromised bone quality of patients treated with KD, research on how to reduce or reverse the side effect of a KD on bone were then conducted, and several antiosteoporosis drugs were reported to be effective. Draaisma et al. conducted a retrospective observational cohort study on epileptic children treated with KD and bisphosphonate for over 6 months; DXA scans were taken to assess the bone mass, and the result showed that bisphosphonate may have a positive effect on the bone mass (34).

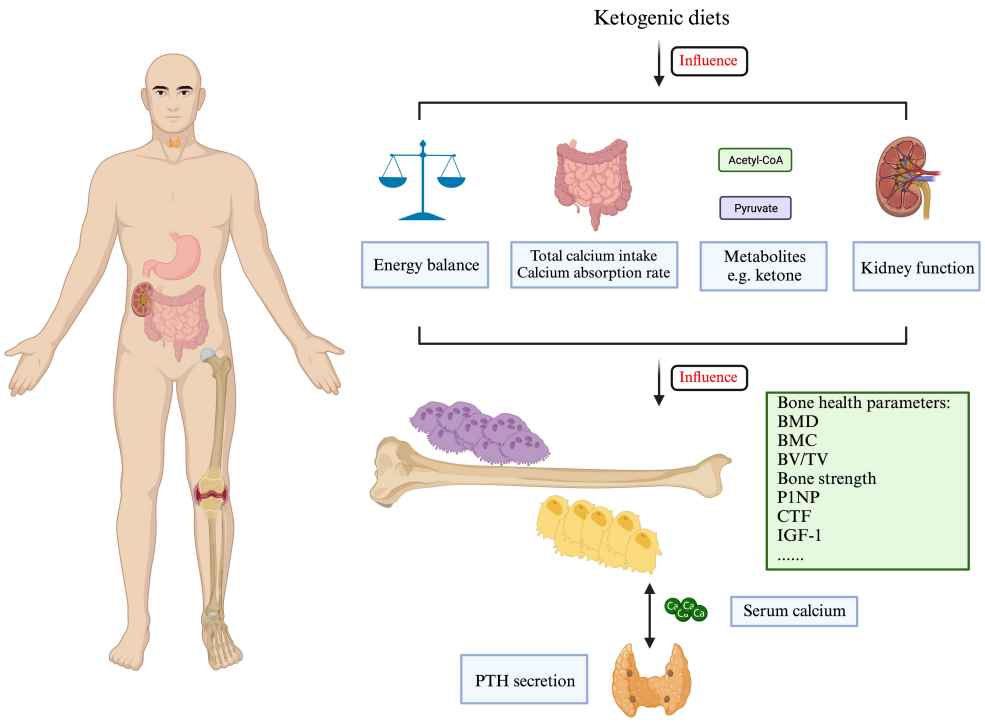

FIGURE 1
The ketogenic diets may affect bone formation and resorption in multiple ways. Created with BioRender.com.

4 Mediterranean diet and bone health

4.1 The definition of a Mediterranean diet

The MD was first defined as being low in saturated fat and high in vegetable oils in the 1960s and has been continuously revised since then. The modern concept of the MD describes it as a diet that includes a high intake of extra virgin olive oil, vegetable, and fruit; a moderate intake of fish and other meat, dairy products, and red wine; and a low intake of eggs and sweets (63). The most recent definition of the MD was released in 2010 by the Mediterranean Diet Foundation (64) (Table 4). The MD has been shown to be effective in a variety of diseases, like cardiovascular disease and cancer, as well as in bone health. Its protective effect was due to its antioxidant and anti-inflammatory active molecules such as polyphenols (65, 66).

4.2 The Mediterranean diet and bone metabolism

4.2.1 Evidence from animal studies

Until now, only a handful of animal studies have been conducted to reveal the effect of the MD on bone health. Olive oil and vitamin D, which are abundant in the MD, were proven to resist the bone loss induced by estrogen deprivation by regulating the inflammation and oxidative stress status in mice (12). A variety of olive compounds have been studied and proven to have a protective effect on bone. Puel et al. injected ovariectomized rats with osteoporosis (15 mg/kg) with oleuropein, a common component of the MD. After 100 days, the injected rats had a doubled BMD compared with the untreated ovariectomized group. Another study published in 2008 focused on tyrosol and hydroxytyrosol, the main olive oil phenolic compounds. The results showed that ovariectomized rats after 84 days of tyrosol and hydroxytyrosol treatment had higher blood concentrations of osteocalcin and BMD than untreated ovariectomized rats (13, 67).

TABLE 4 The definition of the Mediterranean diet.

Food	Frequency
Sweets	≤2 servings weekly
Potatoes	≤3 servings weekly
Red meat	<2 servings weekly
Processed meat	≤1 servings weekly
Dairy (preferably low fat)	2 servings daily
Olives/nuts/seeds	1–2 servings daily
Olive oil	Every main meal
Fruits	1–2 servings every main meal
Vegetables (variety of color/textures)	≥2 servings every main meal
Bread/pasta/rice/couscous/other cereals	1–2 servings every main meal

Serving sizes specified as 25 g of bread, 100 g of potato, 50–60 g of cooked pasta, 100 g of vegetables, 80 g of apple, 60 g of banana, 100 g of orange, 200 g of melon, 30 g of grapes, 1 cup of milk or yoghurt, 60 g of meat, and 100 g of cooked dry beans.

4.2.2 Evidence from human studies

A study about the MD in children with celiac disease found that it could improve bone health. It was found that both bone mineral content and bone mineral density in these children were significantly increased with high MD adherence than those with low MD adherence. The adherence to the MD was evaluated using the Mediterranean Diet Quality Index in Children and Adolescents (KIDMED) survey. Participants are classified into three categories: (1) high MD adherence (≥8 points), (2) medium MD adherence (4–7 points), and (3) low MD adherence (≤3 points) (35). Another study revealed that compared to a basal diet, male adolescents who adopted an MD as the main meal had a significant improvement in calcium absorption and retention (36). In the meantime, Julian et al. showed that the MD was not associated with BMD (37). Several studies have demonstrated that perimenopausal women with MD had more BMD and trabecular density (38) and less probability of osteoporosis (68–72) than women without. Meanwhile, several studies have indicated that the MD was associated with a reduced risk of fracture, especially in hip fracture (73–75). A case–control study in 2014 that included nearly 700 elderly Chinese persons conducted from 2009 to 2013 with hip fracture showed that a high score in diet-quality scales such as aMed was significantly associated with a decreased risk of hip fractures (76), and a high score in diet-quality scales was often associated with the MD. Other studies revealed that high compliance with the MD was associated with higher BMD and less risk of incident falls (39, 77–79). The protective effect of this diet may be related to the intake of vitamin D3, calcium ions, and the elevated levels of parathyroid hormone in the body (80). However, not all studies found benefits in the MD for bone health. A study conducted from 2000 to 2010 on elderly French persons found no link between the diet and the risk of bone fractures, possibly due to race or environment (40). In the study, individuals with an incident fracture at any of the three sites had a higher mean MeDi score, which assesses MD adherence, at baseline than those who remained free of fracture. Specifically, greater fruit consumption (i.e., >14 servings/week) was significantly associated with a doubled 8-year risk of hip fracture, and a lower intake of dairy products (i.e., <17.0 servings/week in men and <17.9 servings/week in women) was associated with a doubled risk of wrist fracture. It has also been suggested that the olive oil in the MD may reduce the risk of osteoporosis by reducing chronic inflammation (81).

