Trends in
Endocrinology & Metabolism

CellPress

Review

Flavonoids in Bone Erosive Diseases: Perspectives in Osteoporosis Treatment

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Imbalance of bone homeostasis, with excessive bone resorption compared with bone formation, leads to the development of progressive osteopenia leading to lower bone resistance to load, with consequent pain and functional limitations. Phytochemicals with therapeutic and preventive effects against bone resorption have recently received increasing attention since they are potentially more suitable for long-term use than traditional therapeutic chemical compounds. In this systematic review of the literature of the past 5 years, comprehensive information is provided on flavonoids with potential antiresorption and pro-osteogenic effects. It aims to highlight the molecular mechanisms of these molecules, often epigenetic, and their possible pharmacological use, which is of great importance for the prevention and treatment of osteoporosis (OP).

Highlights

Flavonoids act to promote bone deposition and inhibit bone resorption.

Flavonoids, through reduction of oxidative stress, favor bone deposition and inhibit bone resorption, regulating bone homeostasis.

Flavonoids act through several mechanisms, such as epigenetic regulation (acetylation/deacetylation, miRNA expression).

Flavonoids are demonstrated to act on osteoblast differentiation and activity.

Flavonoids: Bone Homeostasis and Osteoporosis Treatment

Osteoporosis (OP) is a worldwide chronic progressive bone disease characterized by increased bone resorption leading to bone loss and reduced mineral content, which lead to increased risk of bone fractures (≥8.9 million fractures/year) [1]. OP is often associated with a reduced quality of life, due to disability, pain, and depression, often related to fractures (i.e., hip, vertebra, wrist). Primary OP occurs mainly with aging or during postmenopause, while secondary OP is related to various causes, such as lifestyle, systemic pathologies (i.e., diabetes, hypothyroidism), and long-term drug therapy (i.e., glucocorticoids) [2,3]. Bone fragility in OP is determined by various factors: (i) decreased osteoblast (OB) differentiation and activity (Box 1), resulting in reduced bone deposition [4]; (ii) increased osteoclast (OC) differentiation and activity (Box 2), resulting in excessive bone resorption [4]; and (iii) both phenomena through the modulation of signaling pathways involved in inflammation and oxidative status (Box 3) [5–9].

The increase in life expectancy and the rapid growth of the elderly population, in conjunction with the high prevalence of diseases related to bone loss in the elderly, such as OP, has led to an increase in the financial burden for the health system [10]. Currently, there are few approved drugs for OP treatment, divided into two classes: the antiresorptive drugs, such as bisphosphonates, hormones, raloxifene, and monoclonal antibodies (denosumab, romosozumab), and the anabolic ones, such as teriparatide, that induce new bone formation and increase bone density. Estrogen receptors (ERs) have been used in hormone replacement therapy (HRT) but have been limited due to suspected influence on cancer risk, heart attack, and stroke [11]. These aspects prompted the search for new therapeutic agents and nutritional supplements for the management and promotion of bone health. Calcium, vitamin D, and other nutritional factors help in the maintenance of skeletal health during aging, supporting bone matrix production and mineralization [12].

Recently, phytochemicals, most of which are flavonoids with antioxidative activities, have emerged as potential therapeutic agents in a broad range of degenerative pathologies involving

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Box 1. Osteoblast Differentiation Signaling: Extremely High-Complexity System

OB differentiation and activity are controlled by a dense network of signaling pathways, where Wnt, BMPs, and TGF-β signals interact positively or negatively with other signals, such as bFGF, Hedgehog (Hh), Notch, and IGF-1, in the regulation of osteoblastogenesis, activating RUNX-2, the master regulator of OB differentiation, and Osterix (OSX), a transcription factor of OB effector genes (Figure I) [4]. Wnt signaling through the Frizzled (Fz)/LDL receptor-related protein (LRP)-5/6 interaction lead to axin sequestration, disrupting the complex of axin/adenomatous polyposis coli (APC)/GSK-3β, which is implicated in β -catenin degradation. Nuclear translocation of β-catenin leads to activation of the TGF-β and BMP-2 genes through interaction with the transcription factors TCF and lymphoid enhancer-binding factor (LEF). PTH and E-cadherin pathways help in the activation of β-catenin through its stabilization. Furthermore, Wnt signaling enhances the transcription of both OPG and RANKL, leading to an overall RANKL/OPG ratio reduction, inhibiting OC differentiation. Wnt signaling, through the cofactor ROR2 or RYK, also leads to the activation of RUNX-2 in a noncanonical pathway, leading to OB differentiation commitment and inhibition of the PPAR-γ pathway, which is involved in adipocyte differentiation. Wnt signaling is blocked by the direct binding of Wnt or by molecules that interfere with receptor or coreceptor binding. The Dkk, kremen, and sclerostin factors inhibit the LRP-5/6 cofactor, while secreted Fz-related protein (sFRP) binds Wnt [4]. BMP/BMP receptor (BMPR) interactions induce the autophosphorylation of receptors, leading to the recruitment and activation of the SMA- and MAD-related protein 1/5/8 (Smad-1/5/8) protein complex, the binding of Smad-4, and its consequent translocation into the nucleus, where it leads to the expression of DLX-5, RUNX-2, and OSX. BMP pathway activation is also responsible for the kinase signaling cascade that ends with the activation of p38-kinase, a MAPK family member, which leads to OB maturation through the phosphorylation of RUNX-2, DLX-5, and OSX. The BMP pathway has various repressors: Noggin inhibits BMP binding and Smad-6 induces Smad-1/5/8 degradation, while Smad-7 inhibits BMPR autophosphorylation and then Smad-1/5/8 complex activation. BMPs, activin membrane-bound inhibitor (BAMBI), and cysteine-rich motor neuron-1 (CRIM-1) are other negative regulators of BMP signaling in BMSCs [4]. TGF-β, through interaction with their receptors, activates Smad-2/3 with Smad-4 recruitment, leading to the expression of various factors such as Wnt. Smad-7 and SMURF are TGF-β pathway repressors. Smad-7 inhibits TGF-β receptor autophosphorylation and nuclear translocation of the Smad-2/3–Smad-4 complex. Furthermore, Smad-7 and the SMURF complex lead to Smad-2/3 degradation [4]. Autophosphorylation of the IGF-1R by ligand interaction leads to PI3K activation, which catalyzes the phosphorylation of phosphatidylinositol bisphosphate (PIP2) to phosphatidylinositol trisphosphate (PIP3), activating protein kinase B (Akt) and consequently mammalian target of rapamycin (mTOR), favoring OB survival, and inhibits FOXO1 causing OCN, FGF, and RUNX-2 downregulation. FGF pathway activation leads to phospholipase C (PLC) activity, catalyzing the formation of diacylglycerol (DAG) and inositol trisphosphate (IP3) by PIP3. DAG activates protein kinase C (PKC), which increases RUNX-2 activity by phosphorylation through MAPK activation. Furthermore, the MAPK pathway with ERK protein activation leads to nuclear exportation of the ERF protein, an inhibitor of RUNX-2 activity, improving osteogenesis [4]. Proteolytic cleavage of the Notch receptor after interaction with its ligands (Delta and Jagged) determines Notch intracellular domain (NICD) release and its nuclear translocation, where, with core-binding factor-1 (CBF-1), it leads to the transcription of HES and HEY, which negatively regulate RUNX-2 [4]. The Hh/Patched-1 (PTC-1) interaction leads to smothered (SMO) activation with the consequent liberation of GLI proteins by SUFU, their translocation, and the transcription of BMP-2 and WNT. All pathways converge on the expression and activation of RUNX-2 and OSX, which are the master regulators responsible for OB commitment and the transcription factor for OB effector genes, respectively [4].

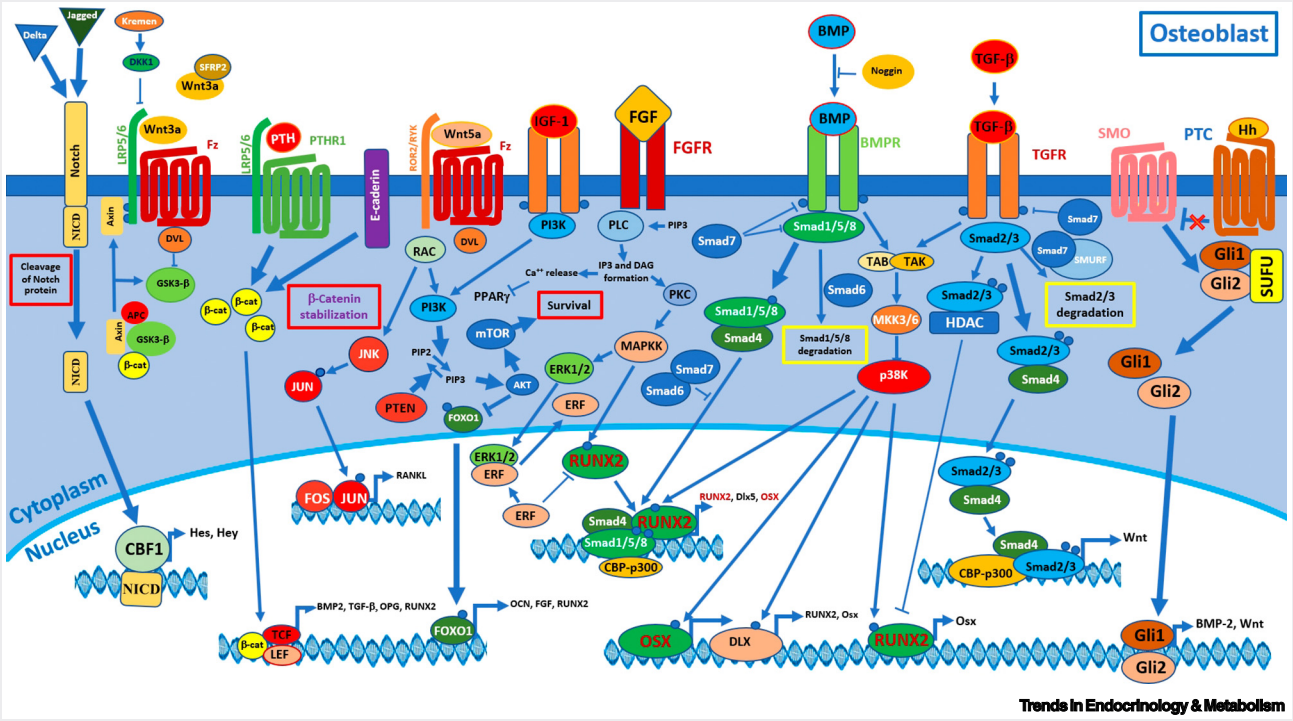

Figure I. Schematic Diagram Representing the Complexity of Network Pathways in Osteoblast (OB) Differentiation.

