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REVIEW ARTICLE

Are Oxidative Stress and Inflammation Mediators of Bone Loss Due to Estrogen Deficiency? A Review of Current Evidence

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Abstract: Osteoporosis is one of the major health issues associated with menopause-related estrogen deficiency. Various reports suggest that the hormonal changes related to menopausal transition may lead to the derangement of redox homeostasis and ultimately oxidative stress. Estrogen deficiency and oxidative stress may enhance the expression of genes involved in inflammation. All these factors may contribute, in synergy, to the development of postmenopausal osteoporosis. Previous studies suggest that estrogen may act as an antioxidant to protect the bone against oxidative stress, and as an anti-inflammatory agent in suppressing pro-inflammatory and pro-osteoclastic cytokines. Thus, the focus of the current review is to examine the relationship between estrogen deficiency, oxidative stress and inflammation, and the impacts of these phenomena on skeletal health in postmenopausal women.

Keywords: Inflammation, menopause, estrogen, osteoporosis, oxidative stress, review.

1. INTRODUCTION

Progressive bone loss and skeletal fragility caused by osteoporosis is an emerging public health problem in a world experiencing a rapid increase in the elderly population. The primary risk factor for bone loss in midlife women is menopause [1, 2]. A high level of estrogen is present in women from the onset of menstrual periods during puberty until menopause, which marks the termination of reproductive age. Most estrogen is produced in the ovaries, released into the circulation and exert their effects on target tissues through endocrine signalling. After menopause, the circulating estrogen levels fall drastically when estrogen production from the ovaries ceases [3]. Approximately 50% of trabecular bone and 30% of cortical bone diminish during the course of a woman's lifetime, of which half is lost during the first 10 years after menopause [4].

The pleiotropic effects of estrogen indicate that its deficiency will impact many signalling pathways in the body [5]. For example, metabolic changes due to menopause, like the accumulation of adipose tissue in the body, is associated with chronic low-grade inflammation and oxidative stress, leading to diseases like cancer [6]. The effects of menopause on oxidative stress and inflammation are also of particular interest in the field of osteoporosis because all of them are contributors to bone loss [7-9].

Therefore, this review aims to provide an overview of the relationship between estrogen deficiency, oxidative stress and inflammation and their combined effects on bone loss in women. The information obtained will be instrumental in planning preventive strategies against postmenopausal bone loss.

2. ESTROGENS AND ESTROGEN SIGNALLING PATHWAY

Estrogen is a group of steroid hormones governing the development of secondary female sex characteristics and regulating the female reproductive system. It also has other non-reproductive physiological roles. Estrogen is produced primarily in the ovaries through the stimulation of follicle-stimulating hormone, and in small amounts by the adrenal glands, breasts, adipose and liver. Endogenous estrogen is converted from androgens in women via a series of enzymatic reactions, which produce estrone (E1), estradiol (E2) and estriol (E3). In the ovary, androstenedione is produced from cholesterol and converted immediately into either E1 or testosterone. Aromatase, a cytochrome P450 enzyme in the endoplasmic reticulum of estrogen producing cells, then converts androstenedione and testosterone into E2. Estradiol is the potent and predominant estrogen present before the first period until the menopause. The strong potency of E2 is attributed to its high affinity towards estrogen receptors compared to other estrogen forms. Estrone is a weak estrogen found in women after menopause, which can be converted to E2 and vice versa. Estriol is the weakest estrogen produced in abundance during pregnancy and cannot be converted to E2 nor E1 [10].

Estrogen is a chemical messenger, which can travel through the circulatory system and interact with cells by

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binding to estrogen receptors. Estrogen signalling occurs via specific nuclear receptors by acting as ligand-activated transcription factors of two isomers of estrogen receptors (ERs), i.e. ER-alpha (ER-α) and ER-beta (ER-β) [11-14]. ER-α is found predominantly in the uterus, bone, adipose, liver, kidney, heart [12, 15, 16], whereas ER-β is found predominantly in the ovary, bladder, prostate, gastrointestinal tract, central nervous system and hematopoietic cells [12, 16]. Estrogen binds to its receptors in the nucleus, causing the receptor to dimerize and bind to estrogen response elements located in the promoters region of target genes. Subsequently, expression of these genes will be modulated, resulting in the biological actions of estrogen. In addition, ERs also regulate gene expression by influencing protein-protein interactions with other DNA-binding transcription factors in the nucleus. Estrogen also acts via nongenomic action through the activation of protein-kinase cascades via membrane-associated ERs [17].

