GLUCOCORTICOID AND BONE: FRIEND OR FOE?

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GLUCOCORTICOID AND BONE: FRIEND OR FOE?

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Table of Contents

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Editorial: Glucocorticoid and bone: Friend or foe

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Editorial on the Research Topic
Glucocorticoid and bone: Friend or foe?

Introduction

Glucocorticoids have potent anti–inflammatory effects and their discovery in 1948 improved therapy for many diseases with chronic inflammation noticeably. However, glucocorticoid treatment causes severe side effects, including bone loss and increased fracture risk. The first studies on the interaction between glucocorticoids and bone were published shortly after their discovery over 60 years ago and interest on the interaction between endogenous and exogenous glucocorticoids and bone grows (Figure 1). The collection of articles on the topic "Glucocorticoid and bone: friend or foe" consists of original and review articles which summarize and elaborate on current knowledge of basic mechanisms of glucocorticoid hormones and their receptors in bone cells and on the clinical aspects of treatment and prevention of glucocorticoid-induced osteoporosis (GIOP).

Exogenous and endogenous glucocorticoid metabolism and bone

Endogenous glucocorticoid hormones are main mediators of stress responses and besides regulating immune responses, they influence whole body homeostasis, metabolism and tissue homeostasis including the skeletal system. The activity of glucocorticoids within the cells is controlled by the enzymes 11β-hydroxysteroid dehydrogenases 1 and 2 (11β-HSD1, 11β-HSD2) acting in opposing manners. 11β-

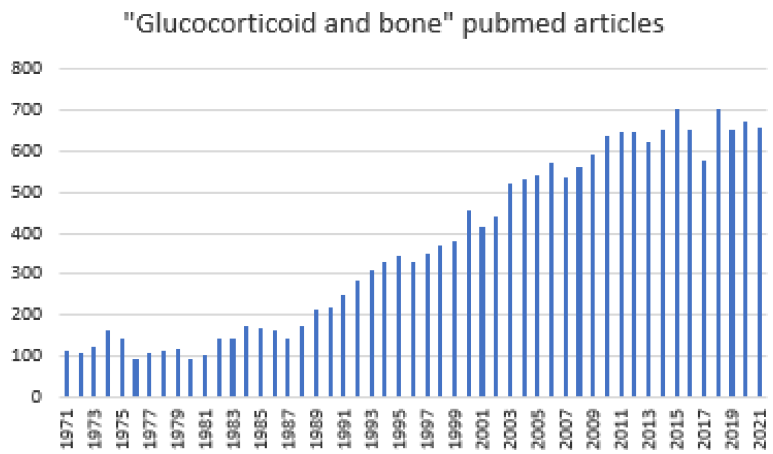

FIGURE 1
Number of publications listed in pubmed on "glucocorticoids and bone" over the last 50 years.

HSD1 converts the inactive hormone cortisone into the active form cortisol, whereas 11β-HSD2 oxidizes cortisol into cortisone. Local pre-receptor metabolism of glucocorticoids by 11ß-HSDs contributes to cell-type and tissue specific actions of glucocorticoids that may influence metabolism and also bone homeostasis. Martin et al. review the role of 11ß-HSD enzymes and local metabolism in bone homeostasis and bone function and discuss strategies how to modulate local glucocorticoid metabolism in order to treat bone diseases.

Activated glucocorticoids bind to the glucocorticoid receptor, a nuclear receptor that induces transactivation or transrepression of different target genes depending on cell types. Lee et al. discuss in their review the complexity of glucocorticoid actions in different bone cell types on a molecular level. They further summarize recent studies on influences of therapeutic glucocorticoids on circadian rhythm of endogenous glucocorticoid levels and the consequent impact of the disturbed circadian rhythm on bone integrity.

The article of Gado et al. summarizes the effects and molecular mechanisms of therapeutic glucocorticoids on bone cells, specifically on osteoblasts and osteocytes and highlight their implications for clinical therapy of GIOP.

The clinical impacts of endogenous hypercortisolism on phosphate homeostasis are investigated by Bosman et al. in a retrospective study on 99 patients with Cushing's syndrome (CS). 16% of patients with CS had hypophosphatemia, which was associated with increased cortisol urinary excretion. In a subset of patients, serum phosphate level increased significantly after CS patients went into remission. The authors postulate that possible mechanisms for urinary phosphate excretion could include FGF23, BMI and parathyroid hormone levels.

Treatment and prevention of glucocorticoid-induced osteoporosis

Hayes et al. addresses the difficulties and uncertainties on starting and stopping bone-protective medication in GIOP. They state that there is a low awareness of GIOP but also a lack of clear guideline recommendations in particular for when to stop osteoporosis treatment. Based on current evidence the advice is to stop bone-protective medication 6-12 months after glucocorticoid discontinuation, since fracture remains elevated for about one year following glucocorticoid treatment cessation. Since it is widely known from the Denosumab and Teriparatide Administration (DATA) extension study that teriparatide followed by denosumab is effective for treatment-naïve postmenopausal osteoporotic women with an increase in femoral neck, total hip and spine BMD (1), it is interesting to see whether this also counts for patients on glucocorticoids. Hirooka et al. investigated sequential treatment strategies in GIOP patients who were pre-treated with bisphosphonates. The study demonstrates that the treatment sequence of two years of teriparatide followed by two years of denosumab leads to higher femoral neck bone mineral density (BMD) gain than with 4 years of continuous denosumab treatment (non-randomized). Only little is described on herbal medicines for GIOP. Zhang et al. review the potential use of herbal compounds. They describe that compounds like escin, ginsenosides and glycyrrhizic acid exert anti-inflammatory properties like glucocorticoids, but without inducing GIOP, and also compounds such as tanshinol and icariin that alleviate GIOP through mechanisms including regulation of Wnt and RANKL/RANK signaling.

GIOP in various diseases

This section includes articles on bone health and fracture risk of rare conditions, which are frequently treated with high dose glucocorticoids.