Box 2. Osteoclast Differentiation Signaling: RANKL/OPG Ratio and the M-CSF/c-FMS System

The osteoclastogenesis process is regulated by two pathways: RANK/RANKL and the macrophage colony-stimulating factor (M-CSF)/colony-stimulating factor-1 receptor (c-FMS) system. The RANK/RANKL/OPG system is the first regulatory system of OC differentiation and activity. Osteotrophic factors, such as PTH and calcitriol, induce OBs to produce RANKL, enhancing osteoclastogenesis through RANK stimulation. This activation is counteracted by the synthesis of OPG, which binds free RANKL interfering with this signal. The second signal pathway is regulated by the M-CSF/c-FMS interaction that leads to MAPK activation, enhancing RANKL production, and the AKT/mTOR pathway that guarantees the survival of differentiating OCs (Figure I) [4]. The RANK/RANKL pathway is the first regulator of OC differentiation. The RANK/RANKL interaction induces the recruitment and activation of the adaptor protein TNF receptor-associated factor 6 (TRAF6), which mediates the activation of several kinases, such as inhibitor of kappa B kinase (IKK), dual-specificity MAPK-1 (MAP2K-1/MEK-1), -6 (MAP2K-6/MKK-6), and -7 (MAP2K-7/MKK-7), the proto-oncogene tyrosine-protein kinase Src (SRC), and PLC. IKK is implicated in the inactivation of NF-κB inhibitor (IKB) by phosphorylation, leading to its degradation and inducing NF-κB activation. MEK-1 activates ERK (MAPK family) and then the transcription factor FOS. MKK-6 activates p38 kinase (MAPK family), implicated in the activation of the microphthalmia-associated transcription factor (MITF), fundamental for the expression of OC effector genes such as the CTSK and TRAP enzymes. MKK-7 activates JNK-1, another member of the MAPK family, which leads to the activation of c-Jun. The SRC protein induces the activity of the PI3K/Akt/mTOR pathway, increasing survival during OC differentiation. The formation of a transcription complex containing dimer activator protein 1 (AP-1), comprising c-Fos and c-Jun, and NF-κB, activates the expression of NFAT-c1, the master regulator of OC activity genes such as TRAP, CTSK, MMP-9, MMP-13, etc. TGF-β is able to induce RANK expression in macrophages, promoting osteoclastogenesis. This pathway is in contrast to the synthesis of OPG, which binds RANKL interfering with signal activation. The M-CSF/c-FMS system interaction leads to the activation of MEK-1, improving the signaling of RANKL in AP-1 activation, and increases PI3K activation of the PI3K/AKT/mTOR pathway that guarantees OC survival.

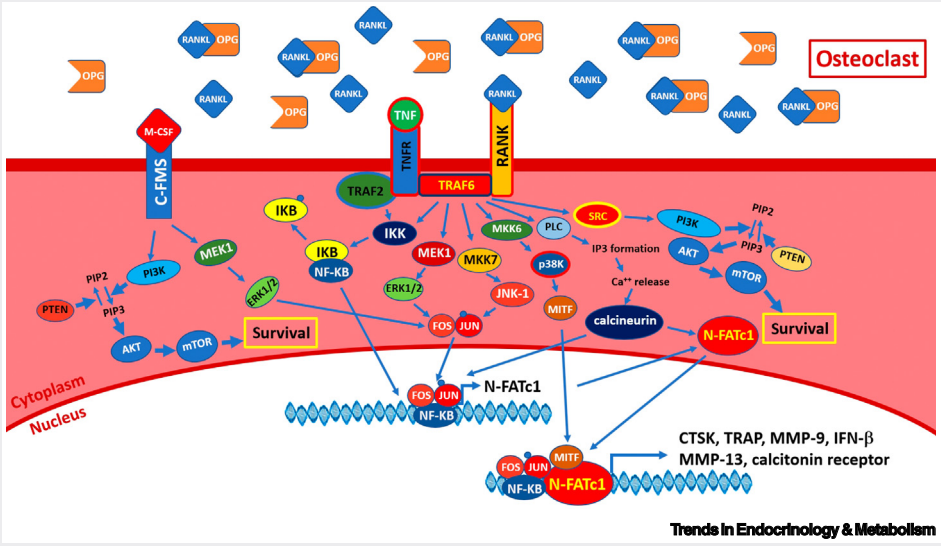

Figure I. Schematic Diagram Representing the Complexity of Network Pathways in Osteoclast (OC) Differentiation.

oxidative stress [13]. Several show specific regulatory effects on bone cells and they seem to be potentially more suitable for long-term use than traditional therapeutic agents [14,15].

This systematic review provides an exhaustive overview of the literature on flavonoids isolated from plants over the past 5 years that show protective effects against OP through their effects on the survival, proliferation, and differentiation of cells implicated in bone homeostasis. The review classifies the flavonoids on the basis of their general molecular structure. Their effectiveness in suppressing osteoclastogenesis and bone resorption as well as in promoting osteogenesis and bone formation are described. In addition, their molecular mechanisms of action are highlighted and discussed in terms of epigenetics, the theory that currently represents the main explanation of diet–DNA interactions [16].

Box 3. Role of Inflammation and Oxidative Stress in Bone Homeostasis

Inflammation and oxidative stress play key roles in the pathogenesis of OP. Inflammation status inhibits OB differentiation as well as accelerating OC differentiation and activity (Figure I). Proinflammatory cytokines such as IL-1β, IL-6, and IL-17 are induced through NF-κB activation and the decrease of the OPG/RANKL ratio, leading to OC differentiation and stimulating the activity of mature OCs. Furthermore, reactive oxygen species (ROS) increase the differentiation, survival, and activity of OCs while reducing those of OBs and osteocytes [5]. In postmenopausal OP, the reduction of estrogen levels leads to modulation of cytokine expression and activity, leading to an inflammatory state and increased ROS production, resulting in excessive bone resorption and a high risk of osteoporotic fracture [6]. The anti-inflammatory cytokines IL-4 and IL-10 are able to inhibit OC differentiation [7]. In particular, IL-10 is important in the modulation of the inflammatory response and its expression is highly regulated at the transcriptional and post-transcriptional levels [8], and in some tissues by epigenetic control of its gene [9]. It is also able to decrease RANKL expression and induce the production of OPG, increasing the OPG/RANKL ratio and inhibiting OC differentiation. Furthermore, IL-10 is able to reduce MMPs expression and increase the production of their inhibitors, tissue inhibitors of metalloproteinases (TIMPs), leading to a reduction of OC activity [7].