5 Caloric restriction diet

5.1 The definition of a caloric restriction diet

Caloric restriction (CR) diet is classically defined as a diet with reduced caloric intake, which is approximately 20%–30% below average and does not cause malnutrition during the diet

intervention. CR was reported to have the ability not only to reduce weight (82) but also to improve aging-related outcomes (83). However, CR was previously considered as a risk factor for compromising bone quality, and the mechanism behind this phenomenon might be the alteration in bone metabolism, hormones, and weight bearing. Consequently, researchers have tried to introduce a number of remedies to reduce dietary bone damage, including vitamin D intake, high-protein intake, and exercise training (84). Studies in the last decade have gained more results on this topic, which will be reviewed below.

5.2 Effects of a caloric restriction diet on bone mass

5.2.1 Evidence from animal studies

Most of the recent animal studies on CR and bone health have once again confirmed the degraded bone mineral condition in rats, mice, and rhesus monkeys. A study on obese female rats implemented a −35% CR diet for 4 months; the BMD, trabecular, and cortical bone volume and bone strength were found to be decreased (14). Another study researched the effect of CR on bone and discovered that the starting age of CR application was found to be crucial to determine its effects. Younger mice showed a more significant loss under CR in terms of cortical bone, cortical BMD, and thickness, compared to senile mice. Long-term CR showed beneficial effects on vertebrae trabecular BMD and BV/TV, which were considered as a reorganization and compensation for the bone loss in cortical bone (15). Issues have also been proposed on whether the decrease of BMD was a pathological process or an adaptation to weight loss. The study on rhesus monkeys indicated that the alteration might be an adaptation process. It was reported that despite the fact that BMD was lower after CR, the alteration of bone turnover markers was not significant; thus, the decreased BMD may be associated with the lower mechanical load generated by a smaller body size, rather than pathological osteopenia (16).

Regarding the mechanism of how CR affects bone health, recent studies focused most on CR-induced bone marrow adipose tissue (BMAT) alterations. BMAT could respond to CR-induced energy imbalance, cause volume expansion and metabolic or endocrinal change (17), and then cause bone loss. The trigger factors of the alterations in BMAT have been widely studied, and the roles of corticoid have been clarified the most. The uprising of serum glucocorticoid as a result of CR was considered to be relevant (85). The effect of leptin was still unclear, and low serum leptin level was found to be insufficient for BMAT expansion (85). Additionally, despite daily leptin supplementation suppression of BMAT formation in CR mice, it does not attenuate BMD loss or the impairment of bone microstructure; thus, the roles of leptin in bone–fat interaction remain unclear (86). Development of insulin resistance during CR was also found to coincide with BMAT expansion (87). Another study revealed that the preservation of BMAT during CR might be related to its characteristics of beta-adrenergic stimuli resistance compared to white adipose tissue (WAT) (88). Furthermore“, it was proposed that bone–hypothalamus–pituitary–adrenal crosstalk might occur, which may regulate BMAT during CR (89). The alteration of BMAT affected bone health in multiple ways. The expansion of BMAT stored fat, which might take up space in bone marrow, and BMAT released biotic factors that modulated bone turnover. However, whether or not BMAT expansion itself was necessitated in bone loss remains controversial since the amount of expansion might not always be related to the extent of bone loss (87, 90, 91). Adiponectin, secreted by WAT and BMAT, was found to be increased in mice and non-obese adults in CR situations, and overexpression of adiponectin might interfere with glucose metabolism and sympathetic tone, which further affects bone cells and induces bone loss (41, 92). Regarding bone metabolic status, a human study revealed the bone metabolic responses to CR, and based on the evaluation of blood samples, P1NP concentration decreased while CTX concentration remained unchanged, and IGF-1 and leptin levels were decreased, which suggested that CR might induce bone loss and decrease bone formation rather than increase bone resorption (53) (Figure 2).

5.2.2 Evidence from human studies

Regarding the determination of whether CR impairs bone quality, while several 2-year studies in non-obese patients, including an RCT study, kept the affirmative opinion (41, 93), other studies found that whether the loss of bone quality existed or not was related to gender differences and the extent of weight loss. An RCT study observed 424 obese and overweight participants, and

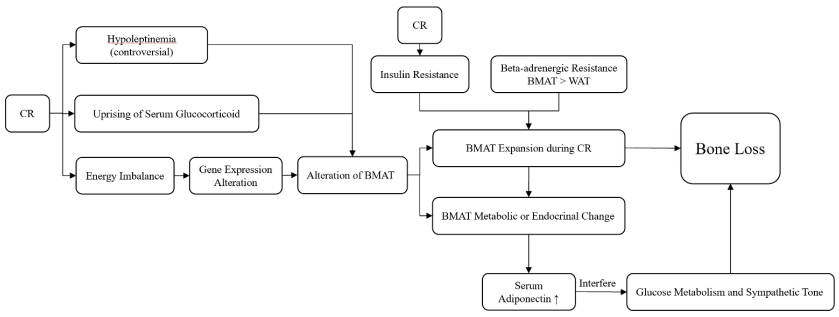

FIGURE 2
The mechanism of bone–fat interaction during CR.

found decreased BMD in the femoral neck in all patients, while postmenopausal women also showed decreased BMD in the spine. Male patients showed increased BMD in the spine. All participants also underwent regular exercise, which may influence the analysis of the results (42). Another study that investigated 38 men showed that moderate weight loss (−7.9 ± 4.4%) using CR would not decrease BMD at any site or decrease cortical and trabecular bone and geometry (43). Meanwhile, studies have also focused on determining the timing of bone loss during CR. A study on postmenopausal women has shown that the BMD loss did not recover in 2 years after a 6-month CR intervention (44). Moreover, evidence has also shown that bone mineral loss and bone turnover would not recover even after weight regain (45). Furthermore, regarding the assessment tools for the evaluation of BMD in humans who underwent CR diet intervention, aside from the gold standard of DXA, other new predictive tools were investigated. A multiple regression analysis study collected data from 107 obese adults with CR. The researchers selected the changes in thigh muscle volume, lean body mass, osteocalcin, P1NP, CTX, and one-repetition maximum strength as variables. After stepwise multiple linear regression analysis, they demonstrated that changes in thigh muscle volume were positively correlated with changes in hip BMD and were its independent predictor (46).

5.3 Methods for attenuating CR-induced bone loss

Considering the potential side effects of CR on bone, researchers have recently focused on interventions that might attenuate such effects, including exercise, vitamin D and calcium supplementation, and other nutrient supplementation.