Box et al. review current evidence and mechanism of bone loss and increased fracture risk in large and small vessel vasculitides with a particular focus on the impact of high dose glucocorticoids on bone health. The article also elaborates on other factors that increase fracture risk including chronic inflammation, organ involvement such as chronic kidney disease and relative immobility. The increasing use of adjunctive glucocorticoid-sparing treatments may have a potential positive impact on fracture risk in patients with vasculitis.

The observational study by Liu et al. presents quantitative computer tomography BMD data of nineteen patients with Duchenne muscular dystrophy (DMD) treated with high dose glucocorticoids. The study shows a gradual overall BMD loss over 2 years at the lumbar spine. A multilevel mixed effect model identified age and functional activity scores but not cumulative glucocorticoid exposure as independent predictors of BMD loss.

Rymuza et al. describes the impact of intravenous methylprednisolone on bone microarchitecture in 15 patients with graves orbitopathy. The study shows that trabecular bone score decreased significantly in 33% of patients treated with high dose intravenous methylprednisolone. The authors highlight the need for fracture risk and BMD assessment in these patients.

In summary, glucocorticoid effects on bone are still not completely understood, thus this topic is still a major research focus. The research topic provides state-of-the-art reviews and novel molecular and therapeutic insights into the dichotomous relationship between glucocorticoids and bone. Overall, novel insights into the pathogenesis of GIOP may provide better prevention and treatment strategies of affected patients.

Author contributions

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

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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REVIEW
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Endogenous Glucocorticoid Metabolism in Bone: Friend or Foe

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The role of tissue specific metabolism of endogenous glucocorticoids (GCs) in the pathogenesis of human disease has been a field of intense interest over the last 20 years, fuelling clinical trials of metabolism inhibitors in the treatment of an array of metabolic diseases. Localised pre-receptor metabolism of endogenous and therapeutic GCs by the 11β-hydroxysteroid dehydrogenase (11β-HSD) enzymes (which interconvert endogenous GCs between their inactive and active forms) are increasingly recognised as being critical in mediating both their positive and negative actions on bone homeostasis. In this review we explore the roles of endogenous and therapeutic GC metabolism by the 11β-HSD enzymes in the context of bone metabolism and bone cell function, and consider future strategies aimed at modulating this system in order to manage and treat various bone diseases.

Keywords: glucocorticoid, bone, 11beta-hydroxysteroid dehydrogenase, osteoclast, osteoblast, osteoporosis, chronic inflammation

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INTRODUCTION TO PRE-RECEPTOR GLUCOCORTICOID METABOLISM

The role of tissue specific metabolism of endogenous glucocorticoids (GCs) in the pathogenesis of human disease has been a field of intense interest over the last 20 years. This has fuelled clinical trials of chemical inhibitors aiming to prevent metabolic side effects associated with corticosteroid excess, such as insulin resistance, cardiovascular disease and hypertension (1–8). Several enzymes, but most prominently 11β-hydroxysteroid dehydrogenase (11β-HSD) type 1 and 2 play a critical role in regulating peripheral exposure to GCs within tissues *via* their pre-receptor enzyme activity (9, 10).

Abbreviation: 11β-HSD1, 11β-hydroxysteroid dehydrogenase type 1; 11β-HSD2, 11β-hydroxysteroid dehydrogenase type 2; BLCs, bone lining cells; CBG, corticosteroid-binding globulin; CTx, carboxy terminal telopeptide of type I collagen; DXA, dual-energy X-ray absorptiometry; GCs, glucocorticoids; GIOP, glucocorticoid induced osteoporosis; GR, glucocorticoid receptor; HIFα, hypoxia-inducible factor alpha; HPA, hypothalamic-pituitary-adrenal; IL-6, interleukin 6; MAPK, mitogen-activated protein kinase; NAD, nicotinamide adenine dinucleotide; NADPH, nicotinamide adenine dinucleotide phosphate; NFκB, nuclear factor kappa-light-chain-enhancer of activated B cells; OPG, osteoprotegerin; P1CP, procollagen type 1 carboxy-terminal propeptide; P1NP, procollagen type 1 amino-terminal propeptide; PDGF-BB, platelet-derived growth factor-BB; RANK, receptor activator of nuclear factor kappa-B; RANKL, receptor activator of nuclear factor kappa-B ligand; Runx2, runt-related transcription factor 2; THE, tetrahydrocortisone; THF, tetrahydrocortisol; TNFα, tumour necrosis factor alpha; VEGF, vascular endothelial growth factor.

To date, therapeutic interventions have primarily focussed on the GC activating enzyme 11β-HSD1 based on its expression in tissues that are themselves targets for GCs such as bone, where therapeutic GCs drive a rapid and sustained reduction in bone formation and increased risk of fracture (11, 12). Here, global transgenic deletion of 11β-HSD1 in murine models of corticosterone excess have protected animals from deleterious side effects, reinforcing the potential clinical utility of pharmacological inhibition (13, 14). However, the role of corticosteroid activation by 11β-HSD1 selectively within bone, and the cell populations that regulate bone metabolism remain cell and context dependant, and the value of selective 11β-HSD1 inhibitors in clinical practice is unclear. However, given the deleterious impact of therapeutic and endogenous corticosterone excess on bone metabolism, a greater understanding of the role of 11β-HSD1 in bone remains paramount (15). This review examines the latest literature relating to both the role of 11β-HSD1 in bone cells and its regulation of bone metabolism, and further explores the value of therapeutic 11β-HSD1 inhibition to treat osteoporosis.