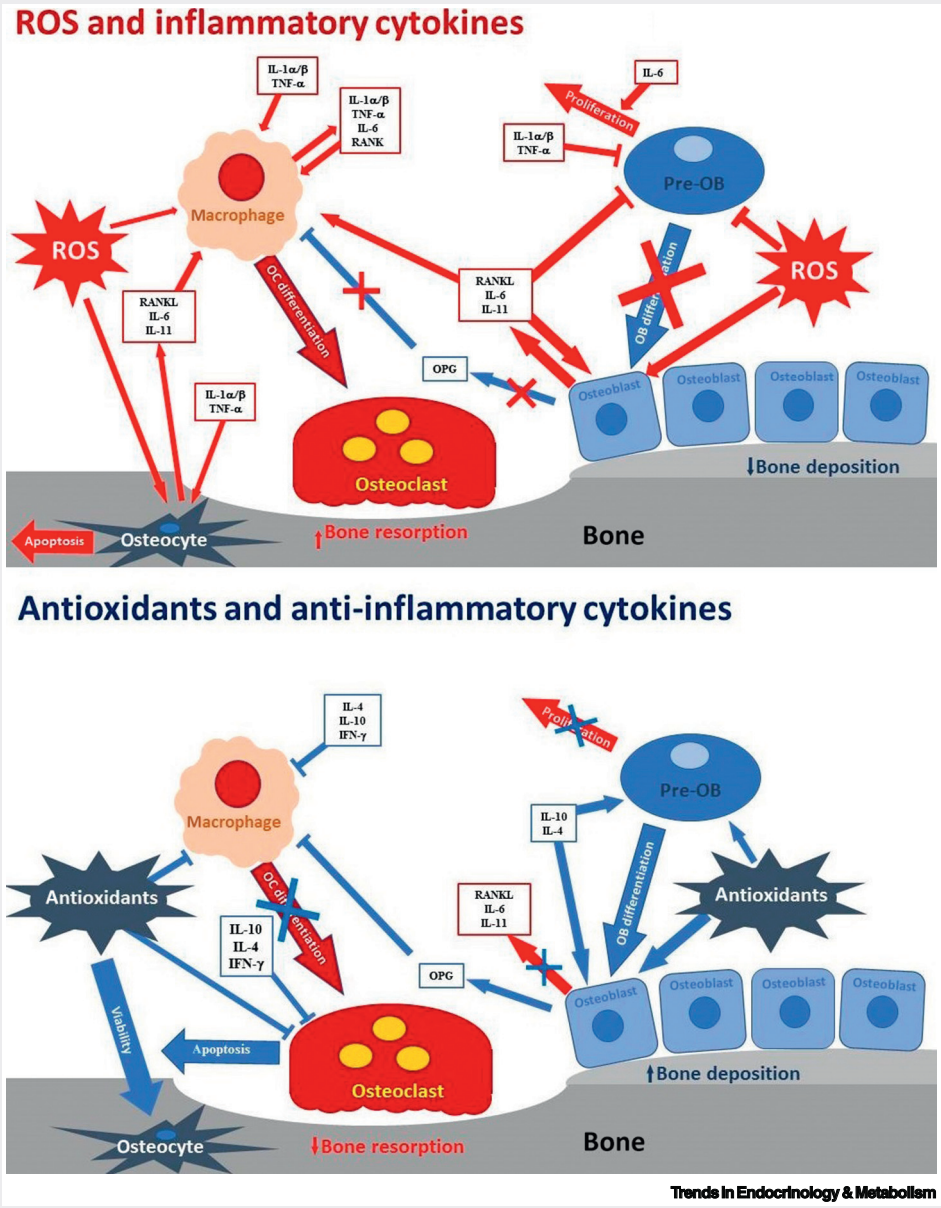

Figure I. Schematic Diagram of the Effects of Oxidant/Antioxidant Molecules and Pro- and Anti-inflammatory Cytokines on Osteoblast (OB) and Osteoclast (OC) Differentiation. (A) Inflammatory conditions [interleukin (IL)-1α/β or tumor necrosis factor-α (TNF-α)] induce macrophages to produce a series of proinflammatory cytokines that act in an autocrine manner inducing OC differentiation. In pre-OBs, inflammation inhibits OB differentiation and induces proliferation, while in OBs and osteocytes it induces the expression of IL-6 superfamily factors (e.g., IL-11) and receptor activator of nuclear factor kappa-B ligand (RANKL) while reducing that of OPG, which increases the inflammatory conditions and the apoptosis of osteocytes and induces OC differentiation through the increase of the RANKL/OPG ratio. Reactive oxygen species (ROS) produced by oxidative stress accelerate OC differentiation and activity while inhibiting that of OBs. Antioxidants and anti-inflammatory cytokines reduce OC differentiation, induce OC apoptosis, and increase OB differentiation and activity, acting also through epigenetic mechanisms. Abbreviation: IFN, interferon

Search Strategies and Results of Collected References

The following literature search was conducted in four different databases: MEDLINE, ScienceDirect, EMBASE, and Web of Science. The strings used in the bibliographic search were: (Osteoporosis AND Flavonoid). In the MEDLINE database (PubMed search engine) 377 articles were retrieved by considering publications written in English (AND "English" [language]) and published after 1 January 2015 (AND ("2015/01/01"[Date - Entrez]: "2020/04/30"[Date - Entrez])). Reviews (NOT Review [Publication Type]) were then excluded, reducing the number of collected articles to 316. In the ScienceDirect, EMBASE, and Web of Science databases, 788, 588, and 192 articles were found, respectively, using the same strings and limitations (year range, language, and research articles). Then, four reviewers manually assessed the titles and abstracts of the collected references and those considered not pertinent (e.g., research articles that did not use isolated compounds) and duplicates were discarded. Then, 34 articles related to the topics of the review were selected. Finally, a further 17 references were cited to add information on some technical aspects for greater understanding of the mechanisms acting in bone regulation and OP development. A detailed flux diagram of search strategies is provided in Figure 1.

Of the 34 selected articles, 17 concerned *in vitro* studies and 18 *in vivo* studies and only two were clinical trials. Considering the *in vitro* studies, the pathway involved in the differentiation of OBs or OCs by flavonoids was identified in only 76.5% (13/17) of the studies; of these 13 articles, in only three studies were the factors with which these compounds interact identified through gain- and/or loss-of-function experiments. Furthermore, 64.7% (11/17) of the studies described the effect of treatment with flavonoids on OBs and 11.8% (2/17) on OCs and only 17.6% (3/17) of the experiments were performed in both cell systems. Of the *in vivo* studies, 27.8% (5/18) reported exclusively *in vivo* experiments while the remaining 72.2% (13/18) used both *in vivo* and *in vitro* experiments to identify the pathways involved. For the latter, the authors described the effect of treatment with flavonoids on OBs in 58.8% (7/13), on OCs in 7.8% (1/13), and on both cell types in the remaining 33.4%. Again, few authors (3/13) identified the factors with which phytochemicals

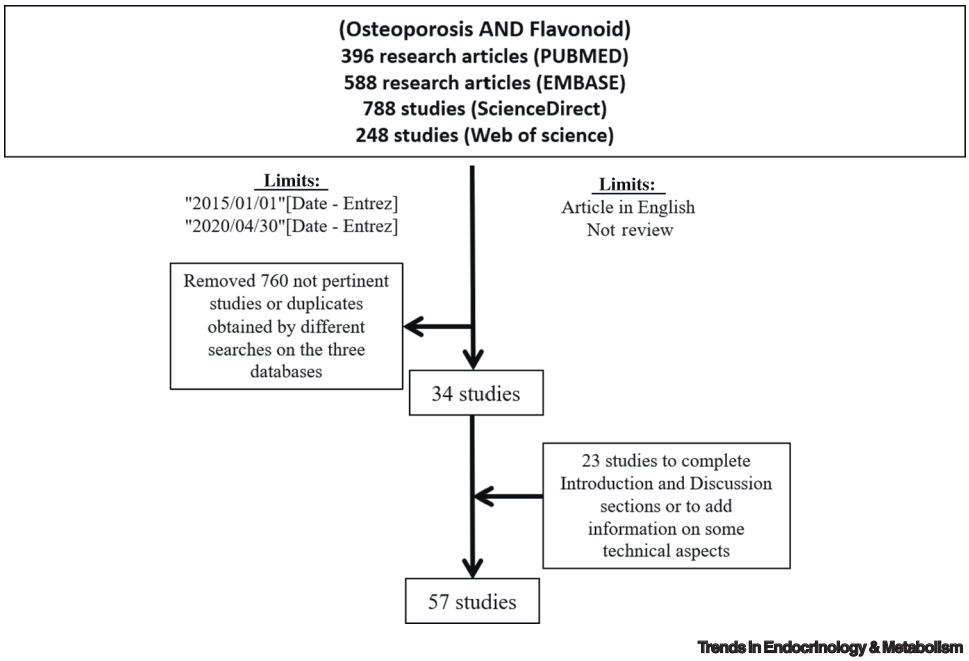

Figure 1. Flowchart of the Search Strategy and Selection of Bibliographic References.

interact, always by means of experiments on gain and/or loss of function. All *in vivo* studies used small-animal models (50% mice and 50% rats), in which various techniques were used to induce osteopenia. Bilateral ovariectomy (OVX) was the most used surgical technique to induce osteopenia (9/18); the other models inducing osteopenia were administration of glucocorticoids (2/18), lipopolysaccharide (LPS) (1/18), induction of type I diabetes mellitus (DM) (1/18), high-fat diet (3/18), administration of the NF-κB ligand (RANKL) (sRANKL) (1/18), and bilateral orchiectomy (ORX) (1/18).

All of the evidence emerging from the literature of the past 5 years on the ability of flavonoids to improve bone homeostasis and limit bone loss are reported in the following paragraphs.

Polyphenols: Flavonoid Classes in Bone Homeostasis

Polyphenols are secondary metabolites produced by plants as a defense against pathogens or UV radiation. Flavonoids are the most studied polyphenols for OP treatment, and numerous molecules have been isolated, identified, and characterized.

The term flavonoid is used to describe a broad collection of plant compounds characterized by a flavone ring that constitutes the backbone of the structure. They are ubiquitous molecules playing an integral role in the growth and development of plants. They are present in significant amounts in many commonly consumed fruits, vegetables, grains, and herbs. These structurally differing compounds exhibit a range of effects in *in vitro* models, including antioxidant, anti-inflammatory, differentiation, and apoptotic effects, that may explain their potential therapeutic properties. Flavonoids have shown potential bone-specific effects in several studies. The results of those studies are reported later separately for each flavonoid subfamily: flavones, isoflavones, flavonols, flavanols, flavanones, and anthocyanins (Figure 2). Table 1 reports all selected articles where flavonoids were involved, highlighting their role in the regulation of bone metabolism and in the reduction of bone loss.