In terms of exercise, more recent literature confirmed the positive effect of exercise during CR, while a few animal studies disagreed with this opinion. In studies with positive opinions on the subject, RCTs involving overweight/obese adults have found that aerobic training (AT) for 3 months (94) and resistance training (RT) for 5 months (95) were beneficial to weight-bearing bones' BMD during CR. However, RT might be more effective in bone quality reservation than AT according to the comparison between the BMDs of RT+CR and AT+CR. The potential mechanism needs to be discussed in the future (95). The level of serum sclerostin was found to be higher in participants with exercise training during CR, which might positively influence bone quality (96, 97). In contrast with human studies, animal studies have revealed varying results. Five-month-old female rats that were fed with CR with exercise for 12 weeks have better BMD, BMC, and lean mass compared to rats only fed with CR (98, 99). Another study found that obese rats fed with CR and subjected to exercise for 3 months can attenuate bone volume decrease at the distal femur (100). Nebot et al. proposed a mixed exercise-training protocol with CR and reported that it induced weight loss while preserving bone quality (19). A few studies also pointed out the negative effects of exercise on bone during CR. Hitori et al. conducted a study that randomly divided 14-week-old mature male rats into a control group, a CR group, an exercise group, and a CR+exercise group, and found no significant difference in femur and tibia BMD and trabecular bone volume between groups after 13 weeks (101). They then conducted a similar study on 4-week-old rats; the results indicated that 13-week exercise with CR was detrimental to bone microstructure and strength (102). A more recent study fed 10-week-old rats with CR and subjected them to exercise for 6 weeks. Bone quality was found to be compromised with increased cortical porosity. Exercise also suppressed MAT formation, interrupting its function as an energy supply source to bone formation during CR (103).

In terms of nutrient supplementation, vitamin D and calcium supplementation provided the most significant findings. Over the last 10 years, four RCT studies have revealed different results. A 6-month supplementation of 400 IU vitamin D and 800 mg calcium per day can improve tibial bone properties, which was measured by quantitative CT in young male jockeys who usually undertake CR and high volumes of physical activities (104). Another RCT study observed that a 6-week intake of 1,200 mg calcium and 400 IU vitamin D supplementation in healthy or obese participants during CR could elevate their osteocalcin level and improve insulin sensitivity, which might benefit bone formation (105). Regarding the dose of vitamin D supplementation, an RCT double-blind study found that when calcium intake is 1,200 mg per day, either 10 or 63 μg of vitamin D per day is sufficient to maintain the calcium balance during CR. Calcium balance was evaluated with the parameter of true fractional calcium absorption (TFCA) (106). However, another RCT study revealed that a 12-month vitamin D supplementation (2000 IU/day) did not result in different changes in BMD from placebo in women participating in a weight loss program with CR. It is worth noting that all participants also took part in 225 min/week of aerobic exercise (107). Other nutrient supplementation researched by recent studies included special protein regimens (soy or casein) and Omega-3 polyunsaturated fatty acid (n-3 PUFA) supplementation. However, both of them could not improve bone quality during CR according to the study results (108, 109).

6 The effect of high-protein diets on bone health

According to the Recommended Dietary Allowances (RDA) published by the National Research Council (US) in 1989, 0.8 g/kg body weight/day of protein is sufficient for adults, while high-protein (HP) diets refer to diets that contain more than 0.8 g/kg body weight/day of protein. HP diets have gained attention since they have been widely used in the treatment of obesity and diabetes. Furthermore, it is believed that HP diets may improve athletes' performance and body posture by increasing muscle mass (110–112). However, controversial topics of whether and how HP diets influence bone health still remain. During HP diets, serum IGF-1 and bone matrix collagen synthesis were upregulated, while PTH secretion was downregulated. These factors were beneficial to bone formation. On the other hand, HP diets could also produce more acid during protein metabolism, which could impair bone formation. Overall, the protective effect appears to outweigh the detrimental effect (113).

Animal studies on a moderate-high protein diet showed its positive effect on bone, while even higher protein diets did not seem to further improve bone quality. In a study involving 5-week-old rats fed with different levels of dietary protein and that underwent different levels of exercise, it was found that the BMD of tibia and femoral breaking force were lower in the low-protein-diet group (18). Another study examined 6-week-old obese Zuker rats for 2 months, showing that the combination of an HP diet (25% protein) and exercise enhances the trabecular bone microarchitecture and BMD, while leaving the bone turnover markers unchanged (114). However, a study that tracked 3-week-old rats for 3 weeks did not find any improvement in bone length and bone formation biomarkers in the high-protein group (26% protein) compared with the low-protein group (12% protein) (20). In another study, the HP diet (40% protein) did not improve bone quality more than the moderate-protein diet (20% protein) in rats with high-level exercise (18).

In human studies, no detrimental effects on bone health were found during an HP diet. Researchers compared the HP diet with habitual diets in 24 exercise-trained women for half a year. The results proved that neither the whole-body BMD nor lumbar BMD were significantly different after intervention (47). In another large-sample study, the protein intake of 12,812 subjects with femoral BMD and T scores from the National Health and Nutrition Examination Survey (NHANES) were analyzed. It was demonstrated that BMD and T-scores were positively correlated with the amount of protein intake (48). Another analysis also studied the NHANES database and extracted data from 4,447 subjects. The result showed that diets with a higher percentage of energy from protein were associated with higher T-scores (49). However, the two studies did not record the duration of HP diets. In another aspect, there are studies that have shown that HP diets do not attenuate bone loss in patients with chronic kidney disease and low energy availability (48, 50).

7 Intermittent fasting and bone health

Intermittent fasting (IF) is defined as dieting with periodic fasting and non-fasting (115), which includes complete alternate-day fasting, modified fasting regimens, time-restricted feeding (TRF), religious fasting, and Ramadan fasting, thus improving metabolic profiles and reducing the risk of obesity and related diseases (116). Although the intake of calcium was reported to be relatively lower in IF (117), the actual effects of IF on bone health were unclear.

In animal studies, evidence showed the detrimental effects of IF on bone mass and bone remodeling. A study that researched 16 pregnant female rats reported that rats fed with an IF regimen showed a decrease in cortical thickness of the vertebra and the ability of bone remodeling according to the osteoclast count (21). They further observed the offspring of eight pregnant rats fed with IF and found thyroid abnormalities that may be associated with the decrease in bone remodeling ability (22). Another study observed Alzheimer's disease-induced estrogen-deficient rats, showing that IF could aggravate BMD loss (23). It was also determined that IF may attenuate the detrimental effects of KD on bone. The combination of IF and KD was named Every-other-day ketogenic diet. It was reported that this diet would not impair bone microstructure and strength compared to a normal KD in a rat study (56).

Current human studies showed no detrimental effects in either BMD measurement or bone turnover markers. In terms of IF without energy restriction, a randomized study enrolled 24 healthy lean midlife/older adults, while 10 participants were randomized to stick to their normal feeding pattern for 6 weeks and then transition to a 6-week TRF. The other 14 participants were randomized to stick to the 6-week TRF and then transition to their normal diet pattern; the TRF required participants to consume all meals within a 8-h time window, and the caloric intake was within a regular range to avoid weight loss. In the study results, researchers did not find significant differences between the normal feeding group and the TRF group (51). In terms of IF with energy restriction, another study investigated 16 lean participants for 3 days; on day 1, they consumed a 24-h diet with or without energy restriction (25% of the estimated energy requirement), followed by a standardized breakfast and *ad libitum* lunch and dinner on day 2, and fasting overnight and return on day 3. Their CTX, PINP, and PTH levels were measured on all 3 days, with no differences found between the groups, which indicated that a 24-h severe energy restriction did not affect bone metabolism (52).