3. DIRECT EFFECTS OF ESTROGEN ON BONE HEALTH

Estrogen exerts a strong influence on skeletal growth and homeostasis. During bone growth, estrogen is responsible for the proper closure of epiphyseal plates [18]. In bone, estrogens act directly via ERs on osteoblasts, osteocytes, osteoclasts, immune cells and other cells in maintaining bone mass [19-23]. Bone is being constantly remodelled via the actions of these bone cells [24]. Osteoblasts perform bone formation by laying down the new bone matrix and mineralize it, while osteoclasts break down the bone during bone resorption. The balance between both of these processes is crucial for sustaining bone mass and maintaining systemic skeletal homeostasis [25]. During puberty, estrogen increases bone mass through increasing number and activity of osteoblast, as well as decreasing osteoclast activity [26]. Estrogen also prevents apoptosis of osteocytes by preserving their autophagy function [27]. The inverse occurs during menopause, whereby the rate of bone resorption overwhelms bone formation, resulting in a decrease in bone mass [28].

Differential expression of ERs has been reported in osteoblasts and osteoclasts. In general, ERα mediates most actions of estrogen on bone cells. Activation of ER signalling pathway stimulates osteoblast differentiation and suppresses osteoclast activity [29]. Estrogen deficiency increases osteoclast formation and prolongs their lifespan. Estrogen deficiency activates the inflammatory cascades, leading to increase production of macrophage colony-stimulating factor (M-CSF) and receptor activator of nuclear factor kappa-B ligand (RANKL), which is an osteoprotegerin (OPG) ligand, by stromal-osteoblast lineage cells [30, 31]. The binding of RANKL to RANK receptors stimulates osteoclast differentiation and activity and prevents their apoptosis. The binding of M-CSF to its receptor also stimulates the proliferation and survival of osteoclast precursors and the mature osteoclasts [32]. OPG produced by the stromal-osteoblast lineage cells binds to RANKL and prevents the activation of the RANK-RANKL signalling pathway [33]. Estrogen is reported to increase both OPG and RANKL expression, but the OPG expression sustains for a longer period compared to RANKL, giving rise to a larger OPG/RANKL ratio [34-36]. ERα-knockout mice had less apoptotic osteoclasts than wildtype mice when estrogen was supplemented, suggesting that ERα is needed to upregulate the pro-apoptotic factor Fas ligands in osteoclasts [37]. Lower bone mass and strength developed in osteoblast-specific ERα-depleted mice, showing that estrogen acts on ERα in osteoblasts to achieve its skeletal protective effects [38].

The mechanical properties of bone are determined by its structural and material characteristics [39]. The deterioration of bone mass, macro and micro-architecture of the bone induced by estrogen deficiency will lead to a reduction of bone strength and increased risk of fracture. Ovariectomized (OVX) rats showed lower femoral or tibial bone volume, trabecular number (Tb.N), and trabecular thickness (Tb.Th) and higher structural model index and trabecular separation (Tb.Sp) compared to the sham group as evaluated by micro-computed tomography or bone structural histomorphometry analysis [40-46]. These degenerative changes result in increased porosity of the bone [47]. Similar changes were observed in OVX model of mice and rabbits [48, 49] The OVX rats also showed a significant reduction in density of the maxillary bone after 12 weeks, which could cause tooth loss [41].

The mineralizing activity of the bone *in vivo* can be measured by dynamic histomorphometry parameters. OVX rats are reported to have a lower double-labelled surface (sites of bone mineralization), bone formation rate and mineral appositional rate compared to the sham group [45, 50]. In bone cellular histomorphometry, lower osteoblast surface, osteoid surface and osteoid volume were observed in OVX rats, suggesting reduced osteoblast number and bone formation activity [41, 45, 51]. On the other hand, OVX rats experienced increased bone resorption as evidenced by increased osteoclast surface and eroded surface (sites of bone resorption) [41, 45, 51]. Alternatively, changes in bone remodelling activities were illustrated by circulating markers. Some studies reported increased bone resorption markers (like C-terminal cross-linking telopeptide type I collagen/CTX-1) and reduced bone formation markers (like alkaline phosphatase/ALP and osteocalcin) in OVX rats [41, 52]. However, examples of concurrent evaluation of both bone formation and resorption markers, suggestive of high bone turnover, are also common [53].