Flavones are a class of flavonoids with the backbone structure of 2-phenylchromen-4-one (2-phenyl-1-benzopyran-4-one). Four articles were retrieved about three flavones: apigenin, a

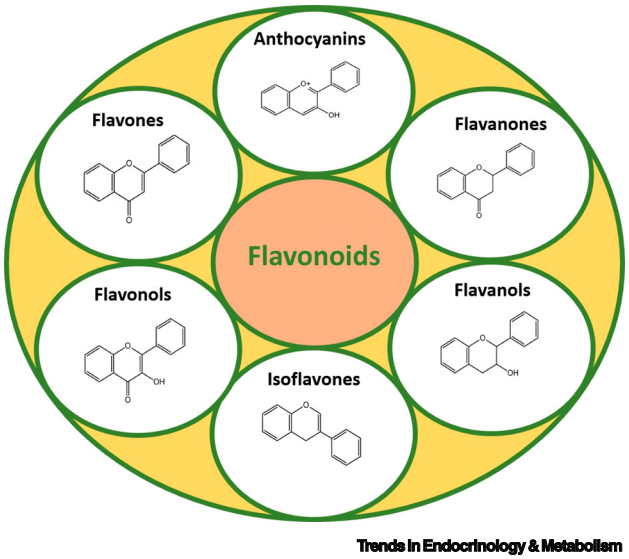

Figure 2. Schematic Diagram of Flavonoid Classes.

Table 1. List of Flavonoids Implicated in Bone Metabolism and in Reduction of Bone Loss

Compound	Flavonoid subgroup	Study type	Cell/*in vivo* model	Dose	Identified pathway	Effect of administration	Refs
Apigenin	Flavones	*In vitro*	MC3T3-E1 cell line; mouse spleen cells	1 μM, 5 μM, 10 μM	–	Inhibited OB and OC differentiation; reduced OVX-related bone loss	[19]
		In vivo	OVX Balb/c mouse	10 mg/kg/2 day			
Luteolin		*In vitro*	MC3T3-E1 cell line	0.05 μM, 0.1 μM, 0.2 μM	MAPK, Wnt	Induced OB differentiation	[17]
		In vivo	GI-OP rat	25 mg/kg/day, 50 mg/kg/day, 100 mg/kg/day (intragastrically)			
Corylin		*In vitro*	Rat OBs	1 μM, 10 μM	ER, Wnt	Induced OB differentiation	[18]
Daidzein	Isoflavones	*In vitro*	MG-63 cell line	0.01 μM, 0.1 μM, 1 μM	ER, MAPK, IGF	Induced OB differentiation	[21]
		In vitro	MC3T3-E1 cell line; RAW264.7 cell line	10^{-3} μM	OPG/RANKL	Inhibited OC differentiation	[22]
		In vitro	MC3T3-E1 cell line	0.1 μM, 1 μM, 10 μM	ER	Induced OB differentiation	[35]
Puerarin		*In vivo*	HFD and exercise in OVX Sprague Dawley rat	400 mg/kg/day	MAPK	Reduced OVX-related bone loss	[25]
Genistein		*In vitro*	MC3T3-E1 cell line; primary rat OBs	0.01 μM, 0.1 μM, 1 μM, 10 μM, 100 μM	ER, OPG/RANKL	Induced OB differentiation	[23]
		In vitro	MC3T3-E1 cell line	0.1 μM, 1 μM, 10 μM	ER	Induced OB differentiation	[35]
		In vivo	ORX male Wistar rat	30 mg/kg/day (subcutaneously)	ER	Reduced ORX-related bone loss	[24]
Equol		*In vitro*	MC3T3-E1 cell line	0.1 μM, 1 μM, 10 μM	ER	Induced OB differentiation	[35]
		In vitro	Primary rat OBs	0.01 μM, 0.1 μM, 1 μM	ER	Induced OB proliferation and differentiation	[33]
Cladrin		*In vitro*	3T3-L1 cell line	1 pM, 100 pM, 10 nM, 1 μM	Wnt, OPG/RANKL	Reduced adipogenesis; inhibited OC activity; reduced HFD-induced bone loss	[26]
		In vivo	HFD C57BL/6 mouse	5 mg/kg/day, 10 mg/kg/day			
		In vitro	Rat OBs; rat BMMs	10^{-2} μM, 10^{-1} μM, 1 μM	MAPK	Induced OB activity	[27]
		In vivo	OVX Sprague-Dawley rat	10 mg/kg/day			
Calycosin		*In vitro*	ST2 cell line	4 μM, 8 μM, 16 μM, 32 μM	BMP, Wnt	Induced OB differentiation and activity	[30]
		In vitro	Rat OBs	0.1 μM, 1 μM, 10 μM	IGF	Induced OB differentiation and activity	[31]
		In vitro	Mouse BMMs	2.5 μM, 5 μM, 10 μM	NF-κB, MAPK	Inhibited OC differentiation.	[32]
Formononetin		*In vitro*	3T3-L1 cell line	100 pM, 10 nM, 1 μM	MAPK, Wnt	Reduced HFD-induced bone loss	[28]
		In vivo	HFD C57BL/6 mouse	0.1 mg/kg/day, 1 mg/kg/day, 10 mg/kg/day			
		In vitro	Rat calvarial OBs; rat BMMs	10^{-2} μM, 10^{-1} μM, 1 μM	MAPK	Induced OB activity	[27]
		In vivo	OVX Sprague Dawley rat	10 mg/kg/day			
		In vivo	OVX Balb/c mouse	10 mg/kg/day	BMP	Increase bone regeneration of femoral drill-hole injury in OVX mouse	[29]

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Table 1. (continued)

Compound	Flavonoid subgroup	Study type	Cell/*in vivo* model	Dose	Identified pathway	Effect of administration	Refs
Icariin	Flavonols	*In vitro*	Mouse OBs; mouse OCs	0.1 μM, 1 μM, 10 μM	BMP, IGF, NF-κB, TGF, Wnt	Induced OB differentiation; reduced OC differentiation; reduced OVX-related bone loss	[36]
		In vivo	OVX ICR mouse	40 mg/kg/day			
		In vitro	BMMs; RAW264.7 cell line	10^{-7} M, 10^{-6} M, 10^{-5} M, 10^{-4} M, 10^{-3} M, 10^{-2} M, 10^{-1} M	MAPK, NF-κB	Reduced RANKL-induced OC differentiation	[37]
		In vitro	SaoS-2 cell line	10^{-6} mM, 10^{-5} mM, 10^{-4} mM, 10^{-3} mM	Wnt	Induced OB differentiation; reduced glucocorticoid-related bone loss	[38]
		In vivo	GI-OP C57BL/6 mouse	250 mg/kg/day			
		In vitro	Rat BMSCs	10^{-6} mM, 10^{-5} mM, 10^{-4} mM, 10^{-3} mM, 10^{-2} mM, 10^{-1} mM, 1 mM	–	Induced OB differentiation; reduced adipocyte differentiation; reduced LPS-related bone loss	[39]
		In vivo	LPS-induced osteonecrosis rat	30 mg/kg/day			
		In vitro	Rat BMSCs	10 μM, 20 μM, 40 μM, 80 μM, 160 μM	ER	Induced OB differentiation; reduced adipocyte differentiation; reduced OVX-related bone loss	[40]
		In vivo	OVX Sprague Dawley rats	20 mg/kg/day			
Quercetin		*In vitro*	Rat BMSCs	0.1 μM, 1 μM, 10 μM	MAPK	Induced OB differentiation	[41]
		In vitro	Rat BMSCs	1 μM	NF-κB, Wnt	Induced OB differentiation; reduced inhibition of TNF-α-dependent OB differentiation	[42]
		In vivo	OVX Sprague Dawley rats	50 mg/kg/day			
Rutin		*In vitro*	SAOS-2 cell line	1 μM, 10 μM, 100 μM	–	Induced OB differentiation	[43]
EGCG	Flavanols	*In vitro*	Dedifferentiated fat cells	1.25 μM, 10 μM	–	Induced OB differentiation	[44]
		In vitro	MC3T3-E1 cell line	10 μM, 30 μM, 50 μM	MAPK	Induced OB differentiation	[45]
EAF		*In vitro*	MC3T3-E1 cell line; RAW 264.7 cell line	10^{-4} μM, 10^{-3} μM, 10^{-2} μM, 10^{-1} μM, 1 μM	NF-κB, OPG/RANKL	Induced OB differentiation; reduced OC activity; reduced OVX-related bone loss	[46]
		In vivo	OVX C57BL/6J mouse	500 μg/kg/day			
ECAP		*In vitro*	RAW 264.7 cell line	10 μg/ml, 50 μg/ml, 100 μg/ml	NF-κB	Reduced OC differentiation; reduced OVX-related bone loss	[47]
		In vivo	OVX osteoporotic mouse	50 mg/kg/day, 100 mg/kg/day			
Hesperidin	Flavanones	*In vitro*	Alveolar OBs	0.1 μM, 1 μM, 10 μM, 100μM	Wnt	Induced OB differentiation	[48]
		In vivo	Streptozotocin induced type I DM male albino rat	200 mg/kg/day	NF-κB	Reduced TNF-α and NF-κB expression induced by type I DM; ameliorates bone structure	[49]
Baicalein		*In vivo*	Osteotomy in OVX Sprague Dawley rat	1 mg/kg/day; 10 mg/kg/day; 100mg/kg	–	Reduced OC differentiation; promotes in late- but not in early-stage bone healing	[50]
Delphinidin	Anthocyanins	*In vitro*	Human adipose MSCs	25 μM, 50 μM, 100 μM, 200 μM	–	Reduced adipocyte differentiation; induced chondrocyte differentiation	[51]
Cyanidin		*In vitro*	Human adipose MSCs	25 μM, 50 μM, 100 μM, 200 μM	–	Induced chondrocyte differentiation	[51]
		In vitro	Mouse BMMs; mouse OBs	5 μM, 10 μM, 25 μM, 50 μM, 100 μM, 200 μM	NF-κB, MAPK	Reduced OC differentiation; induced OB differentiation	[52]

Table 1. (continued)

Compound	Flavonoid subgroup	Study type	Cell/*in vivo* model	Dose	Identified pathway	Effect of administration	Refs
Malvidin		*In vitro*	Human adipose MSCs	25 μM, 50 μM, 100 μM, 200 μM	BMP	Induced OB differentiation	[51]
Petunidin		*In vitro*	MC3T3-E1 cell line; RAW 246.7 cell line	0.25 μg/ml, 1 μg/ml, 4 μg/ml, 5 μg/ml, 8 μg/ml, 16 μg/ml, 20 μg/ml, 32 μg/ml	BMP, MAPK, OPG/RANKL	Induced OB differentiation; reduced OC differentiation and activity; reduced sRANKL-induced bone loss	[53]
		In vivo	sRANKL-induced osteopenic mouse	7.5 mg/kg/day			

common flavone found in chamomile and parsley; luteolin, originally obtained from *Reseda luteola* and used as a source of yellow dye since at least the first millennium BC [17]; and corylin flavones contained in *Psoralea corylifolia* [18].