8 Conclusion

This review presents an overview of the current knowledge on the effects of a KD, an MD, an HP diet, IF, and CR on bone health. We suggest that several problems should be solved first before further addressing the following: (i) Related studies lack standardization of the dietary intervention, which includes the proportion and type of fat in a KD, the energy restriction rate and the nutrition structure of CR, the proportion and resources of protein in HP, the types of IF, the duration of the intervention, and whether calcium supplementation can meet the minimum daily requirement. (ii) The method used for measuring bone quality also lacks standardization, which includes the bone site of measurement and the selection of the assessment tool such as x-ray, computed tomography (CT), or DXA. (iii) Sometimes, studies displayed conflicting results in human and animals; further explanation is needed to address this. (iv) More high-level evidence studies, such as an RCT and meta-analysis of different forms of dietary interventions, should be carried out with a standardized protocol and long-term follow-up. In summary, in a KD and CR, detrimental effects on bone quality were more significant, and attenuation methods were proposed. In contrast, most of the relevant studies on MDs and HP diets showed a positive or non-effective impact on bone health. In IF, recent human studies and animal studies showed different results. Although numerous researchers have been working on this topic for a long period of time, current lines of

evidence on human and animal studies were still not sufficient to reach a final solid conclusion.

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References

1. Kopp W. How western diet and lifestyle drive the pandemic of obesity and civilization diseases. *Diabetes Metab Syndr Obes* (2019) 12:2221–36. doi: 10.2147/DMSO.S216791

2. Meira ID, Romao TT, do Prado HJP, Kruger LT, Pires MEP, da Conceicao PO. Ketogenic diet and epilepsy: what we know so far. *Front Neurosci* (2019) 13. doi: 10.3389/fnins.2019.00005

3. Satija A, Hu FB. Plant-based diets and cardiovascular health. *Trends Cardiovasc Med* (2018) 28(7):437–41. doi: 10.1016/j.tcm.2018.02.004

4. Giugliano D, Esposito K. Mediterranean diet and metabolic diseases. *Curr Opin Lipidol* (2008) 19(1):63–8. doi: 10.1097/MOL.0b013e3282f2fa4d

5. Merlotti D, Cosso R, Eller-Vainicher C, Vescini F, Chiodini I, Gennari L, et al. Energy metabolism and ketogenic diets: what about the skeletal health? A narrative review and a prospective vision for planning clinical trials on this issue. *Int J Mol Sci* (2021) 22(1):435. doi: 10.3390/ijms22010435

6. Wu X, Huang Z, Wang X, Fu Z, Liu J, Huang Z, et al. Ketogenic diet compromises both cancellous and cortical bone mass in mice. *Calcified Tissue Int* (2017) 101(4):412–21. doi: 10.1007/s00223-017-0292-1

7. Aikawa Y, Yamashita T, Nakai N, Higashida K. Low-carbohydrate, high-fat diet, and running exercise influence bone parameters in old mice. *J Appl Physiol* (2022) 132 (5):1204–12. doi: 10.1152/japplphysiol.00789.2021

8. Zengin A, Kropp B, Chevalier Y, Junnila R, Sustarsic E, Herbach N, et al. Low-carbohydrate, high-fat diets have sex-specific effects on bone health in rats. *Eur J Nutr* (2016) 55(7):2307–20. doi: 10.1007/s00394-015-1040-9

9. Ding J, Xu X, Wu X, Huang Z, Kong G, Liu J, et al. Bone loss and biomechanical reduction of appendicular and axial bones under ketogenic diet in rats. *Exp Ther Med* (2019) 17(4):2503–10. doi: 10.3892/etm.2019.7241

10. Liu Q, Wang X, Huang Z, Liu J, Ding J, Xu X, et al. Ketogenic diet delays spinal fusion and decreases bone mass in posterolateral lumbar spinal fusion: an *in vivo* rat model. *Acta Neurochirurgica* (2018) 160(10):1909–16. doi: 10.1007/s00701-018-3616-7

11. Liu Q, Zhou J, Yang Z, Xie C, Huang Y, Ling L, et al. The ginsenoside exhibits antiosteoporosis effects in ketogenic-diet-induced osteoporosis via rebalancing bone turnover. *Front Pharmacol* (2020) 11:593820. doi: 10.3389/fphar.2020.593820

12. Tagliaferri C, Davicco MJ, Lebecque P, Georgé S, Amiot MJ, Mercier S, et al. Olive oil and vitamin D synergistically prevent bone loss in mice. *PloS One* (2014) 9 (12):e115817. doi: 10.1371/journal.pone.0115817

13. Puel C, Mathey J, Agalias A, Kati-Coulibaly S, Mardon J, Obled C, et al. Dose-response study of effect of oleuropein, an olive oil polyphenol, in an ovariectomy/inflammation experimental model of bone loss in the rat. *Clin Nutr* (2006) 25(5):859–68. doi: 10.1016/j.clnu.2006.03.009

14. Shen CL, Zhu WB, Gao WM, Wang S, Chen LX, Chyu MC. Energy-restricted diet benefits body composition but degrades bone integrity in middle-aged obese female rats. *Nutr Res* (2013) 33(8):668–76. doi: 10.1016/j.nutres.2013.05.008

15. Behrendt AK, Kuhla A, Osterberg A, Polley C, Herlyn P, Fischer DC, et al. Dietary restriction-induced alterations in bone phenotype: effects of lifelong versus short-term caloric restriction on femoral and vertebral bone in C57BL/6 mice. *J Bone Mineral Res* (2016) 31(4):852–63. doi: 10.1002/jbmr.2745

16. Colman RJ, Beasley TM, Allison DB, Weindruch R. Skeletal effects of long-term caloric restriction in rhesus monkeys. *Age* (2012) 34(5):1133–43. doi: 10.1007/s11357-011-9354-x

17. Li ZR, Bowers E, Zhu JX, Yu H, Hardij J, Bagchi DP, et al. Lipolysis of bone marrow adipocytes is required to fuel bone and the marrow niche during energy deficits. *eLife* (2022) 11:32. doi: 10.7554/eLife.78496.sa2

18. Takeda S, Kobayashi Y, Park JH, Ezawa I, Omi N. Effect of different intake levels of dietary protein and physical exercise on bone mineral density and bone strength in growing male rats. *J Nutr Sci Vitaminol* (2012) 58(4):240–6. doi: 10.3177/jnsv.58.240

19. Nebot E, Martinez R, Kapravelou G, Sanchez C, Llopis J, Aranda P, et al. Combination of caloric restriction and a mixed training protocol as an effective strategy to counteract the deleterious effects in trabecular bone microarchitecture caused by a diet-induced obesity in sprague dawley rats. *Nutrients* (2022) 14(18):18. doi: 10.3390/nu14183672

20. Tirapegui J, Ribeiro SML, Pires ISD, Rogero MM. Effects of two different levels of dietary protein on body composition and protein nutritional status of growing rats. *Nutrients* (2012) 4(9):1328–37. doi: 10.3390/nu4091328

21. Kamel M, Badraoui R, Sahnoun Z, Rebai T, Aouidet A. Intermittent fasting effect on bone maturation and remodeling in young rats. *Nutr Clin Metab* (2008) 22(2):44–51. doi: 10.1016/j.nupar.2008.04.001

22. Kamel M, Barkia A, Nasri M, Rebai T, Sahnound Z, Kassis M, et al. Effects of food restriction imposed to adult rats on the bone growth and the histological structure of the thyroid in their youth. *Pathol Biol* (2012) 60(5):296–300. doi: 10.1016/j.patbio.2011.08.001