GLUCOCORTICOID METABOLISM BY THE 11β-HSD1 AND 11β-HSD2 ENZYMES

The 11β-HSDs are intracellular enzymes that interconvert endogenous GCs between their inactive and active forms (**Figure 1**). There are two 11β-HSD enzymes. 11β-HSD1, in the presence of its cofactor nicotinamide adenine dinucleotide phosphate (NADPH), primarily converts the inactive adrenal corticosteroid cortisone to its active counterpart cortisol *via* its oxoreductase activity (converting a ketone on the 11 position of ring C to a hydroxyl group) and conferring increased affinity for the glucocorticoid receptor (GR). This promotes downstream GR signalling (16). In contrast, 11β-HSD2, in the presence of its cofactor nicotinamide adenine dinucleotide (NAD), potently inactivates cortisol to cortisone *via* its dehydrogenase activity, protecting mineralocorticoid receptors in responsive tissues such as kidney, colon and placenta from inappropriate activation by cortisol (17, 18). Final metabolism and urinary clearance of cortisol and cortisone occurs following their metabolism by 5α and 5β-reductases. This, in combination with 3α-hydroxysteroid dehydrogenase activity yields tetrahydrocortisone (THE)/ tetrahydrocortisol (THF) and allo THF. The ratio of THF to THE metabolites excreted in the urine can be utilised as a surrogate measure of systemic 11β-HSD1 activity (2). The 11β-HSD enzymes also metabolise several synthetic GCs with 11β-HSD1 activating GCs such as prednisone and 11β-HSD2 inactivating hydrocortisone and prednisolone. However, other synthetic steroids such dexamethasone and methylprednisolone are resistant to metabolism by the 11β-HSD enzymes due to fluorine and methyl group substitutions that significantly reduce metabolic clearance and increase half-life (19, 20). Whilst 11β-HSD2 clearly plays a central role in mineralocorticoid responsive tissues and in determining the circulating cortisol/cortisone ratio, its basal expression outside of these tissues is limited and

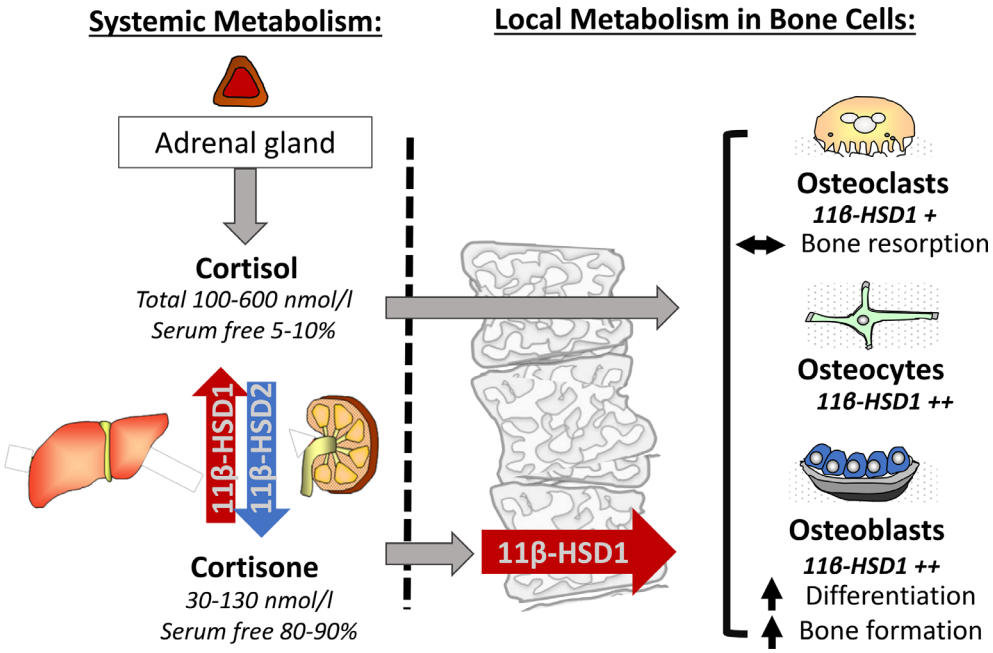

FIGURE 1 | The adrenal corticosteroids cortisol and cortisone achieve a circulating equilibrium predominantly through their renal inactivation by 11β-HSD2 and reactivation by hepatic 11β-HSD1. Both active and inactive serum free corticosteroids are able to enter bone cells. Here 11β-HSD1 can further activate and amplify the actions of corticosteroids through the conversion of cortisone to cortisol where, in combination with serum free cortisol, it influences basal bone metabolism in osteoclasts, osteocytes and osteoblasts.

its importance is less clear. Consequently, this review will primarily focus on the roles of GC metabolism by 11β-HSD1 in the context of bone metabolism and bone cell function.