Ultimately, all of these structural and mineral degenerative changes result in a reduction in bone biomechanical strength in OVX rats. Reduced maximum/ultimate force to break the bone, elastic modulus and stress were observed in OVX rats compared to the sham group [40-49, 54].

Estrogen deficiency renders postmenopausal women vulnerable to osteoporosis [55]. Bone mineral density (BMD) by dual-energy X-ray absorptiometry (DXA) is considered the gold standard in determining osteoporosis [56]. Based on the World Health Organization (WHO), osteoporosis is defined by a BMD T-score ≤ -2.5. In multiple studies, DXA assessment revealed that osteoporosis prevalence was higher among the postmenopausal women compared to their men counterparts [57-60]. Decreased BMD

was found to be significantly associated with age, menopause age and the year since menopause [57].

Mederle *et al*. reported that the decrease of E2 level in postmenopausal women was associated with a significant decrease in BMD at the lumbar spine and femoral neck [61]. Concurrent reduction of BMD and high bone turnover indicated by high circulating bone turnover markers, such as ALP, osteocalcin and CTX-1, are commonly observed among postmenopausal women [62]. Thompson *et al*. revealed that within 5 years of menopause, alveolar bone density loss was associated with elevated circulating levels of matrix metalloproteinase-2 (MMP-2) indicative of osteoclastic bone resorption [63]. Menopausal women were observed to have a higher level of bone resorption markers, like N-terminal propeptide of type I procollagen and CTX-l, and lower trabecular bone score, which correlated with skeletal microarchitecture deterioration [64].

4. ESTROGEN DEFICIENCY AND OXIDATIVE STRESS

Oxidative stress is defined as a disparity between the generation of reactive oxygen species (ROS) from various oxidation pathways and the antioxidant defence system in the body. The excess free radicals overwhelm the normal antioxidant capacity of the body, leading to damage of cellular macromolecules, such as enhanced lipid peroxidation, protein modification and DNA breakage, ultimately affecting cellular functioning [65].

Generally, ROS are short-lived but highly reactive chemical species containing oxygen [66]. The nicotinamide adenine dinucleotide phosphate (NADPH) oxidase is a membrane-bound enzyme complex that generates ROS, including oxygen-derived free radicals like superoxide anion (O^-) and the hydroxyl radical (H^-), or non-radical molecules like hydrogen peroxide (H_2O_2) [67]. Free radicals react with oxygen to produce O^-, which react with nitric oxide (NO) to produce peroxynitrite. The oxygen singl*et al*so undergoes dismutation in a process catalysed by superoxide dismutase (SOD) to produce H_2O_2, which is detoxified into a water molecule by antioxidants enzymes such as catalase (CAT) and glutathione peroxidase (GSH-Px/GPX). GPX neutralizes H_2O_2 by taking hydrogens from two glutathione (GSH) molecules, thus forming two water molecules and one glutathione disulfide (GSSG). An increase in oxidative stress will cause intracellular GSSG accumulation and a decrease in GSH/GSSG ratio level [68, 69]. Therefore, the ratio of reduced GSH and oxidize GSSG is important in determining redox status and serves as useful indicators of oxidative stress markers. Glutathione-S-transferase (GST) catalyzes the conjugation of glutathione (GSH) to form endogenous and exogenous electrophilic compounds. GST plays a regulatory role in the mitogen-activated protein (MAP) kinase pathway involved in cellular survival and death signals [70-72].

In the culture of human bone marrow cells, H_2O_2 was shown to stimulate a significant increase in the formation and activity of osteoclast-like cells expressing tartrate-resistant acid phosphatase (TRAP). H_2O_2 also increased the expression of M-CSF, RANKL and RANKL/OPG ratio. Treatment with CAT significantly suppressed the formation of TRAP multinucleated cells as well as M-CSF and RANKL expression [73]. A study conducted by Lean *et al*. demonstrated that 17β-estradiol increased the expression of GPX in osteoclasts and antiestrogen ameliorated this effect. Overexpression of GPX in RAW 264.7 cells suppressed osteoclastic differentiation associated with inhibition of NFκB-activation mediated by RANKL [74]. In MLO-Y4 osteocyte-like cells, 17β-estradiol significantly increased the total glutathione S-transferase P expression and suppressed RANKL and sclerostin expression, RANKL release, and RANKL/OPG ratio [75]. Therefore, these studies demonstrated that estrogen can regulate the activity of bone cells by altering their redox status.