23. Shin BK, Kang S, Kim DS, Park S. Intermittent fasting protects against the deterioration of cognitive function, energy metabolism and dyslipidemia in Alzheimer's disease-induced estrogen deficient rats. *Exp Biol Med* (2018) 243(4):334–43. doi: 10.1177/1535370217751610

24. Xu XL, Ding JY, Wu XH, Huang ZC, Kong GG, Liu Q, et al. Bone microstructure and metabolism changes under the combined intervention of ketogenic diet with intermittent fasting: an *in vivo* study of rats. *Exp Animals* (2019) 68(3):371–80. doi: 10.1538/expanim.18-0084

25. Hahn TJ, Halstead LR, Devivo DC. Disordered mineral metabolism produced by ketogenic diet therapy. *Calcified Tissue Int* (1979) 28(1):17–22. doi: 10.1007/BF02441213

26. Simm P, Bicknell-Royle J, Nation J, Draffin K, Stewart KG, Cameron F, et al. The effect of the ketogenic diet on the developing skeleton. *Epilepsia* (2013) 54:5–. doi: 10.1016/j.eplepsyres.2017.07.014

27. Svedlund A, Hallbook T, Magnusson P, Dahlgren J, Swolin-Eide D. Longitudinal study of bone mass in swedish children treated with modified ketogenic diet. *Hormone Res Paediatrics* (2018) 90:169–70. doi: 10.1159/000492307

28. Gomez-Arbelaez D, Bellido D, Castro AI, Ordonez-Mayan L, Carreira J, Galban C, et al. Body composition changes after very-low-calorie ketogenic diet in obesity evaluated by 3 standardized methods. *J Clin Endocrinol Metab* (2017) 102(2):488–98. doi: 10.1210/jc.2016-2385

29. Athinarayanan SJ, Adams RN, Hallberg SJ, McKenzie AL, Bhanpuri NH, Campbell WW, et al. Long-term effects of a novel continuous remote care intervention including nutritional ketosis for the management of type 2 diabetes: A 2-year non-randomized clinical trial. *Front Endocrinol* (2019) 10. doi: 10.3389/fendo.2019.00348

30. Bertoli S, Trentani C, Ferraris C, De Giorgis V, Veggiotti P, Tagliabue A. Long-term effects of a ketogenic diet on body composition and bone mineralization in GLUT-1 deficiency syndrome: A case series. *Nutrition* (2014) 30(6):726–8. doi: 10.1016/j.nut.2014.01.005

31. Vargas-Molina S, Carbone L, Romance R, Petro JL, Schoenfeld BJ, Kreider RB, et al. Effects of a low-carbohydrate ketogenic diet on health parameters in resistance-trained women. *Eur J Appl Physiol* (2021) 121(8):2349–59. doi: 10.1007/s00421-021-04707-3

32. Carter JD, Vasey FB, Valeriano J. The effect of a low-carbohydrate diet on bone turnover. *Osteoporosis Int* (2006) 17(9):1398–403. doi: 10.1007/s00198-006-0134-x

33. Heikura IA, Burke LM, Hawley JA, Ross ML, Garvican-Lewis L, Sharma AP, et al. A short-term ketogenic diet impairs markers of bone health in response to exercise. *Front Endocrinol* (2020) 10. doi: 10.3389/fendo.2019.00880

34. Draaisma JMT, Hampsink BM, Janssen M, van Houdt NBM, Linders ETAM, Willemsen MA. The ketogenic diet and its effect on bone mineral density: A retrospective observational cohort study. *Neuropediatrics* (2019) 50(6):353–8. doi: 10.1055/s-0039-1693059

35. Nestares T, Martín-Masot R, de Teresa C, Bonillo R, Maldonado J, Flor-Alemany M, et al. Influence of mediterranean diet adherence and physical activity on bone health in celiac children on a gluten-free diet. *Nutrients* (2021) 13(5):1636. doi: 10.3390/nu13051636

36. Seiquer I, Mesías M, Hoyos AM, Galdó G, Navarro MP. A Mediterranean dietary style improves calcium utilization in healthy male adolescents. *J Am Coll Nutr* (2008) 27 (4):454–62. doi: 10.1080/07315724.2008.10719725

37. Julián C, Huybrechts I, Gracia-Marco L, González-Gil EM, Gutiérrez Á, González-Gross M, et al. Mediterranean diet, diet quality, and bone mineral content in adolescents: the HELENA study. *Osteoporos Int* (2018) 29(6):1329–40. doi: 10.1007/s00198-018-4427-7

38. Pérez-Rey J, Roncero-Martín R, Rico-Martín S, Rey-Sánchez P, Pedrera-Zamorano JD, Pedrera-Canal M, et al. Adherence to a mediterranean diet and bone mineral density in spanish premenopausal women. *Nutrients* (2019) 11(3):555. doi: 10.3390/nu11030555

39. Cervo MMC, Scott D, Seibel MJ, Cumming RG, Naganathan V, Blyth FM, et al. Adherence to Mediterranean diet and its associations with circulating cytokines, musculoskeletal health and incident falls in community-dwelling older men: The Concord Health and Ageing in Men Project. *Clin Nutr* (2021) 40(12):5753–63. doi: 10.1016/j.clnu.2021.10.010

40. Feart C, Lorrain S, Ginder Coupez V, Samieri C, Letenneur L, Paineau D, et al. Adherence to a Mediterranean diet and risk of fractures in French older persons. *Osteoporos Int* (2013) 24(12):3031–41. doi: 10.1007/s00198-013-2421-7

41. Villareal DT, Fontana L, Das SK, Redman L, Smith SR, Saltzman E, et al. Effect of two-year caloric restriction on bone metabolism and bone mineral density in non-obese younger adults: A randomized clinical trial. *J Bone Mineral Res* (2016) 31(1):40–51. doi: 10.1002/jbmr.2701

42. Tirosh A, de Souza RJ, Sacks F, Bray GA, Smith SR, LeBoff MS. Sex differences in the effects of weight loss diets on bone mineral density and body composition: POUNDS LOST trial. *J Clin Endocrinol Metab* (2015) 100(6):2463–71. doi: 10.1210/jc.2015-1050

43. Pop LC, Sukumar D, Tomaino K, Schlussel Y, Schneider SH, Gordon CL, et al. Moderate weight loss in obese and overweight men preserves bone quality. *Am J Clin Nutr* (2015) 101(3):659–67. doi: 10.3945/ajcn.114.088534

44. Von Thun NL, Sukumar D, Heymsfield SB, Shapses SA. Does bone loss begin after weight loss ends? Results 2 years after weight loss or regain in postmenopausal women. *Menopause-J N Am Menopause Soc* (2014) 21(5):501–8. doi: 10.1097/GME.0b013e3182a76fd5

45. Hinton PS, Rector RS, Linden MA, Warner SO, Dellsperger KC, Chockalingam A, et al. Weight-loss-associated changes in bone mineral density and bone turnover after partial weight regain with or without aerobic exercise in obese women. *Eur J Clin Nutr* (2012) 66(5):606–12. doi: 10.1038/ejcn.2011.212

46. Armamento-Villareal R, Aguirre L, Napoli N, Shah K, Hilton T, Sinacore DR, et al. Changes in thigh muscle volume predict bone mineral density response to lifestyle therapy in frail, obese older adults. *Osteoporosis Int* (2014) 25(2):551–8. doi: 10.1007/s00198-013-2450-2