Apigenin has been shown to reduce the viability and differentiation of OBs, as demonstrated by the reduced collagen-1A1 (Col-1A1), alkaline phosphatase (ALP) synthesis, and calcium deposition in a treated OB-like cell line (MC3T3-E1). However, these effects seem to be unrelated to antioxidant activities of apigenin and their mechanisms of action were not investigated. Furthermore, apigenin treatment inhibited the formation of RANKL-induced multinucleated (MNC) OCs in a dose-dependent manner. OVX mice treated with apigenin showed a reduction of trabecular bone loss, while cortical bone was unaffected, as demonstrated by bone mineral density (BMD) measurement through micro–computed tomography (μ-CT) analysis [19].

Luteolin is a common plant flavone with anti-inflammatory activity; it markedly enhances the activation of Wnt and mitogen-activated protein kinase (MAPK) pathways, upregulating the expression and activity of low-density lipoprotein (LDL) receptor-related protein 5 (LRP-5), glycogen synthase kinase-3β (GSK-3β), β-catenin, and extracellular signal-regulated kinase 1 (ERK-1). This yellow flavone reduced the apoptotic effect on OBs due to dexamethasone treatment and increased the osteoprotegerin (OPG/RANKL ratio, inhibiting OC differentiation and thus OC bone resorption activity [17]. *In vivo* experiments in a glucocorticoid-induced OP (GI-OP) mouse model showed that luteolin reverted induced bone loss, as demonstrated by μ-CT analysis, biomechanical properties, and serum bone-turnover markers [17]. Both apigenin and luteolin were identified as epigenetic modulators of class I histone deacetylases (HDACs) through *in silico* studies; both molecules docked stably in the binding pocket of these HDACs as determined by a molecular dynamics simulation study [20].

Corylin exerts osteogenic effects through ER and Wnt/β-catenin pathway activation [18]. Yu *et al.* showed an increase of the OB markers RUNX-2 and Col-1A1. Osteogenic induction was also demonstrated in an *ex vivo* system where micromass cultures incubated with corylin displayed induction of osteogenesis with robust staining patterns, as indicated by intense ALP staining and Alizarin Red S staining [18].

Isoflavones, the most-studied flavonoid subclass, differ from flavones with the phenyl group in the C3 position instead of C2. Fourteen collected research articles report information on six isoflavones: daidzein [21,22] and genistein [23,24], present in various plants, particularly *Glycine max* (soy bean), *Pueraria mirifica*, *Pueraria lobata*, *Lupinus*, etc.; puerarin, isolated from *P. lobata* [25]; cladrin, a 3′,4′-dimethoxy analog of daidzein [26,27]; formononetin, a methoxy-isoflavone isolated from *Butea monosperma*, *Astragalus mongholicus*, and *Trifolium pretense* [27–29];

calycosin-7-*O*-β-D-glucoside (calycosin), an isoflavone isolated from *Astragalus membranaceus* [30–32]; and equol (4′,7-isoflavandiol), another active metabolite of daidzein [33,34].

Daidzein is structurally similar to 17β-estradiol and exerts its actions through stimulation of ERs. It was found that daidzein is able to stimulate the proliferation and differentiation of an OB-like cell line (MG-63) through the activation of ER-dependent ERK and AKT, demonstrated by cell viability MTT assays, ALP activity, and Col-1A1 expression [21]. Daidzein treatment of MG-63 cells was also able to reduce apoptosis through increased synthesis of the antiapoptotic factor B cell lymphoma extra-large (BCL-XL), similar to estrogen treatment, as highlighted by protection against cisplatin-induced programmed cell death. The use of ER antagonists and short hairpin RNA (shRNA) through ER-α and ER-β confirmed the action of daidzein through the ER [21]. Park *et al.* showed that daidzein is able to inhibit OC differentiation by various mechanisms, including an increase in OPG and a reduction in the production of RANKL in the pre-OB MC3T3-E1 cell line and thus, similar to estrogen, through the increase in the OPG/RANKL ratio. To confirm this action, the authors treated the murine cell line RAW264.7 (pre-OC) with conditioned medium of cultured MC3T3-E1 (OB-like cell line) treated with daidzein, reducing OC differentiation through the RANKL signaling block and suppression of the activation of nuclear factor of activated T cells-c1 (NFAT-c1), similar to treatment with OPG, the latter confirmed with the use of siRNA specific for NFAT-c1 [22].

Similarly, genistein induced OB differentiation in the MC3T3-E1 cell line and primary rat calvarial OBs through stimulation of ER pathways, increasing MAPK and ERK pathway activation, and enhancing ER-α and ER-β expression (positive feedback on genistein actions). Furthermore, it induces OPG synthesis, leading to an increased OPG/RANKL ratio and inhibiting OC differentiation [23]. Genistein treatment of ORX rats showed its protective effect on trabecular bone by ER stimulation of bone cells. Marked changes of structural parameters were observed, such as increases in bone area, trabecular thickness, and number and a decrease in trabecular separation. In addition, Filipović *et al.* found that genistein influenced the activity of thyroid follicular cells, with a decrease in circulating thyroid hormones that they hypothesized to act indirectly on bone formation [24]. Co-treatment with the soy isoflavones genistein and daidzein, administered in middle-aged OVX mice, increased OB activity and inhibited OC activity by maintaining high ALP and low tartrate-resistant acid phosphatase (TRAP) levels, respectively. The combined effect of genistein, daidzein, and β-carotene on osteogenesis and OB activity was investigated by Nishide *et al.*, who highlighted that their administration led to greater ALP activity than isoflavones administered alone, suggesting that co-treatment has a synergistic effect on the different pathways related to bone modeling [35]. However, although β-carotene enhanced RUNX-2, secreted phosphoprotein 1 (SPP-1), and ALP expression, isoflavones had no significant effects on these genes. By contrast, β-carotene did not show any effect on Osterix (OSX) expression enhanced by isoflavones [35].

In Ok *et al.*'s study, puerarin administration had a positive effect on estrogen-deficiency bone loss in an OVX rat model. They evaluated puerarin treatment in association with a low-fat diet (LFD) or high-fat diet (HFD) and the presence/absence of physical exercise. HFD exacerbated OVX-related bone loss, as indicated by μ-CT analysis, serum bone-turnover markers, and RANKL mRNA expression in bone cells. Morphometric parameters of the femur indicated a significant reduction of trabecular bone loss with puerarin treatment. Puerarin's mechanism was related to a reduction of RANKL expression without any influence on OPG expression, resulting in an increase of the OPG/RANKL ratio and a consequent inhibition of OC differentiation. Exercise enhanced puerarin's mitigation of these molecular factors in bone loss [25].

Cladrin has antiadipogenic and antiobesity effects, which could play a positive role in adipogenesis-related bone loss with HFD. Recently, studies have shown that cladrin is able to: (i) promote osteogenesis, increasing the expression of OB genes; (ii) inhibit OC differentiation, increasing the OPG/RANKL ratio; and (iii) inhibit adipocyte differentiation, reducing the levels of major adipogenic transcription factors such as peroxisome proliferator-activated receptor-γ (PPAR-γ) and CCAAT enhancer-binding protein A (C/EBP-a) [26,27]. Gautam *et al.* showed that cladrin prevented bone loss in a HFD mouse model, improving bone quality as indicated by trabecular bone parameters and bone mechanical strength analysis. Bone marrow cells isolated from mice fed a HFD showed a reduced ability to differentiate into OBs that was partially reverted by the administration of cladrin to these mice, as demonstrated by increases in ALP and Alizarin Red staining. Furthermore, Gautam *et al.* reported that cladrin inhibited lipid accumulation in a preadipocyte 3T3-L1 cell line, reducing adipocyte differentiation through the inhibition of adipogenic gene regulators such as PPAR-γ, C/EBP-a, and sterol regulatory element-binding protein (SREBP) and the adipogenic marker leptin, without affecting their cell viability [26]. The same authors compared cladrin and formononetin's actions on OB differentiation and activity [27]. Cladrin stimulated OB proliferation and induced an increase in OB differentiation compared with untreated cells as highlighted by ALP production, and mineralized nodule formation at 21 days. Cladrin's actions were abolished by co-treatment with U0126, a MAPK kinase 1/2 (MEK) inhibitor, while co-treatment with LY294002, an inhibitor of phosphatidylinositol-3-kinase (PI3K), had no effect. This suggested that cladrin acts on OB proliferation through MEK/ERK-1 but not the AKT pathway [27].