In animal studies, OVX rats exhibited a significant decrease in the activity of SOD, CAT, GPX, GST and GSH level, as well as an increase in malondialdehyde (MDA) level [76-78]. A previous study showed that estrogen deficiency compounded the effects of ageing on the oxidative status in rats, wherein LPO and NO level, as well as inducible NO synthase protein expression, were increased in the old rats compared to the young animals, and the changes were more prominent in the OVX group [79]. Mitochondria are a major source of ROS. Estrogen deficiency was shown to inhibit mitochondrial β-oxidation of fatty acid and increase ROS production. As evidence, the liver mitochondrial and peroxisomal H_2O_2 generation in OVX mice increased significantly, while the antioxidant enzyme activities decreased [80]. In OVX rats fed with high-fat diet, the expressions of antioxidant enzymes SOD and GPX were significantly suppressed whereas the expression of pro-oxidative enzyme NADPH oxidase was elevated compared to the control group. The resultant oxidative stress-activated mitogen-activated protein kinases (MAPK) pathway and upregulated ERK 1/2 and p38, leading to metabolic derangement of the rats in conjunction with high-fat diet [81]. Additionally, ovariectomy also increased the level of homocysteine (an indicator of cardiovascular disease), along with elevated oxidative stress markers MDA, oxidized-low density lipoprotein and GSSG levels in the rats [82]. A study by He *et al*. reported that lower BMD was associated with lower SOD and GPX levels and OPG expression in OVX rats compared to the control group. Ovariectomy also increased serum osteocalcin, ALP, MDA levels and RANKL expression in the rats [83]. Estrogen treatment was shown to normalize redox stress and preserve the bone health of the OVX rats [84]. In a study by Yang *et al*., the depleted antioxidant status due to ovariectomy was associated with declined autophagy and increased apoptosis of osteocytes. Treatment with estrogen was able to reverse these changes [85].

The link between oxidative stress and estrogen deficiency has been demonstrated by several human studies. Oxidative stress is hypothesized as one of the causes of physiological changes due to postmenopausal and ageing [86, 87]. Signorelli *et al*. reported that postmenopausal women (n=51, aged 52.1±1.3 years old) experienced a higher level of oxidative stress compared to fertile women (n=50, 32.5±1.1 years old), indicated by higher serum MDA, 4-hydroxynonenal and oxidized lipoprotein levels [7]. Another study also demonstrated that serum GSH levels decreased

significantly while serum MDA and γ-glutamyltransferase levels increased significantly in the postmenopausal women group (n=16) compared to the premenopausal women group (n=17) [88]. Increased hydroperoxide (ROOH) level, a marker of lipid peroxidation, was negatively and independently associated with decreased BMD and increased bone resorption rate marked by CTX-1 levels in postmenopausal women [89]. Other studies showed that hormone replacement therapy (tibolone [90], oestradiol alone or in combination with medroxyprogesterone [91]) was able to reverse the decline in GSH, GPX and non-enzymatic antioxidants (alpha-tocopherol) while suppressing the level of lipid peroxidation markers in postmenopausal women [90, 91].

5. ESTROGEN DEFICIENCY AND INFLAMMATION

Inflammation involves the coordinated action of many cell types and mediators in response to various harmful stimuli, such as damaged cells, pathogens, and toxicants, leading to the elimination of the insult and restoration of homeostasis [92]. Interleukins (ILs) are the most well-profiled inflammation markers in diseases. Some of the examples include IL-6, a proinflammatory cytokine produced in response to tissue injury [93], tumour necrosis factor-alpha (TNF-α), an important mediator of the inflammation [94] and interferon-gamma (IFN-γ), produced by activated T lymphocytes in response to inflammation [95]. Intracellular signalling pathways, including MAPK, NF-κB, Janus kinase (JAK)-signal transducer and activator of transcription (STAT) are involved in the regulation of inflammation in disease state [96, 97]. Toll-like receptors (TLR) mediated immune response through the induction of MMPs or inhibiting the expression of certain structural proteins. Animal studies showed that inhibition of TLR4 signalling is a pharmacological avenue for retarding the progression of osteoporosis [98].