SYSTEMIC ENDOCRINE METABOLISM *VERSUS* LOCAL AUTOCRINE METABOLISM

The roles of 11β-HSD1 span 'endocrine' regulation of circulating corticosteroid availability and systemic GC exposure, and the fine-tuning of local tissue and cell specific exposure *via* its 'autocrine' activation of cortisol, independently of circulating cortisol. The regulation of systemic endocrine cortisol activation is primarily determined by hepatic 11β-HSD1, which is constitutively and highly expressed within the liver (21, 22). In contrast, the regulation of 11β-HSD1 in tissues, such as adipose, muscle, bone and within sites of inflammation is dynamically regulated in a highly cell and context specific fashion (23–28). Whilst 11β-HSD1 within these tissues also influences circulating endocrine metabolism (albeit to a much lesser extent than hepatic 11β-HSD1), the overwhelming role of 11β-HSD1 in this context is mediated through its autocrine influence on local cortisol exposure independently of circulating cortisol levels. The hypothalamic pituitary adrenal (HPA) axis determines both ultradian and circadian regulation of systemic cortisol levels, with stressors such as inflammation activating production of cortisol by the adrenal gland and negative feedback from GCs suppressing this. The expression of 11β-HSD1 in the suprachiasmatic nucleus, or the "biological clock of the brain", and the hypothalamus imply a role for this enzyme in circadian regulation of the HPA axis by the negative feedback of active GCs (29). Additionally, 11β-HSD1 in the hippocampus, visceral adipose tissue and subcutaneous adipose tissue has been identified to exhibit circadian variation in gene expression and enzyme activity (30, 31). Whilst circadian and ultradian rhythm have not been shown to be substantially affected in murine models with transgenic 11β-HSD1 deletion, its precise role in central circadian regulation in response to stress and inflammation remain poorly defined. Given its potent inflammatory regulation within myeloid and mesenchymal derived population, it has been hypothesised that chronic activation of 11β-HSD1 may represent a causative factor in the dysregulation of the HPA axis in inflammatory disease (32). Systemic GC activation by hepatic 11β-HSD1 and inactivation by renal 11β-HSD2 play a central role in establishing the circulating ratio of cortisol to cortisone, with levels of active cortisol (typically ranging from 100-600 nmol/l) being 5-6 times higher than for cortisone (30-130 nmol/l) (27). Whilst this circulating ratio helps determine endocrine GC signalling, its function extends to the provision of cortisone as a substrate for local 11β-HSD1 cortisol activation in peripheral tissues. For both aspects of circulating corticosteroid action, one further factor, corticosteroid-binding globulin (CBG) should be considered. Here, approximately 90-95% of total circulating cortisol is sequestered by CBG, and to a lesser extent serum albumin, preventing cell entry and GR mediated cell signalling (33–35). Its relevance to local 11β-HSD1 signalling arises due to a greatly reduced affinity of CBG (roughly 10-fold less) for the inactive corticosteroid, cortisone (33, 36, 37). Consequently, whilst total cortisone circulates at a 5-6-fold lower concentration than total cortisol, serum free cortisone levels available for local autocrine amplification by 11β-HSD1 can match or exceed that of serum free cortisol (**Figure 1**). To appreciate the roles that 11β-HSD1 plays in utilising and activating this abundant pool of circulating serum free cortisone within peripheral tissues such as bone, one must first delineate its cellular expression and distribution within bone itself.

THE ROLE OF 11β-HSD1 IN BONE CELLS AND BONE HEALTH IN NORMAL PHYSIOLOGY

The Role of Endogenous GCs in Bone Cell Differentiation and Activity

Despite their well-known deleterious effects on bone at therapeutic doses, endogenous GCs play key roles in the formation and maintenance of bone under homeostatic conditions. Continued GC signalling is required for maintenance of adequate bone mass, as seen in models with targeted deletion of GR in osteoblast progenitors or ectopic expression of 11β-HSD2 in mature osteoblasts and osteocytes which exhibit reductions in bone density (38–40). At the individual cell level, GCs promote differentiation of osteoblasts from mesenchymal cells *via* the Wnt/β-catenin pathway. Ectopic expression of the 11β-HSD2 gene in mature osteoblasts *via* the Col2.3 promoter or abrogation of Wnt signalling instead induces adipocyte lineage commitment (41–43). GCs have also been identified to drive differentiation of osteoclasts from mesenchymal precursors and enhance the bone resorption activity of mature osteoclasts (44–46). As well as direct effects on bone cells, GCs influence bone metabolism *via* paracrine signalling. GC stimulation of osteoblasts and osteocytes induces production of receptor activator of nuclear factor kappa-B ligand (RANKL) while suppressing expression of the RANKL decoy receptor osteoprotegerin (OPG), resulting in survival and activation of local osteoclasts (47–50). Within normal physiological conditions, this GC-mediated regulation of bone metabolism functions under strict homeostatic control to carefully balance anabolic and catabolic effects on target cells. Whilst GCs have stimulatory effects on osteoblasts at low doses, they are inhibitory at higher doses, where they instead promote apoptosis of osteoblasts (51, 52). Similarly, GC regulation of mature osteoblast function *via* expression of Wnt proteins functions in a dose-dependent 'biphasic' manner (53). Local activation of GCs by the enzyme 11β-HSD1, at both the autocrine and paracrine levels, helps determine available GC for normal physiological responses, as well as potential roles in states of inflammation and GC excess. The functional impact of 11β-HSD1 in bone metabolism has been demonstrated in normal physiology and states of GC excess (54, 55). Systemic

11β-HSD1 activity, and the inactive 11β-HSD1 substrate cortisone, negatively correlated with measures of anabolic bone formation by osteoblasts in cross sectional population studies and was shown to increase with ageing. Furthermore, enzymatic activity of 11β-HSD1 in *ex vivo* grown bone cells was found to increase with donor age (56). These changes were independent of circulating 'endocrine' cortisol, suggesting that 11β-HSD1 within cells such as osteoblasts underpinned these observations. Whilst osteoblasts were highlighted as a primary anti-anabolic target of GC metabolism by 11β-HSD1, its dynamic regulation of expression across multiple cell types, including osteocytes, osteoclasts and endothelial cells, hint at a highly cell and context specific role of 11β-HSD1 in bone metabolism *in vivo*.