47. Antonio J, Ellerbroek A, Evans C, Silver T, Peacock CA. High protein consumption in trained women: bad to the bone? *J Int Soc Sport Nutr* (2018) 15:5. doi: 10.1186/s12970-018-0210-6

48. Lee CL, Tsai SF. The impact of protein diet on bone density in people with/without chronic kidney disease: An analysis of the National Health and Nutrition Examination Survey database. *Clin Nutr* (2020) 39(11):3497–503. doi: 10.1016/j.clnu.2020.03.014

49. Gao SH, Qian X, Huang SC, Deng WX, Li Z, Hu YY. Association between macronutrients intake distribution and bone mineral density. *Clin Nutr* (2022) 41 (8):1689–96. doi: 10.1016/j.clnu.2022.05.019

50. Murphy C, Bilek LD, Koehler K. Low energy availability with and without a high-protein diet suppresses bone formation and increases bone resorption in men: A randomized controlled pilot study. *Nutrients* (2021) 13(3):12. doi: 10.3390/nu13030802

51. Martens CR, Rossman MJ, Mazzo MR, Jankowski LR, Nagy EE, Denman BA, et al. Short-term time-restricted feeding is safe and feasible in non-obese healthy midlife and older adults. *Geroscience* (2020) 42(2):667–86. doi: 10.1007/s11357-020-00156-6

52. Clayton DJ, James LJ, Sale C, Templeman I, Betts JA, Varley I. Severely restricting energy intake for 24 h does not affect markers of bone metabolism at rest or in response to re-feeding. *Eur J Nutr* (2020) 59(8):3527–35. doi: 10.1007/s00394-020-02186-4

53. Papageorgiou M, Martin D, Colgan H, Cooper S, Greeves JP, Tang JCY, et al. Bone metabolic responses to low energy availability achieved by diet or exercise in active eumenorrheic women. *Bone* (2018) 114:181–8. doi: 10.1016/j.bone.2018.06.016

54. Wu X, Ding J, Xu X, Wang X, Liu J, Jiang J, et al. Ketogenic diet compromises vertebral microstructure and biomechanical characteristics in mice. *J Bone Mineral Metab* (2019) 37(6):957–66. doi: 10.1007/s00774-019-01002-2

55. Liu Q, Yang Z, Xie C, Ling L, Hu H, Cao Y, et al. The hyperglycemia and hyperketonemia impaired bone microstructures: A pilot study in rats. *Front Endocrinol (Lausanne)* (2020) 11:590575. doi: 10.3389/fendo.2020.590575

56. Xu X, Ding J, Wu X, Huang Z, Kong G, Liu Q, et al. Bone microstructure and metabolism changes under the combined intervention of ketogenic diet with intermittent fasting: an *in vivo* study of rats. *Exp Animals* (2019) 68(3):371–80. doi: 10.1538/expanim.18-0084

57. Liu Q, Xu X, Yang Z, Liu Y, Wu X, Huang Z, et al. Metformin alleviates the bone loss induced by ketogenic diet: an *in vivo* study in mice. *Calcif Tissue Int* (2019) 104 (1):59–69. doi: 10.1007/s00223-018-0468-3

58. Yang Z, Liu Q, Xu X, Liu Y, Wu X, Huang Z, et al. Simvastatin alleviates the bone loss caused by ketogenic diet: an *in vivo* study. *Chin J Osteoporosis* (2019) 25 (7):899–904,74. doi: 10. 3969 /j.issn.1006-7108. 2019. 07. 003

59. Sugiyama M, Kodama T, Konishi K, Abe K, Asami S, Oikawa S. Compactin and simvastatin, but not pravastatin, induce bone morphogenetic protein-2 in human osteosarcoma cells. *Biochem Biophys Res Commun* (2000) 271(3):688–92. doi: 10.1006/bbrc.2000.2697

60. Shellhaas RA, Joshi SM. Vitamin D and bone health among children with epilepsy. *Pediatr Neurol* (2010) 42(6):385–93. doi: 10.1016/j.pediatrneurol.2009.12.005

61. Hawkes CP, Levine MA. Ketotic hypercalcemia: A case series and description of a novel entity. *J Clin Endocrinol Metab* (2014) 99(5):1531–6. doi: 10.1210/jc.2013-4275

62. Hawkes CP, Roy SM, Dekelbab B, Frazier B, Grover M, Haidet J, et al. Hypercalcemia in children using the ketogenic diet: A multicenter study. *J Clin Endocrinol Metab* (2021) 106(2):E485–E95. doi: 10.1210/clinem/dgaa759

63. Davis C, Bryan J, Hodgson J, Murphy K. Definition of the mediterranean diet; a literature review. *Nutrients* (2015) 7(11):9139–53. doi: 10.3390/nu7115459

64. Bach-Faig A, Berry EM, Lairon D, Reguant J, Trichopoulou A, Dernini S, et al. Mediterranean diet pyramid today. Science and cultural updates. *Public Health Nutr* (2011) 14(12A):2274–84. doi: 10.1017/S1368980011002515

65. Romero Pérez A, Rivas Velasco A. Adherence to Mediterranean diet and bone health. *Nutr Hosp* (2014) 29(5):989–96.

66. Tresserra-Rimbau A, Rimm EB, Medina-Remón A, Martínez-González MA, López-Sabater MC, Covas MI, et al. Polyphenol intake and mortality risk: a re-analysis of the PREDIMED trial. *BMC Med* (2014) 12:77. doi: 10.1186/1741-7015-12-77

67. Puel C, Mardon J, Agalias A, Davicco MJ, Lebecque P, Mazur A, et al. Major phenolic compounds in olive oil modulate bone loss in an ovariectomy/inflammation experimental model. *J Agric Food Chem* (2008) 56(20):9417–22. doi: 10.1021/jf801794q

68. Correa-Rodríguez M, El Mansouri-Yachou J, Tapia-Haro RM, Molina F, Rus A, Rueda-Medina B, et al. Mediterranean diet, body composition, and activity associated with bone health in women with fibromyalgia syndrome. *Nurs Res* (2019) 68(5):358–64. doi: 10.1097/NNR.0000000000000361

69. Silva TRD, Martins CC, Ferreira LL, Spritzer PM. Mediterranean diet is associated with bone mineral density and muscle mass in postmenopausal women. *Climacteric* (2019) 22(2):162–8. doi: 10.1080/13697137.2018.1529747

70. Rivas A, Romero A, Mariscal-Arcas M, Monteagudo C, Feriche B, Lorenzo ML, et al. Mediterranean diet and bone mineral density in two age groups of women. *Int J Food Sci Nutr* (2013) 64(2):155–61. doi: 10.3109/09637486.2012.718743

71. Kontogianni MD, Melistas L, Yannakoulia M, Malagaris I, Panagiotakos DB, Yiannakouris N. Association between dietary patterns and indices of bone mass in a sample of Mediterranean women. *Nutrition* (2009) 25(2):165–71. doi: 10.1016/j.nut.2008.07.019

72. Aparicio VA, Ruiz-Cabello P, Borges-Cosic M, Andrade A, Coll-Risco I, Acosta-Manzano P, et al. Association of physical fitness, body composition, cardiometabolic markers and adherence to the Mediterranean diet with bone mineral density in perimenopausal women. The FLAMENCO project. *J Sports Sci* (2017) 35(9):880–7. doi: 10.1080/02640414.2016.1196825