In animals, T cells harvested from OVX mice produced insufficient TNF-α to induce RANKL-independent osteoclastic formation, but sufficient to increase osteoclastogenesis caused by M-CSF and RANKL through the engagement of TNF-α receptor p55 [99]. Reports showed that 17β–estradiol caused a 1.7 to 3.2-fold increase in osteoclast apoptotic proportion [100]. Incubation of normal human osteoblastic-like cells with 17β–oestradiol revealed a significant increase in tumour growth factor-beta (TGF-β) level after 24 hours [101]. The effects of TGF-β on osteoclasts suggest the involvement of estrogen in the direct inhibition of osteoclast resorption activity [102]. A study also showed that estrogen hastened the resolution of inflammation in RAW 264.7 cells through SOCS3 and STAT3 signalling pathways [103]. Thus, this could be one of the mechanisms estrogen prevents chronic inflammation in the body.

In vivo studies indicated that estrogen deficiency caused a significant increment in serum TNF-α and IL-6 levels in OVX animals compared to the sham group [104-106]. Ovariectomy also caused increased expression of cell adhesion molecules in blood vessels and circulating proinflammatory cytokines in rats [107, 108]. Since estrogen deprivation is associated with an increase in cytokine level, administration of estrogen is expected to decrease cytokine level. Estrogen administration significantly reduced IL-6, TNF-α and IL-1β expression while increasing IL-10 level in OVX rats [79]. OVX also enhanced inflammation at the visceral adipose tissue, as evidenced by reduced IL-10 level (an anti-inflammatory cytokine) and increased TNF-α level [109]. Estrogen replacement improved the inflammatory status of OVX rats by decreasing the TNF-α concentration level by 18% [109].

The pro-inflammatory effects of estrogen deficiency are mediated by multiple mechanisms. A study by Xu *et al*. showed that ovariectomy caused neuroinflammation by significantly increasing TLR-2 and TLR-4, active NF-κB, pro-IL-1β and pro-IL-18 level in the hippocampus of rats [110]. OVX and ER-α knockout mice demonstrated the deregulation of TLR2 signalling in the heart, resulting in a 5.7-fold increase in IL-6 and a 4.7-fold increase in phospho-Stat3 levels. This observation suggests an over-activation of the JAK/STAT3 pathway [111]. ArKO mice suffering from estrogen deficiency expressed significantly higher serum IL-6, TNF, MCP-1 and IFN-γ induced by LPS. These changes were significantly abrogated by the administration of selective agonists of ER-α [112]. TNF-overexpressing transgenic mice showed a dramatic loss of metaphyseal trabecular bone mass marked by significant decreases in both Tb.N and Tb.Th and cortical thickness bone compared to wild type (WT). These skeletal alternations corresponded to higher gene expression of TNF, IL-1β and RANKL in the transgenic mice compared to the WT [113]. In contrast, chronic E2 administration in OVX mice markedly increased the expression of IL-1β, IL-6 and IL-12p40 by lipopolysaccharides-stimulated resident peritoneal macrophages *in vivo*. This effect was attributed to inhibition of phosphoinositide 3-kinase (PI3K) pathway, which acts as a negative regulator to TLR4 signalling [114].

A large body of evidence suggests estrogen deficiency in postmenopausal women is related to an altered immune profile. Hot flash commonly experienced by postmenopausal women was associated with low-grade systemic inflammation indicated by a higher level of circulating IL-8 and TNF-α [115]. Modest weight gain among postmenopausal women was associated with a pro-inflammatory state indicated by increased intercellular adhesion molecule-1 (ICAM-1) and TNF-α [116]. Postmenopausal women free from any pro-inflammatory conditions had higher levels of IL-1, IL-6, and TNF-α compared to premenopausal women [117]. High levels of cytokines (IFN-α2, IFN-γ, IL-12p70, IL-33) and MCP-1 in apparently healthy postmenopausal women were associated with a decrease in hip BMD [118]. *In vitro* studies showed that TNF-α promotes RANKL-induced osteoclast formation through activation of PI3K/ protein kinase B (Akt) signalling, which ultimately contributes to bone loss in postmenopausal women who possessed an increased level of TNF-α [119].