The Role of 11β-HSD1 in Osteoblasts and Osteocytes in Normal Physiology

Osteoblasts were initially shown to possess the highest levels of 11β-HSD1 in bone by immunohistochemistry and *in situ* hybridisation (57, 58). Whilst patient studies had reported a potential anti-anabolic role of 11β-HSD1 with ageing, corticosteroid excess and post menopause, initial *in vitro* studies revealed that the upregulation of 11β-HSD1 in immature osteoblast precursors facilitated their differentiation into osteoid producing osteoblasts (59). These findings fit with the well characterised *in vitro* actions of GCs on cultures of osteoblasts, where they stimulate differentiation *via* regulation of specific growth factors and Wnt signalling molecules (41–43). Consequently, these data revealed a potential anabolic role for autocrine cortisol production by 11β-HSD1 in osteoblasts in normal physiology. Murine models in the DBA-1 strain in which GC activation was blocked selectively within osteoblasts supported this hypothesis. Specifically, the overexpression of 11β-HSD2 selectively within osteoblasts, under control of the 2.3Kb Col1a1 promoter, resulted in potent cortisol inactivation to offset endogenous 11β-HSD1 activity (38, 60–62). These animals presented with reduced vertebral bone density and attenuated cranial ossification and reduced periosteal circumference indicating that 11β-HSD1 was required for normal osteoblastic bone formation. Interestingly, similar experiments in the C57BL/6 strain using the osteocalcin promoter, and C57BL/6 animals with a global deletion of 11β-HSD1 failed to reproduce these findings raising doubts as to this explanation (13, 63). Here, the selection of the mouse strain itself may explain this discrepancy, since C57BL/6 mice are reported to have reduced responsiveness to the action of GCs on anabolic bone formation (64). Whilst this can be overcome at higher exogenous corticosteroid doses, these findings suggest that the C57BL/6 strain may not be suited to examining the actions of endogenous GCs in this setting (13). Ultimately, osteoblast targeted deletion of 11β-HSD1 in an appropriate murine strain is still required to address these questions. The anabolic role of 11β-HSD1 in osteoblast differentiation has been less clear in human population studies. This may reflect the respective cohorts examined, where factors such as ageing and exogenous corticosteroid administration may see 11β-HSD1 move from an anabolic role to one mediating corticosteroid excess and facilitating osteoblast autophagy and apoptosis (65, 66). Certainly, in this context measures of 11β-HSD1 activity negatively correlate with markers of bone formation such as osteocalcin and procollagen type 1 amino-terminal propeptide (P1NP) (54–56). It may be that the anabolic actions of 11β-HSD1 may be more apparent in a younger population. Certainly, trials using therapeutic inhibitors of 11β-HSD1 have yet to identify significant changes on bone metabolism in phase II trials (1–8). However, their penetrance within bone has yet to be validated, and so their role in osteoblast differentiation *in vivo* cannot yet be ruled out in humans. Ultimately, more targeted approaches are still required to examine the anabolic roles of 11β-HSD1 *in vivo* in regulating bone metabolism in normal physiology. However, whether therapeutic inhibition of 11β-HSD1 is able to prevent the reported anti-anabolic effects of GCs in osteoblasts in ageing, or post menopause has yet to be determined.

The Role of 11β-HSD1 in Osteoclasts in Normal Physiology

Analysis of human bone samples confirmed that osteoclasts also express functional 11β-HSD1, but, similar to other bone cell populations, not 11β-HSD2. To assess the functional importance of pre-receptor metabolism of GCs by 11β-HSD1 healthy volunteers were treated with the nonspecific 11β-HSD inhibitor carbenoxolone. After 7 days of treatment, urinary analysis showed normal levels of bone formation markers C- and N-terminal pro-peptides of type I collagen (P1CP and P1NP, respectively) but decreased pyridinoline and deoxypyridinoline, metabolites of bone degraded by osteoclasts. This finding implies a role for local activation of GCs in homeostatic bone resorption (67). In support of this, the selective 11β-HSD1 inhibitor KR-67500, which was found to ameliorate disease in a mouse model of type 2 diabetes, promoted osteoblast maturation of C2C12 cells while blocking RANKL-induced differentiation of murine bone marrow derived macrophages to osteoclasts. Specifically 11β-HSD1 inhibition was found to decrease the genes *Ctsk*, *Fos*, *Nfatc1* and *Dcstamp*, which are required for cellular fusion and multinucleation and bone resorption (68). Despite these findings, clinical trials of 11β-HSD1 inhibitors in diseases such as diabetes, metabolic syndrome, Alzheimer's, and glaucoma appear to have a favourable safety profile in terms of bone health, with minimal adverse effects reported (1, 3, 69). A trial of 11β-HSD1 inhibition in idiopathic intracranial hypertension specifically assessed serum levels of osteocalcin and sclerostin and measured bone mineral content by dual-energy X-ray absorptiometry (DXA) and found no differences in bone metabolism with treatment (8). Blockade of 11β-HSD1 activation of GCs by ectopic expression of the 11β-HSD2 gene in osteoclasts did not drive any negative skeletal phenotype in mice, with Jia et al. reporting normal bone development, mass and cell numbers (70). Similarly, the 11β-HSD1 knock-out mouse does not appear to have any defects in bone development or structure including under conditions of ageing (13, 71). This suggests that though 11β-HSD1 may play a role in increasing local GC levels for osteoclast differentiation and

function, there is inbuilt redundancy in GC regulation of bone metabolism in normal physiological conditions. There is therefore no clear role for 11β-HSD1 in mediating bone homeostasis *via* modulation of endogenous GCs in osteoblasts and osteoclasts, at least under healthy steady state conditions. However, ageing has been shown to increase levels of circulating GCs as well as expression of 11β-HSD1, where dysregulated bone homeostasis frequently presents as conditions such as osteoporosis (72).

Together, whilst these studies suggest that 11β-HSD1 inhibition may have some limited actions on osteoblast and osteoclast maturation and function, its impact on total bone metabolism in normal physiology appear negligible. However, further examination of their effects on bone metabolism across factors such as ageing warrant further investigation.

11β-HSD1 MEDIATES THE ANTI-ANABOLIC ACTIONS OF GLUCOCORTICOID EXCESS IN BONE

Therapeutic GCs are widely utilised in the treatment of both acute and chronic inflammation, and they are the second most common cause of secondary osteoporosis and increased fracture risk (73, 74). Rapid bone loss over several months occurs after initiation of GCs, followed by a more gradual loss with long term use (11). The dose and duration are significant factors in determining the rate and severity of glucocorticoid-induced osteoporosis (GIOP), and suppression of bone formation (75). The underlying pathology of disease for which GCs is utilised invariably influences this process, with chronic inflammation being a well-described driver of systemic bone loss (75). However, the independent action of glucocorticoids in the absence of inflammation has been explored in healthy volunteers and, in patients with Cushing's disease and in patients receiving excessive corticosteroid replacement in conditions of adrenal insufficiency. In these situations a potent suppression of anabolic bone formation is evident, as seen by a marked decrease in circulating markers of osteoblastic bone formation, such as P1NP and osteocalcin (76). This reflects a wider uncoupling of formation and resorption in bone where changes in osteoclastic resorption in response to GCs are less evident or entirely absent (76–78). In patients with Cushing's disease, GC excess increases the risk of fractures secondary to suppressed bone formation (79, 80). The direct and indirect mechanisms whereby exogenous GCs influence bone formation by osteoblasts are reviewed in greater detail elsewhere (81). This review will now examine studies that have aimed to delineate the contribution of GC metabolism by 11β-HSD1 to GIOP.