73. Malmir H, Saneei P, Larijani B, Esmaillzadeh A. Adherence to Mediterranean diet in relation to bone mineral density and risk of fracture: a systematic review and meta-analysis of observational studies. *Eur J Nutr* (2018) 57(6):2147–60. doi: 10.1007/s00394-017-1490-3

74. Byberg L, Bellavia A, Larsson SC, Orsini N, Wolk A, Michaëlsson K. Mediterranean diet and hip fracture in swedish men and women. *J Bone Miner Res* (2016) 31(12):2098–105. doi: 10.1002/jbmr.2896

75. Palomeras-Vilches A, Viñals-Mayolas E, Bou-Mias C, Jordà-Castro M, Agüero-Martínez M, Busquets-Barceló M, et al. Adherence to the mediterranean diet and bone fracture risk in middle-aged women: A case control study. *Nutrients* (2019) 11(10):989–96. doi: 10.3390/nu11102508

76. Zeng FF, Xue WQ, Cao WT, Wu BH, Xie HL, Fan F, et al. Diet-quality scores and risk of hip fractures in elderly urban Chinese in Guangdong, China: a case-control study. *Osteoporos Int* (2014) 25(8):2131–41. doi: 10.1007/s00198-014-2741-2

77. Chen GD, Dong XW, Zhu YY, Tian HY, He J, Chen YM. Adherence to the Mediterranean diet is associated with a higher BMD in middle-aged and elderly Chinese. *Sci Rep* (2016) 6:25662. doi: 10.1038/srep25662

78. García-Gavilán JF, Paz-Graniel I, Babio N, Romaguera D, Martínez JA, Martin V, et al. Inflammatory potential of diet and bone mineral density in a senior Mediterranean population: a cross-sectional analysis of PREDIMED-Plus study. *Eur J Nutr* (2022) 61(3):1445–55. doi: 10.1007/s00394-021-02751-5

79. Erkkilä AT, Sadeghi H, Isanejad M, Mursu J, Tuppurainen M, Kröger H. Associations of Baltic Sea and Mediterranean dietary patterns with bone mineral density in elderly women. *Public Health Nutr* (2017) 20(15):2735–43. doi: 10.1017/S1368980017001793

80. Jennings A, Cashman KD, Gillings R, Cassidy A, Tang J, Fraser W, et al. A Mediterranean-like dietary pattern with vitamin D3 (10 μg/d) supplements reduced the rate of bone loss in older Europeans with osteoporosis at baseline: results of a 1-y randomized controlled trial. *Am J Clin Nutr* (2018) 108(3):633–40. doi: 10.1093/ajcn/nqy122

81. Loued S, Berrougui H, Componova P, Ikhlef S, Helal O, Khalil A. Extra-virgin olive oil consumption reduces the age-related decrease in HDL and paraoxonase 1 anti-inflammatory activities. *Br J Nutr* (2013) 110(7):1272–84. doi: 10.1017/S0007114513000482

82. Liu DY, Huang Y, Huang CSH, Yang SY, Wei XY, Zhang PZ, et al. Calorie restriction with or without time-restricted eating in weight loss. *New Engl J Med* (2022) 386(16):1495–504. doi: 10.1056/NEJMoa2114833

83. Ravussin E, Redman LM, Rochon J, Das SK, Fontana L, Kraus WE, et al. A 2-year randomized controlled trial of human caloric restriction: feasibility and effects on predictors of health span and longevity. *J Gerontol Ser A-Biol Sci Med Sci* (2015) 70 (9):1097–104. doi: 10.1093/gerona/glv057

84. Shapses SA, Sukumar D. Bone metabolism in obesity and weight loss. In: Cousins RJ, editor. *Annual Review of Nutrition, Vol 32*. Palo Alto: Annual Reviews (2012). p. 287–309.

85. Cawthorn WP, Scheller EL, Parlee SD, Pham HA, Learman BS, Redshaw CMH, et al. Expansion of bone marrow adipose tissue during caloric restriction is associated with increased circulating glucocorticoids and not with hypoleptinemia. *Endocrinology* (2016) 157(2):508–21. doi: 10.1210/en.2015-1477

86. Devlin MJ, Brooks DJ, Conlon C, van Vliet M, Louis L, Rosen CJ, et al. Daily leptin blunts marrow fat but does not impact bone mass in calorie-restricted mice. *J Endocrinol* (2016) 229(3):295–306. doi: 10.1530/JOE-15-0473

87. Walji TA, Turecamo SE, Sanchez AC, Anthony BA, Abou-Ezzi G, Scheller EL, et al. Marrow adipose tissue expansion coincides with insulin resistance in MAGP1-deficient mice. *Front Endocrinol Editorial Mater* (2016) 7:9. doi: 10.3389/fendo.2016.00087

88. Scheller EL, Khandaker S, Learman BS, Cawthorn WP, Anderson LM, Pham HA, et al. Bone marrow adipocytes resist lipolysis and remodeling in response to beta-adrenergic stimulation. *Bone* (2019) 118:32–41. doi: 10.1016/j.bone.2018.01.016

89. Pierce JL, Ding KH, Xu JR, Sharma AK, Yu KL, Arbona ND, et al. The glucocorticoid receptor in osteoprogenitors regulates bone mass and marrow fat. *J Endocrinol* (2019) 243(1):27–42. doi: 10.1530/JOE-19-0230

90. Hardouin P, Rharass T, Lucas S. Bone marrow adipose tissue: to be or not to be a typical adipose tissue? *Front Endocrinol* (2016) 7:11. doi: 10.3389/fendo.2016.00085

91. Rendina-Ruedy E, Rosen CJ. Bone-fat interaction. *Endocrinol Metabol Clin North Amer* (2017) 46(1):41. doi: 10.1016/j.ecl.2016.09.004

92. Zhu JX, Liu C, Jia JL, Zhang CG, Yuan WQ, Leng HJ, et al. Short-term caloric restriction induced bone loss in both axial and appendicular bones by increasing adiponectin. *Ann NY Acad Sci* (2020) 1474(1):47–60. doi: 10.1111/nyas.14380

93. Romashkan SV, Das SK, Villareal DT, Ravussin E, Redman LM, Rochon J, et al. Safety of two-year caloric restriction in non-obese healthy individuals. *Oncotarget* (2016) 7(15):19124–33. doi: 10.18632/oncotarget.8093

94. Hosny IA, Elghawabi HS, Younan WBF, Sabbour AA, Gobrial MAM. Beneficial impact of aerobic exercises on bone mineral density in obese premenopausal women under caloric restriction. *Skeletal Radiol* (2012) 41(4):423–7. doi: 10.1007/s00256-011-1196-1

95. Beavers KM, Beavers DP, Martin SB, Marsh AP, Lyles MF, Lenchik L, et al. Change in bone mineral density during weight loss with resistance versus aerobic exercise training in older adults. *J Gerontol Ser A-Biol Sci Med Sci* (2017) 72(11):1582–5. doi: 10.1093/gerona/glx048

96. Armamento-Villareal R, Sadler C, Napoli N, Shah K, Chode S, Sinacore DR, et al. Weight loss in obese older adults increases serum sclerostin and impairs hip geometry but both are prevented by exercise training. *J Bone Mineral Res* (2012) 27(5):1215–21. doi: 10.1002/jbmr.1560