CONCLUSION

The current literature supports the multifaceted role of estrogen in preserving bone via direct binding to ERα primarily and ERβ, regulating redox status and

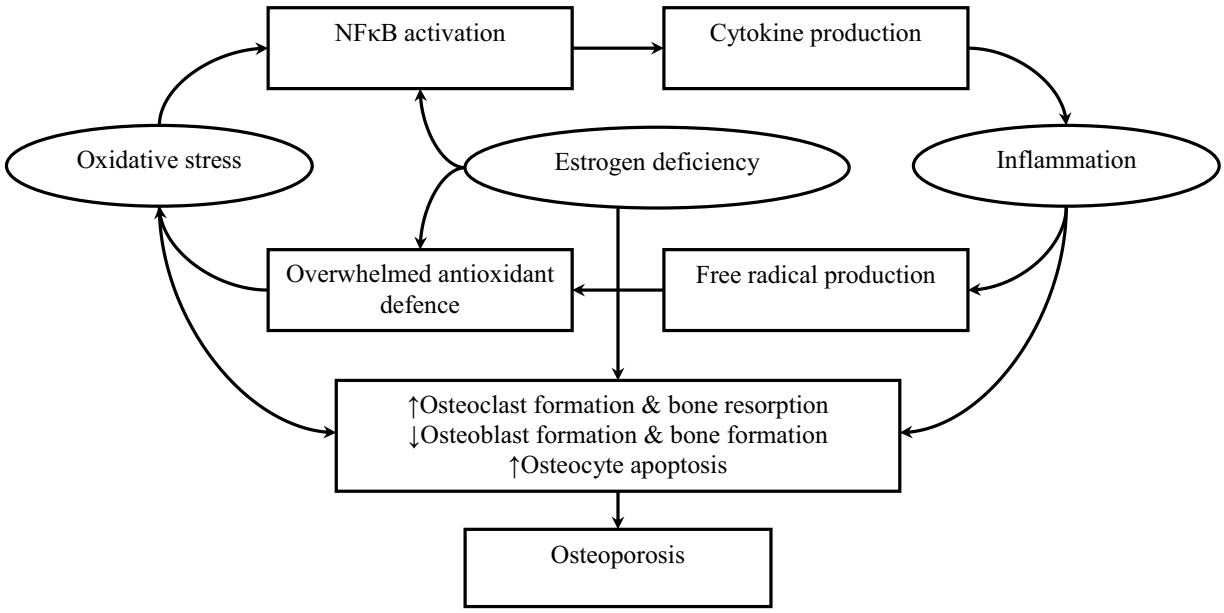

Fig. (1). The effects of estrogens deficiency on bone loss through oxidative stress and inflammation.

inflammation. Estrogen deprivation due to menopause brings physiological challenges to women as the skeletal protective effects of estrogen are lost. The upswing in the ROS level and the release of pro-inflammatory cytokines adversely affect the survival and activity of osteoblasts but promote the formation and activity osteoclasts (Fig. **1**). The corresponding unfavourable changes in bone remodelling skewing towards bone resorption, structural and mineral alternations are the main factors of postmenopausal osteoporosis. These mechanisms are potential avenues for interventions. For instance, phytoestrogens with antioxidants and anti-inflammatory activities may be used to prevent further bone loss of women, in conjunction with sufficient calcium and vitamin intake, as well as other lifestyle interventions. These approaches, however, should not supplant proper pharmacological agents for osteoporosis if the patients are at high risk of fracture. The use of hormone replacement therapy (HRT) is a rational approach to counter osteoporosis induced by estrogen deficiency [120, 121]. Estrogen replacement therapy is the Food and Drug Administration (FDA)-approved treatment to prevent osteoporosis in postmenopausal women. Studies showed that HRT can preserve BMD at all skeletal sites in postmenopausal women [122, 123]. The controversial Women's Health Initiative raised safety concerns of HRT, whereby an association between HRT and cardiovascular diseases and breast cancer was reported [124, 125]. Nonetheless, the data have been re-analysed and it was revealed that the HRT is effective and appropriate to prevent osteoporosis related-fracture [126, 127]. Even so, the choice of different approaches to rehabilitation and therapy still needs to be considered based on treatment feasibility, patient risk, and treatment cost-effectiveness.

CONSENT FOR PUBLICATION

Not applicable.

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CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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