The Role of 11β-HSD1 in Osteoblasts and Osteocytes in Glucocorticoid Excess

Several clinical studies have identified links between dysregulated bone metabolism and 11β-HSD1 activity in patients receiving therapeutic GCs (54, 67, 82). Increasing 11β-HSD1 activity and its inactive GC substrate availability were shown to correlate with decreased serum markers of bone formation, P1NP and osteocalcin. These data indicate that the pre-receptor activation of therapeutic GCs by 11β-HSD1 mediate this suppression of bone formation, either directly within the osteoblast themselves or through an alternative indirect pathway. A direct role for 11β-HSD1 within osteoblasts and osteocytes in mediating these effects is supported by evidence of significant expression and activity in these cell subsets when examined in human bone and primary cultures (67, 83). However, further insights into the mechanisms underpinning this have been limited to *in vitro* studies in primary human and murine osteoblasts cultures. In this context supra-physiological levels of corticosteroids promote osteoblast differentiation and support osteoid deposition (41–43). Whether these findings reflect a failing of these *in vitro* models or are instead evidence of an indirect mechanism whereby 11β-HSD1 indirectly regulates bone formation, such as through influencing circulating anabolic and anti-anabolic factors (such as androgens or parathyroid hormone) at alternative sites have yet to be adequately answered. Further insights have instead come from murine models of corticosterone excess. Global genetic deletion of 11β-HSD1 protects against the anti-anabolic effects of therapeutic GCs in bone (13). This is characterised by preservation of trabecular volume, serum measures of bone formation and preservation of osteoblast and osteocyte numbers following 4 weeks of GC exposure. These findings mirror observations in similar animal models of GIOP with osteoblast targeted blockade of GC signalling, supporting the concept that 11β-HSD1 directly mediates the anti-anabolic actions of GCs in bone (84). Studies utilising osteoblast targeted transgenic deletion of 11β-HSD1 are now still required to validate these findings. Regardless, these studies reveal a critical role for 11β-HSD1 in the suppression of bone formation in GIOP and provide evidence for the efficacy of therapeutic inhibitors of 11β-HSD1 in conditions of GC excess.

The Role of 11β-HSD1 in Osteoclasts in Glucocorticoid Excess

Whilst the actions of therapeutic GCs have been shown to promote early osteoclast differentiation, and suppress bone resorption by mature osteoclast, the role of 11β-HSD1 within the osteoclast in GIOP is less well defined in the context (76–78, 85). Whilst a decrease in bone resorption markers have been reported in healthy volunteers receiving the 11β-HSD inhibitor carbenoxolone and then the inactive GC prednisolone, there has been limited evidence to support a role for 11β-HSD1 in any increases in bone resorption markers in GIOP. Whilst *in vitro* examination of the role of 11β-HSD1 in osteoclasts in GIOP are lacking, further insights are apparent from murine models examining animals with transgenic deletion of 11β-HSD1 (13). Here, oral GCs result in only a minor trend towards increased osteoclast numbers and bone resorption markers with no protection from this conferred in animals lacking 11β-HSD1. Together, these studies imply that 11β-HSD1 plays a limited role in mediating increased bone resorption in conditions of GC excess. However, these observations may be hampered by the relatively small contribution that osteoclasts play in mediating GIOP, relative to the impact on bone formation.

Collectively, these studies suggest that in conditions of endogenous and therapeutic GC excess, inhibition of 11β-HSD1 prevents the anti-anabolic actions of GCs and preserves bone mass, whilst their impact on osteoclast numbers and activity appear minimal.

GLUCOCORTICOID ACTIVATION BY 11β-HSD1 PREVENTS OSTEOCLASTOGENESIS AND BONE RESORPTION IN INFLAMMATORY DISEASE

Systemic bone loss and increased risk of fracture at sites such as the femoral neck, trochanter and spine are hallmarks of patients with many chronic inflammatory diseases (86, 87). Here, circulating inflammatory mediators such as tumour necrosis factor α (TNFα) and interleukin-6 (IL-6) are predictors of decreased bone mineral density and increased fracture risk. Both bone formation and resorption show dysregulation in chronic inflammatory disease such as rheumatoid arthritis, with decrease in P1NP and increases in carboxy (C) terminal telopeptide of type I collagen (CTx) showing strong correlation with markers of disease activity (88, 89). At the cellular level, pro-inflammatory factors act on osteoblasts to directly suppress differentiation and osteoid deposition, whilst their actions on osteoclasts are mediated both directly to increase activity, and indirectly through the RANKL/OPG signalling pathway to increase both numbers and activity (90). The *in vitro* mechanisms underpinning these actions are diverse and the subject of numerous reviews (81, 91). Interest in the role of endogenous GC activation by 11β-HSD1 in this context have been fuelled by studies reporting marked increases in enzyme activity, both systemically and at sites of inflammation (92–96). In this context 11β-HSD1 has been shown to mediate anti-inflammatory GC signalling, supporting resolution and tissue repair. However, in the context of persistent and chronic autoimmune inflammation, its role has been hypothesised to switch to driving ongoing localised GC excess. To date, clinical studies have yet to examine the interaction between 11β-HSD1, bone metabolism and fracture risk in an inflammatory disease cohort. Similarly, clinical trials of 11β-HSD1 inhibitors have yet to examine their impact on bone metabolism in the context of inflammatory disease. To date, no aberrant observations of altered inflammatory responses or dysregulated bone metabolism have been reported in these studies (1–8). At present, significant gaps are present in our knowledge of the role of inflammatory 11β-HSD1 activity in bone cells in chronic inflammatory disease.