97. Murphy C, Koehler K. Caloric restriction induces anabolic resistance to resistance exercise. *Eur J Appl Physiol* (2020) 120(5):1155–64. doi: 10.1007/s00421-020-04354-0

98. Metzger CE, Baek K, Swift SN, De Souza MJ, Bloomfield SA. Exercise during energy restriction mitigates bone loss but not alterations in estrogen status or metabolic hormones. *Osteoporosis Int* (2016) 27(9):2755–64. doi: 10.1007/s00198-016-3590-y

99. Metzger CE, Swift SN, Baek K, De Souza MJ, Bloomfield SA. Fat and lean mass predict bone mass during energy restriction in sedentary and exercising rodents. *Front Physiol* (2018) 9:11. doi: 10.3389/fphys.2018.01346

100. Cao JJ. Caloric restriction combined with exercise is effective in reducing adiposity and mitigating bone structural deterioration in obese rats. *Ann NY Acad Sci* (2018) 1433(1):41–52. doi: 10.1111/nyas.13936

101. Hattori S, Park JH, Agata U, Akimoto T, Oda M, Higano M, et al. Influence of food restriction combined with voluntary running on bone morphology and strength in male rats. *Calcified Tissue Int* (2013) 93(6):540–8. doi: 10.1007/s00223-013-9787-6

102. Hattori S, Park JH, Agata U, Oda M, Higano M, Aikawa Y, et al. Food restriction causes low bone strength and microarchitectural deterioration in exercised growing male rats. *J Nutr Sci Vitaminol* (2014) 60(1):35–42. doi: 10.3177/jnsv.60.35

103. McGrath C, Sankaran JS, Misaghian-Xanthos N, Sen B, Xie ZH, Styner MA, et al. Exercise degrades bone in caloric restriction, despite suppression of marrow adipose tissue (MAT). *J Bone Mineral Res* (2020) 35(1):106–15. doi: 10.1002/jbmr.3872

104. Silk LN, Greene DA, Baker MK, Jander CB. Tibial bone responses to 6-month calcium and vitamin D supplementation in young male jockeys: A randomised controlled trial. *Bone* (2015) 81:554–61. doi: 10.1016/j.bone.2015.09.004

105. Sukumar D, Shapses SA, Schneider SH. Vitamin D supplementation during short-term caloric restriction in healthy overweight/obese older women: Effect on glycemic indices and serum osteocalcin levels. *Mol Cell Endocrinol* (2015) 410(C):73–7. doi: 10.1016/j.mce.2015.01.002

106. Shapses SA, Sukumar D, Schneider SH, Schlussel Y, Sherrell RM, Field MP, et al. Vitamin D supplementation and calcium absorption during caloric restriction: a randomized double-blind trial. *Am J Clin Nutr* (2013) 97(3):637–45. doi: 10.3945/ajcn.112.044909

107. Mason C, Tapsoba JD, Duggan C, Imayama I, Wang CY, Korde L, et al. Effects of vitamin D-3 supplementation on lean mass, muscle strength, and bone mineral density during weight loss: A double-blind randomized controlled trial. *J Am Geriatr Soc* (2016) 64(4):769–78. doi: 10.1111/jgs.14049

108. Duque G, Al Saedi A, Rivas D, Miard S, Ferland G, Picard F, et al. Differential effects of long-term caloric restriction and dietary protein source on bone and marrow fat of the aging rat. *J Gerontol Ser A-Biol Sci Med Sci* (2020) 75(11):2031–6. doi: 10.1093/gerona/glaa093

109. Razny U, Goralska J, Calder PC, Gruca A, Childs CE, Kapusta M, et al. The Effect of Caloric Restriction with and without n-3 PUFA Supplementation on Bone Turnover Markers in Blood of Subjects with Abdominal Obesity: A Randomized Placebo-Controlled Trial. *Nutrients* (2021) 13(9):20. doi: 10.3390/nu13093096

110. Westerterp-Plantenga MS, Lemmens SG, Westerterp KR. Dietary protein - its role in satiety, energetics, weight loss and health. *Br J Nutr* (2012) 108:S105–S12. doi: 10.1017/S0007114512002589

111. Ajala O, English P, Pinkney J. Systematic review and meta-analysis of different dietary approaches to the management of type 2 diabetes. *Am J Clin Nutr Rev* (2013) 97 (3):505–16. doi: 10.3945/ajcn.112.042457

112. Devries MC, Phillips SM. Supplemental protein in support of muscle mass and health: advantage whey. *J Food Sci* (2015) 80:A8–A15. doi: 10.1111/1750-3841.12802

113. Cao JJ. High dietary protein intake and protein-related acid load on bone health. *Curr Osteoporos Rep Rev* (2017) 15(6):571–6. doi: 10.1007/s11914-017-0408-6

114. Nebot E, Aparicio VA, Coll-Risco I, Camiletti-Moiron D, Schneider J, Kapravelou G, et al. Effects of a moderately high-protein diet and interval aerobic training combined with strength-endurance exercise on markers of bone metabolism, microarchitecture and turnover in obese Zucker rats. *Bone* (2016) 92:116–23. doi: 10.1016/j.bone.2016.08.017

115. Veronese N, Reginster JY. The effects of calorie restriction, intermittent fasting and vegetarian diets on bone health. *Aging Clin Exp Res Rev* (2019) 31 (6):753–8. doi: 10.1007/s40520-019-01174-x

116. Patterson RE, Sears DD. Metabolic effects of intermittent fasting. In: Stover PJ, Balling R, editors. *Annual Review of Nutrition, Vol 37*. Palo Alto: Annual Reviews (2017). p. 371–93.

117. Scholtens EL, Krebs JD, Corley BT, Hall RM. Intermittent fasting 5:2 diet: What is the macronutrient and micronutrient intake and composition? *Clin Nutr* (2020) 39 (11):3354–60. doi: 10.1016/j.clnu.2020.02.022

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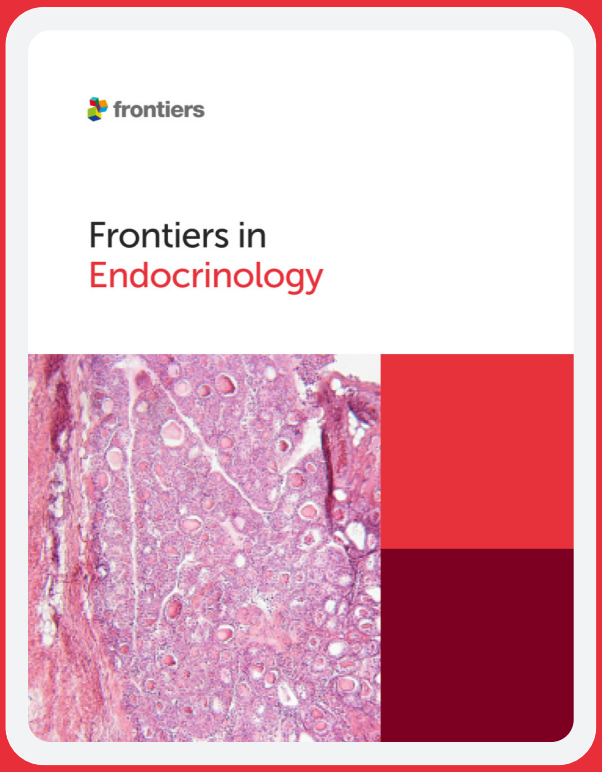

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