Unlike cladrin, formononetin did not modify OB proliferation, but stimulated OB differentiation, as shown by ALP production and mineralized nodule formation. Formonectin's actions were determined by p38-MAPK activation, as confirmed by co-treatment with the p38-MAPK inhibitor SB203580, which was able to block its activity. The c-Jun N-terminal kinase (JNK) and ERK inhibitors were unable to abolish formononetin-stimulated ALP production [27]. In *in vitro* experiments using 3T3-L1 preadipocytes, formononetin treatment limited adipogenesis through reduction of the principal adipogenic transcription factors PPAR-γ, C/EBP-a, and SREBP. This effect was related to AMPK activation that lead to the inhibition of GSK-β activity (phosphorylation), and β-catenin stabilization. β-Catenin activation induces the expression of LRP-5 and Wnt-10 and the inhibition of Dickkopf-2 (DKK-2), increasing the Wnt pathway response. The AMPK-dependent formononetin action was confirmed using an AMPK-specific inhibitor that reverted all actions. *In vivo* formononetin treatment exerts antiobesity effects, modulating plasma lipid parameters such as total cholesterol, high-density lipoprotein (HDL), LDL, and very-low-density lipoprotein (VLDL) levels. Furthermore, formononetin administration reduced HFD-dependent bone loss and ameliorated bone quality, as demonstrated by histomorphometric measurements, μ-CT imaging, and biomechanical bone strength. This phenomenon is due to enhanced osteogenesis and reduced adipogenesis of bone marrow stem cells (BMSCs) isolated from HFD-fed mice administered formononetin. These cells showed increased ALP activity and high RUNX-2, osteocalcin (OCN), and Col-1A1 expression. Formononetin also represses RANKL and TRAP expression and increases the OPG/RANKL ratio, indicating repression of resorptive genes in bone tissue [28]. The bone regeneration ability of formononetin was also tested in OVX mice where a drill-bone defect was made in the femur. Singh *et al.* found that formononetin treatment was able to accelerate bone healing in terms of improvement of histomorphometric parameters, similar to parathyroid hormone (PTH) treatment taken as a positive control because of its proven fracture-healing effect. In the bone tissue of the region surrounding the defect site, expression of the RUNX-2, OCN, and bone morphogenic protein 2 (BMP-2) osteogenic markers increased. Interestingly, the expression of BMP-2, an important factor in bone regeneration, was higher in formononetin-treated OVX mice than in the PTH-treated group [29].

Calycosin induced OB differentiation in an ST2 cell line through increased activity of the BMP and Wnt pathways, as demonstrated by the enhanced expression of BMP-2, the β-catenin and RUNX-2 factors, ALP activity, and Alizarin Red S staining. The effects of calycosin were reversed by treatment with Noggin and Dkk, specific inhibitors of the BMP and Wnt pathway, respectively [30]. Furthermore, OB calycosin treatment produced OB differentiation, as demonstrated by the effects on cell proliferation, ALP expression and activity, expression of the Col-1A1, RUNX-2, OSX, and BMP-2 osteogenic markers, and the formation of calcified nodules. Calycosin exerts its actions through insulin-like growth factor 1 (IGF-1) receptor (IGF-1R)/PI3K/AKT signaling activation, demonstrated using selective inhibitors of both IGF-1R (GSK1904529A) and PI3K (LY294002) [31]. Calycosin also acts on OC differentiation. Calycosin treatment of bone marrow macrophages (BMMs) inhibited the number of RANKL-induced MNC OCs identified through a TRAP staining assay and reduced their resorption activity, measured on a calcium phosphate-coated Corning Osteo Assay Surface plate, in a dose-dependent manner. RNA analysis of treated BMMs also showed reduced expression of the OC transcription factors NF-κB, cellular FBJ murine osteosarcoma viral oncogene homolog (c-FOS), and NFAT-c1, as well as genes for OC activities such as the proteolytic enzymes TRAP, matrix metalloproteinase 9 (MMP-9), and cathepsin K (CTSK). The authors showed that calycosin treatment was also able to inhibit ERK1/2, p38-MAPK, and JNK phosphorylation, relating these pathway inhibitions responsible for decreased OC formation [32].

Finally, equol has a structure like 17β-estradiol and consequently activates ERs. Equol treatment of pre-OBs induced proliferation and osteogenesis as indicated by the MTT assay and measurement of ALP activity. Wang *et al.* demonstrated equol's interaction with ER through the use of a specific ER inhibitor, ICI182780, that reverts all actions of equol treatment [33]. It was reported that soy isoflavones, including the gut microbial metabolite of daidzein *S*-(–)equol, have the potential to influence enzymes that write or erase epigenetic marks; in some cases, they are able to counteract certain hallmarks of cancer. However, no studies on the epigenetic influences of soy isoflavones on bone erosive diseases were found until now [34].

Flavonols are a class of flavonoids that have a 3-hydroxyflavone backbone. Nine articles were found on three phytochemical compounds belonging to this subclass of flavonoids: icariin, the most abundant active component of several species belonging to the *Epimedium* genus [36–40]; quercetin, a common flavonol rich in red onions and kale [41,42]; and rutin, isolated initially from *Chrozophora tinctoria* [43].

Icariin possesses numerous pharmacological effects, including anti-OP, antioxidation, antitumor, and antiaging properties. As a bone-protective agent, icariin was shown to increase OB cell proliferation and differentiation through the activation of pathways such as BMP, transforming growth factor-β (TGF-β), IGF-1, and Wnt signaling and to inhibit bone resorption by decreasing OC differentiation and activity. In addition, icariin inhibits apoptosis and improves OB survival by reducing caspase-3 expression [36]. Icariin treatment in OCs impaired inhibitor of NF-κB (IκB) degradation, influencing RANKL-induced IκB degradation and resulting in the inhibition of NF-κB. Furthermore, all three major MAPK pathways, ERK, p38-MAPK, and JNK, were suppressed by icariin treatment. These icariin actions inhibited osteoclastogenesis, as demonstrated by the decrease in the formation of a highly polarized f-actin ring, and downregulated the expression of master regulators of osteoclastogenesis (c-FOS and NFAT-c1) and of related effector genes (TRAP and CTSK) essential for OC differentiation and activity [37]. It was found that icariin interferes with glucocorticoid-induced inhibition of osteogenesis in the OB-like cell line SAOS-2; dexamethasone and icariin treatment of SAOS-2 decreased and increased osteogenesis, respectively. Dexamethasone and icariin co-treatment showed that icariin antagonizes the impairment of

OB differentiation, as demonstrated by reduced ALP activity and mineralization staining. *In vivo* experiments confirmed these effects, but the use of a specific glucocorticoid receptor antagonist, RU-486, did not abolish icariin's effects, indicating that icariin determines osteogenesis independent of glucocorticoid receptor antagonism. Molecular analysis seemed to indicate that icariin exerts its effects through differentiated embryonic chondrocyte expressed gene 1 (DEC-1), which is a critical player in the osteogenic process of the Wnt/β-catenin signaling pathway. This action was confirmed in DEC-$1^{-/-}$ mice, which showed a tendency for OP due to an impaired Wnt pathway [38]. In Huang *et al.*'s study, it was demonstrated that icariin treatment enhances the proliferation and OB differentiation of BMSCs, as indicated by cell proliferation and ALP activity. Icariin treatment was able to revert the inhibition of osteogenic differentiation by glucocorticoid treatment (methylprednisolone), as indicated by an increase in Alizarin Red S staining and the expression levels of RUNX-2, OCN, ALP, and BMP-2. Furthermore, icariin reduces the adipogenic differentiation of BMSCs induced by methylprednisolone treatment. In an *in vivo* experiment on methylprednisolone-induced femoral head osteonecrosis, icariin treatment reduced bone loss through enhanced osteogenesis, as indicated by μ-CT evaluation of the subchondral region [39]. Xu *et al.* demonstrated the ability of icariin to reduce estrogen-deficiency bone loss in OVX rats; they found that icariin induced OB differentiation of BMSCs, as shown by an increase in Alizarin Red and ALP staining, and gene expression analysis of RUNX-2, ALP, Col-1A1, OPG, OCN, and bone sialoprotein (BSP) confirmed the enhancement of OB differentiation by icariin. Xu *et al.* highlighted an increase in the activation of signal transducer and activator of transcription 3 (STAT-3), a mediator of icariin's actions; with the use of AG490, a specific inhibitor of STAT-3, OCN was shown to be influenced by STAT-3. However, STAT-3 activation did not influence RUNX-2 expression, probably indicating that STAT-3 cooperates with RUNX-2 in OCN expression, which was confirmed by the activation of OCN in HEK293t cells co-transfected with RUNX-2 and STAT-3. Finally, icariin-treated OVX mice partially recovered estrogen deficiency-induced alveolar bone loss only via osteogenesis activation, as indicated by μ-CT analysis [40].

Quercetin has positive pharmacological effects on bone. Its administration in primary BMSCs increased OB differentiation by upregulation of ALP, Col-1A1, and OCN expression. It exerts its effects by enhancing MAPK activity leading to RUNX-2 expression and activation. This evidence was confirmed by specific inhibitors of p38-MAPK (SB203580), ERK1/2 (SP600125), and JNK (PD98059) on quercetin-induced RUNX-2 expression [41]. The anti-inflammatory effect of quercetin was highlighted by Yuan *et al.*, finding reduced tumor necrosis factor-α (TNF-α)-induced inhibition of the osteogenesis of BMSCs. In addition, an *in vivo* experiment in an OVX rat model showed partial rescue of estrogen-deficiency bone loss with quercetin treatment, as indicated by histomorphometric analyses. It was found that quercetin acts by interfering with TNF-α-induced NF-κB activation and promoting β-catenin activity [42].