The Role of 11β-HSD1 in Osteoblasts and Osteocytes in Inflammatory Disease

In vitro studies have revealed a potent upregulation of 11β-HSD1 in mesenchymal derived cell populations, including osteoblasts by pro-inflammatory factors such as TNFα and

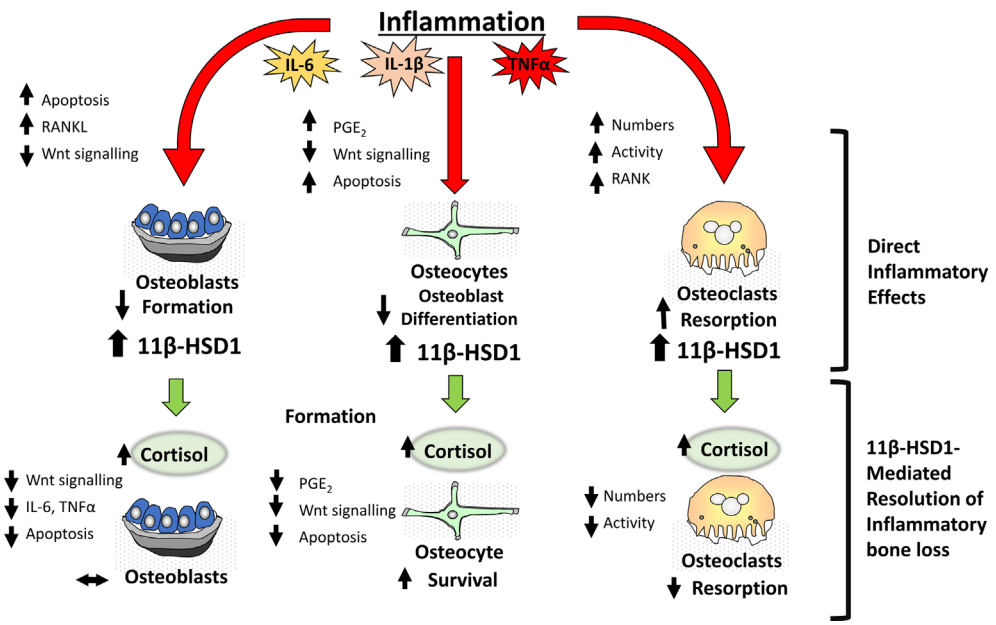

FIGURE 2 | During inflammation within bone, pro-inflammatory factors including IL-6, IL-1β and TNFα have direct effects on bone cells including osteoblasts, osteocytes and osteoclasts resulting in a net loss in bone in addition to a significant induction of the corticosteroid activating enzyme 11β-HSD1. This in turn drives an anti-inflammatory resolution response where increased cortisol activation by 11β-HSD1 promotes a reduction in osteoclastic bone resorption thus suppressing inflammatory bone loss.

IL-1β (25, 83) (**Figure 2**). This inflammatory induction of 11β-HSD1 is in turn synergistically upregulated in combination with GCs, through a mechanism involving the suppression of p38-mitogen-activated protein kinase (MAPK) and upregulation of nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) signalling (25). Together, this leads to a potent increase in local GC activation in osteoblasts in response to inflammation within bone, where they then suppress local inflammatory mediators in a feedback mechanism that appears to support resolution of inflammation (83). The impact of this synergistic inflammatory regulation on bone metabolism in chronic inflammatory diseases such as rheumatoid arthritis is less clear. *In vitro* experiments using human primary osteoblast cultures reveal that these endogenous GCs can suppress osteoblast maturation and collagen deposition. *In vivo* rodent models of polyarthritis offer further insights into these processes. Here, global deletion of 11β-HSD1 severely exacerbates systemic bone loss, and suppresses anabolic bone formation, with a marked reduction in markers of mature osteoblasts, including runt-related transcription factor 2 (Runx2) and OPG (26). These findings would suggest that the anti-inflammatory actions of local GC activation by 11β-HSD1 in this context outweigh their detrimental anti-anabolic actions in osteoblasts. However, targeted mesenchymal genetic deletion of 11β-HSD1 (including in osteoblasts), failed to reproduce this systemic bone loss phenotype, indicating that the expression of 11β-HSD1 in osteoblasts may not play a critical role in mediating this inflammatory bone loss phenotype. More targeted studies, examining osteoblast specific deletion of 11β-HSD1, are now required to further explore these findings. This is particularly since global deletion of 11β-HSD1 exacerbates the severity of systemic inflammation, which is itself a confounder mediating increased bone loss. Overall, these studies point to an important role for 11β-HSD1 in regulating bone formation in chronic inflammatory diseases and bone inflammation.

The Role of 11β-HSD1 in Osteoclasts in Inflammatory Disease

Whilst attenuated bone formation undoubtedly plays a role in abnormal bone metabolism in systemic inflammatory diseases, such as polyarthritis, a marked increase in osteoclastic bone resorption remains the primary mediator of the rapid bone loss observed in this context (90). Whilst osteoclasts, and their myeloid precursors express 11β-HSD1, its inflammatory regulation *in vivo* is less well characterised (67). Numerous studies examining myeloid precursors demonstrate a robust upregulation of 11β-HSD1 over differentiation and in response to inflammatory mediators such as TNFα, whilst similar studies in osteoclasts are lacking (97, 98). However, murine models have significantly advanced our understanding of the contribution of 11β-HSD1 in osteoclasts in this setting. In murine models of polyarthritis, osteoclast mediated bone loss is markedly exacerbated in animals with global deletion of 11β-HSD1, where it is the overriding factor driving inflammatory bone loss through a shift in RANKL/OPG signalling (9, 26, 99). These studies reveal a critical role for 11β-HSD1 in protecting against inflammatory bone resorption and supporting resolution and repair in bone (**Figure 2**). As in osteoblasts, whether these actions are mediated directly by 11β-HSD1 expression within the osteoclast, or indirectly through altered expression of local or systemic inflammatory mediators that drive this increase in osteoclast mediated bone resorption has yet to be elucidated and can only be addressed with osteoclast targeted models of 11β-HSD1 deletion.