Rutin treatment of the OB-like cell line SAOS-2 was able to induce the expression of osteogenesis- and ossification-related markers, similar to estrogen treatment. Furthermore, rutin treatment reduced ACP enzyme activity, a bone-resorption marker, compared with treatment with estrogen, indicating rutin to be a compound with antiosteoporotic effects [43].

Flavanols or flavan-3-ols derive from flavans and contain the 2-phenyl-3,4-dihydro-2*H*-chromen-3-ol skeleton. Four articles were found on this subclass regarding three molecules: epigallocatechin-3-gallate (EGCG), the most abundant catechin in green tea [44,45]; (–)-epiafzelechin (EAF), a common plant flavanol obtained from several species, including *Celastrus orbiculatus*, *Cassia sieberiana*, *Typha capensis*, *Drynariae fortunei*, and *Camellia sinensis* [46]; and (–)-epicatechin-3-*O*-β-D-allopyranoside (ECAP), obtained from *Davallia formosana* Hayata extract [47].

EGCG is considered one of the most active health-promoting molecules due to its anti-inflammatory, anticancer, antioxidant, and antiatherogenic properties. It has been demonstrated to have a role in controlling multiple signaling pathways (i.e., ERK, JNK, STAT-3, and PI3K/AKT) involved in cell proliferation, differentiation, survival, and cytokine production. It was shown that EGCG is able to reduce bone resorption by hindering osteoclastogenesis and to modulate bone formation by increasing ALP activity in OBs and then bone formation [44,45]. In addition, it was found that EGCG induces OB differentiation in MSCs, activates bone-like cells [44], and suppresses interleukin-6 (IL-6) synthesis stimulated by platelet-derived growth factor BB (PDGF-BB), basic fibroblast growth factor (bFGF), or endothelin-1 (ET-1) in MC3T3 E1 OB-like cells [45]. By contrast, there are few data on the specific role of EAF and ECAP in bone. Mainly *in vitro* or *in vivo* studies (mouse models) conducted with extracts of the plants from which these molecules have been isolated have shown their role in stimulating OB proliferation and differentiation (EAF) or in inhibiting OC differentiation through the inhibition of NF-κB activation (ECAP) [47]. The *in vitro* study of Kaida *et al.* demonstrated that low EGCC supplementation of osteogenic medium induces the OB differentiation and mineralization of primary human dedifferentiated adipose tissue (DFAT) cells by the expression of the OB markers RUNX-2, Col-1A1, OSX, and Distal-less homeobox 5 (DLX-5). They found also that the presence of dexamethasone in osteogenic medium altered the ERK-1/2, JNK, and p38-MAPK signaling pathways in the EGCG-induced mineralization of DFAT cells. They could not define the mechanism of EGCG's osteogenic ability, but they did not exclude that it might be epigenetic [44]. Sakai *et al.* showed in an *in vitro* model (OB-like MC3T3 E1 cells) that EGCG treatment induces OPG production through prostaglandin F2α (PGF2α) stimulation and increases the OPG/RANKL ratio, inhibiting OC differentiation [45]. These authors highlighted that EGCG hardly affected the PGF2α-induced phosphorylation of the p44/p42 ERK, p38-MAPK, or stress-activated protein kinase (SAPK)/JNK cascade, but it might directly influence the cellular function of OBs through mediating p44/p42 MAPK signaling [45].

Regarding EAF, Wong *et al.* observed in an *in vitro* model (MC3T3-E1 cells) that it can induce OB differentiation, exerting anabolic effects in pre-OB cells (increases in cell proliferation, ALP activity, the Col-1A1 content of the extracellular matrix, and mineralized nodules) and inhibit OC differentiation acting on NF-κB pathway [46]. The authors also investigated the effect of EAF treatment on OVX mice compared with 17β-estradiol. EAF was shown to be protective against OVX-induced bone loss in a way similar to 17β-estradiol, by suppressing weight gain, urinary Ca^{2+} excretion, and the expression of bone-turnover markers and improving the BMD and microarchitecture of both the proximal tibia and the lumbar spine [46]. Finally, the potential therapeutic benefits of ECAP in OP were evaluated by Hsiao *et al.* in *in vitro* (RAW 264.7 cell) and *in vivo* (OVX mouse) models, researching the molecular mechanisms that mediate ECAP's effects on the differentiation and activity of OCs [47]. They found that ECAP plays a dual role as an antiosteoclastogenic agent, inhibiting RANKL expression and preventing NF-κB and NFAcT-1 pathway activation, and in suppressing the bone resorption activity of mature OCs, modulating their resorption-related genes (i.e., MMP-9). The OVX model allows an understanding of the ability of ECAP to reduce bone loss [47].

Flavanones comprise a flavan molecule with an oxo substituent at position 4. Three articles on two molecules, hesperidin, a flavanone-glycoside present in *Citrus aurantium* [48,49], and baicalein, originally isolated from the roots of *Scutellaria baicalensis* [50], were retrieved.

Hesperidin exhibits several pharmacological effects, such as anti-inflammatory, antioxidation, and overall beneficial effects on bone, increasing the anabolic process and increasing bone production. Hesperidin treatment of alveolar OBs promoted their differentiation through several

factors implicated in this process, such as RUNX-2, BMP-2, OSX, and OCN, as demonstrated by an increase in ALP activity and Alizarin Red staining. Hong *et al.* showed that hesperidin's effect is exerted through the activation of Wnt/β-catenin signaling, because treatment of alveolar OBs with the DKK-1 protein, an antagonist of the Wnt/β-catenin signaling pathway, reverted the acceleration of OB differentiation by hesperidin [48]. Shehata *et al.* showed that hesperidin's antioxidative effects reduce bone loss in type I DM-induced (streptozotocin) young male albino rats. Their analysis of bone tissue showed the presence of degenerated bone with large lacunae in untreated diabetic rats, while insulin-treated DM rats showed relatively large areas of immature bone and these areas appeared as matrix not layered in osteonal arrays. Bone tissue analysis of insulin-treated DM rats showed nearly a normal architecture after hesperidin administration, with most of the osteocytes appearing normal, with few degenerated cells, regular outer and inner bone surfaces, and the presence of basophilic cement lines suggesting bone repair. Hesperidin treatment reduced the serum TNF-α level increased by diabetes induction, restoring it to normal levels. NF-κB expression in bone tissue showed very high levels in untreated diabetic rats compared with an insulin-treated group. This level was restored to normal (not significantly different to the non-diabetic rat group) by hesperidin treatment. Hesperidin's anti-inflammatory effects ameliorated bone structure through the induction of OB differentiation and osteocyte activity and the inhibition of OC differentiation and activity [49].

Baicalein is a flavanone that exhibits lipoxygenase inhibitory activity, which produces a reduction of oxidative status favoring bone regeneration with increased osteogenesis and reduced osteoclastogenesis. Saul *et al.* evaluated bone repair using osteotomy conducted in OVX rats administered baicalein. This study indicated that baicalein accelerated bone regeneration, as indicated by μ-CT analysis, although the administration of a high concentration of this molecule produced a decrease in biomechanical properties in early bone callus formation, indicating that baicalein administration is not advisable in early bone healing [50].

Anthocyanins are a large flavonoid family shown to provide a wide range of health-promoting benefits through their antioxidative properties, greater than all other flavonoids. Three articles on anthocyanins describing four molecules were collected in the literature search: malvidin [51], cyanidin [51,52], delphinidin [51,53], and petunidin [53], which are abundant in various plants, particularly in berry fruits, and show potent antioxidative effects. [54]. Some studies on adipocyte-derived MSC differentiation showed that the different anthocyanins have differing effects on the adipogenesis, chondrogenesis, and osteogenesis of these cells. In particular, *in vitro* experiments by Saulite *et al.* showed the following. (i) Delphinidin is a potent adipogenesis inhibitor, demonstrated by reduced expression of adipose tissue-related genes, fatty acid-binding protein-4 (FABP-4), lipoprotein lipase (LPL), and adiponectin and through Oil Red O staining for lipid accumulation. (ii) Cyanidin and delphinidin promote chondrogenesis, as proved by the strong expression of articular chondrocyte markers, such as Col2a1 and aggrecan, and Alcian blue staining of spheroids. (iii) Malvidin has no impact on the chondrogenic differentiation of MSCs but significantly improves OB differentiation, enhancing RUNX-2 and BMP-2 expression, and promotes calcium accumulation in MSC-differentiated osteocytes through Alizarin Red staining [51].

Cyanidin 3-glucoside treatment reduces osteoclastogenesis, demonstrated by TRAP staining, TRAP activity, and the number of $TRAP^+$ MNC OCs, without affecting the OPG/RANKL ratio, indicating a direct effect on OCs. The antiosteoclastogenic effects are due to the suppression of NF-κB, c-FOS, and NFAT-c1 expression, probably through a dramatic decrease in MAPK activation (ERK, JNK, and p38-MAPK). Furthermore, OB treatment leads to an increase of OB

differentiation, as indicated by the high expression of osteogenic markers, such as ALP, OPG, Col-1A1, RUNX-2, and OSX, and Alizarin Red and ALP staining [52].

Nagaoka *et al.* compared the effect of petunidin with that of delphinidin on OC differentiation; treatment of the RAW264.7 cell line showed a dose-dependent decrease of osteoclastogenesis as demonstrated by TRAP staining. Petunidin decreases NF-κB, c-FOS, and NFAT-c1 expression and consequently reduces the expression of OC effector genes such as MMP-9, CTSK, and DC-STAMP. It significantly stimulated mineralized matrix formation in the MC3T3-E1 cell line, as indicated by Alizarin Red S staining, upregulating the expression of BMP-2, an inducer of OB differentiation, and OCN, a marker of mineralization. The mechanisms of petunidin involve inhibition of NF-κB translocation, as indicated by immune staining of NF-κB co-treated with LPS and petunidin. LPS-induced translocation of NF-κB is inhibited by petunidin. In an *in vivo* experiment using s-RANKL to induce osteopenia in mice, petunidin treatment reverted partially the induced bone loss identified by μ-CT imaging and the measurement of bone morphometric parameters [53].

The Roles of Flavonoids in Bone Tissue Homeostasis

Numerous flavonoids regulate bone homeostasis by interacting with the different signal transduction pathways of bone tissue cells. Effects favoring osteoblastogenesis and an increase of OB activity with improved bone deposition are mainly exerted by flavonoids; some of these compounds also act by inhibiting osteoclastogenesis and OC activity.

During OB differentiation, some isoflavones, flavonols, and anthocyanins cause the upregulation of BMP-2 expression, some flavones, isoflavones, flavonols, and flavanones stimulate the Wnt signaling pathway, and the MAPK pathway is activated by most of the flavonoids, resulting in overexpression of the RUNX-2 transcription factor and of OSX, a transcription factor for OB effector genes, increasing OB activity. Flavones, isoflavones, and flavonols interact with the ER, leading to increased OB differentiation, although some exhibit estrogenic effects only in certain cell types, behaving as selective ER modulators. OB differentiation and activity are also stimulated by the activation of the IGF-1 pathway, through the promoting action of some isoflavones and flavonols. Finally, some flavonols, flavanols, and anthocyanins are able to inhibit NF-κB, indirectly improving OB activity.

During OC differentiation, only a few flavonoids, such as isoflavones, flavanols, and anthocyanins, have been tested and shown to inhibit osteoclastogenesis, upregulating OPG expression and then influencing the OPG/RANKL ratio. Although the NF-κB transduction pathway is clearly inhibited in OBs by flavonoid treatment, only a few flavonoids have demonstrated specific inhibitory effects on NF-κB leading to a decrease of NFAT-c1 expression and then of OC differentiation and activity.

Furthermore, all reported flavonoids showed high antioxidative activities, leading to an increase in OB and a decrease in OC differentiation and activity, through epigenetic regulation including DNA methylation, histone (de)acetylation, and miRNA expression. In particular, SIRT-1, an important deacetylase, is activated by the NAD^+/NADH ratio, and all phytochemical compounds with their antioxidative effects contribute to its activation, enhancing the survival, proliferation, and differentiation of OB as well as the induction of OC apoptosis and inhibition of OC differentiation and activity.

Perspectives on the Use of Flavonoids in OP

Flavonoids have beneficial and protective effects on pathological bone loss and OP development. Despite the continuing increase in knowledge on their mechanisms of action, mostly derived from *in vitro* and *in vivo* preclinical studies, to our knowledge there are still no purified phytochemical compounds marketed as supplements or drugs for OP treatment.

Among the possible treatment strategies with the use of purified compounds, it might be useful to administer compounds that simultaneously have an effect both on bone deposition and remodeling. Some flavonoids, such as the isoflavones (daidzein, genistein, puerarin, cladrin, calycosin), flavonols (icariin), flavanols (EAF), anthocyanins (cyanidin, petunidin), act both by stimulating OBs and inhibiting OCs.

Although the retrieved studies showed that only seven of the 22 isolated compounds have effects on both OBs and OCs, many of the pathways involved in the actions of all compounds are common (ER, MAPK, NF-κB) to both cell types and thus it is plausible that they act on both cell types. Many of the included studies consider only one cell type (OB or OC) and did not exclude interaction with the other, and often the effects of a chemical compound on OBs and OCs are highlighted by different publications, confirming our hypothesis. In particular, regarding flavonoid compounds, we have noted that these molecules are tested primarily in OB differentiation, rather than OC differentiation. Therefore, further preclinical studies are needed to compensate for the deficiencies in knowledge on the mechanisms of action of these compounds on both OBs and OCs, before a clinical trial on one of these compounds could be considered. Some of these compounds (cladrin, icariin, EAF, and petunidin) have almost completed preclinical studies confirming their potential role in OP, while the others must be tested in *in vivo* models (daidzein, calycosin, cyanidin, genistein, and puerarin). For the former, it would be mandatory to perform specific clinical trials to evaluate the administration modalities and doses of these compounds in OP treatment.

Concluding Remarks

The flavonoids reported in this review have been shown to have predominantly protective actions aimed at hindering pathological bone loss, highlighting specific effects primarily on OBs, but also on OC differentiation and activity through the same interactors (Figure 3); however, few clinical trials have been conducted. Several preclinical studies have demonstrated the protective effects of flavonoids. Although the epigenetic roles of these compounds are certain [55], few studies have highlighted these mechanisms.

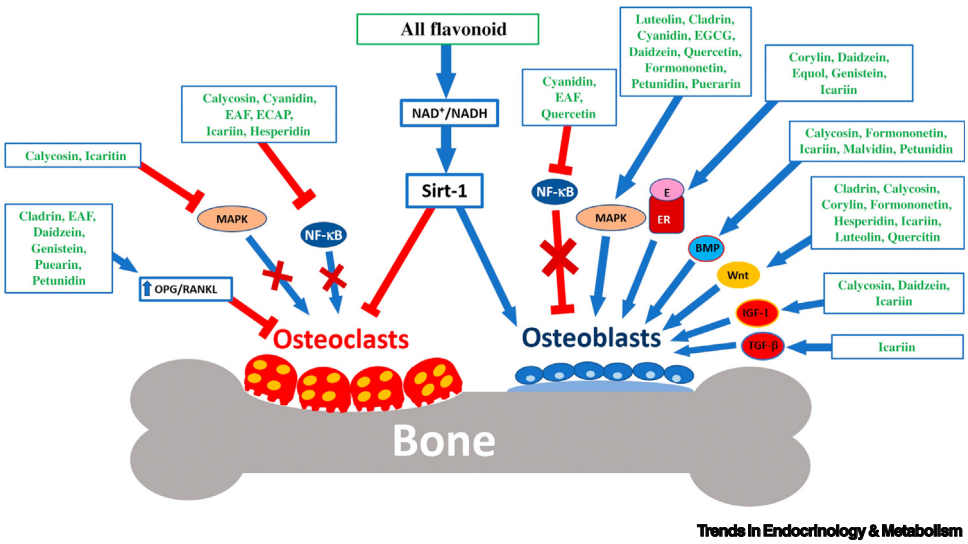

Figure 3. Schematic Diagram of Flavonoid Effects on Osteoblast (OB) and Osteoclast (OC) Cells. Abbreviations: BMP, bone morphogenic protein; E, estrogen; EAF, (-)-epiafzelechin; ECAP, (-)-epicatechin-3-*O*-β-D-allopyranoside; EGCG, epigallocatechin-3-gallate; ER, estrogen receptor; IGF, insulin-like growth factor; MAPK, mitogen-activated protein kinase; NF-κB, nuclear factor kappa-B; OPG, osteoprotegerin; RANKL, receptor activator of NF-κB ligand; SIRT-1, sirtuin-1; TGF, transforming growth factor; WNT, wingless-type MMTV integration site family member.

Outstanding Questions

Many flavonoids affect bone mass, but the mechanisms often remain unsolved.

What are the effects of flavonoids on bone homeostasis?

Are the epigenetic mechanisms common to all classes of flavonoids?

Do the different classes of these compounds act through the same mechanisms and same pathways on OBs and OCs?

Could flavonoids be used as a supplement or therapy to prevent or heal bone fractures by regulating bone homeostasis?

Could these molecules be used to recover bone loss and heal osteoporotic fractures?

Could combined therapy with these phytochemicals and other drugs improve the effects on OP?

Although many *in vivo* studies show a reduction of bone loss in various osteoporotic models (OVX-OP, GI-OP, etc.) by treatment with flavonoids, only a few studies highlight the cellular and molecular mechanisms underlying this effect.

Despite several studies demonstrating unequivocally the beneficial effects of flavonoids on bone health, the lack of detailed mechanisms responsible for bone protection, and of clinical trials on these molecules with large cohorts of osteoporotic patients, is the principal limitation on their current clinical use. Among our bibliographic collection, only two clinical studies on the effects of isoflavone treatment in postmenopausal women were found, highlighting contrasting results on their effects in reducing bone loss and OP development [56,57].

The new omics science nutrigenomics, which describes the epigenetic mechanisms of action of those phytochemicals that can be consumed with the diet, might open new frontiers in counteracting imbalances in bone homeostasis. In the future, several molecules reported here could be taken as phytochemical drugs or phytoepidrugs for the prevention of bone diseases and associated therapy. Further preclinical and clinical studies on the role of phytochemicals in bone homeostasis are mandatory to improve knowledge on the mechanisms of actions, elective targets, and possible side effects of treatments (see Outstanding Questions).

Author Contributions

D.B. and E.D. conceived and designed the search strategy for articles, selected the bibliography, and wrote the manuscript. Vi.C., Va.C., A.D.L., and L.R. revised the section 'Polyphenols: Flavonoid Classes in Bone Homeostasis'. C.G. provided pharmacological feedback. F.C. provided feedback in the epigenetics field. M.F. and G.G. provided critical feedback on the entire manuscript. D.B., E.D., Vi.C., A.D.L., Va.C., L.R., C.G., M.F., F.C., and G.G. approved the submitted version of the manuscript.

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