Together, these studies indicate that systemic inflammation in conditions such as polyarthritis, 11β-HSD1 inhibition appears to have limited affects in osteoblasts, but significantly exacerbates inflammatory mediated osteoclast bone resorption and promotes systemic bone loss.

11β-HSD1 PROTECTS AGAINST INFLAMMATION-INDUCED BONE RESORPTION IN RESPONSE TO THERAPEUTIC GLUCOCORTICOIDS

In regard to bone metabolism, the effect of therapeutic GCs in both acute and chronic inflammatory disease settings remains of significant interest. Understanding the opposing actions of GCs on bone metabolism in systemic inflammatory diseases, such as rheumatoid arthritis, where they suppress the inflammatory mediators that drive bone loss, whilst also acting directly on bone cells to drive GC-induced bone loss, remains paramount. Clinical studies examining this facet of GC action in chronic inflammatory disease are limited by confounding factors such as concurrent anti-inflammatory therapies, patient variation and disease pathophysiology, and differences in steroid dose and duration. Therefore, it is perhaps unsurprising that responses to GCs in this context report divergent outcomes, including both improvements in and worsened bone outcomes in patients with inflammatory disease (100–103). Murine models of chronic inflammatory arthritis receiving therapeutic GCs have examined this process under more controlled experimental conditions and revealed that GCs play an important role in protecting against acute inflammatory bone loss mediated by osteoclastic bone resorption, with both osteoclast numbers and activity being significantly reduced (99) (**Figure 3**). Anabolic bone formation was also significantly suppressed with GC administration, however its actions on total bone metabolism were subtle relative to the rapid inflammatory osteoclast mediated bone loss. Whilst studies examining the contribution of 11β-HSD1 to these findings are limited, several *ex vivo* studies provide insight into this process.

The Role of 11β-HSD1 in Osteoblasts and Osteocytes in Response to Therapeutic GCs in Inflammatory Disease

Whilst therapeutic GCs suppress bone formation by osteoblasts, in models of chronic inflammatory polyarthritis, the relative contribution of 11β-HSD1 to these phenotypes are yet to be reported (9, 99). Animal models of chronic inflammatory disease with both global and osteoblast targeted transgenic deletion of

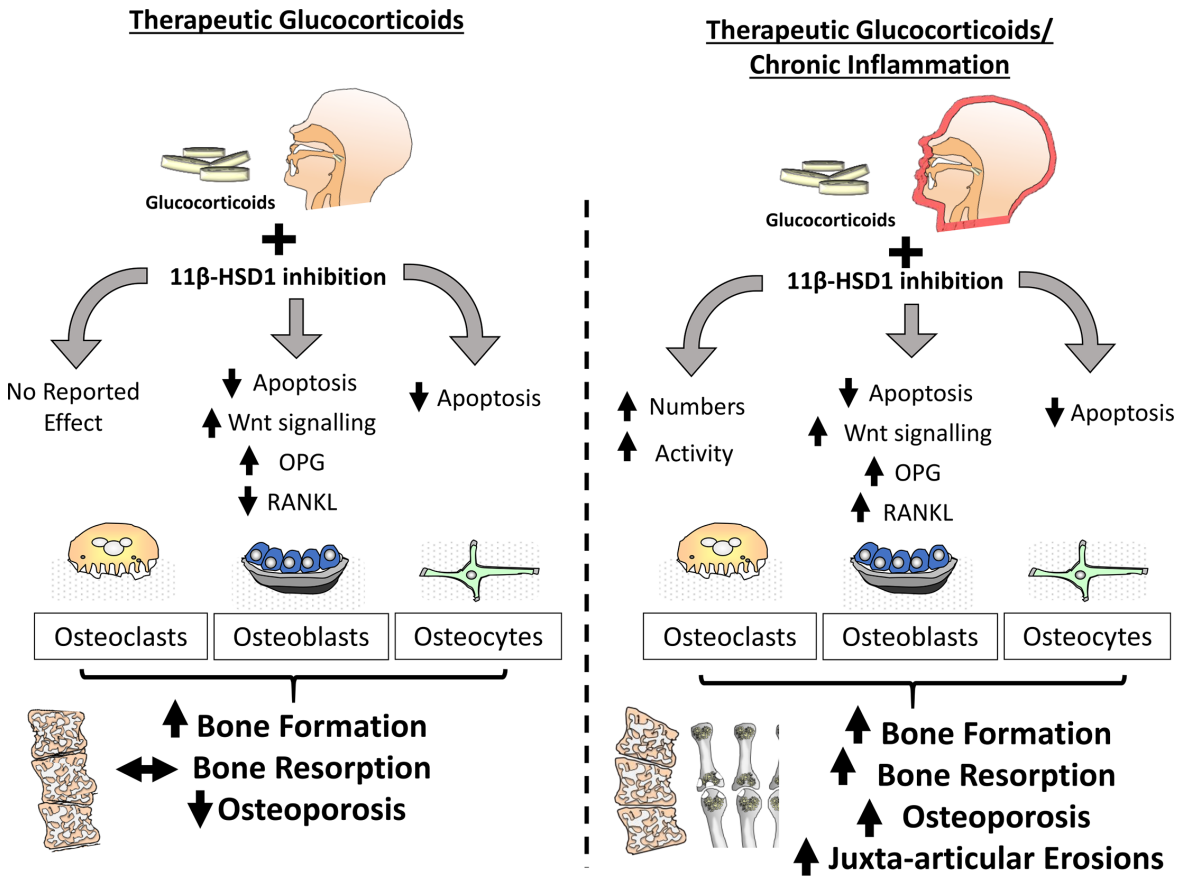

FIGURE 3 | In the absence of inflammation within bone, inhibition of 11β-HSD1 protects against the actions of exogenous glucocorticoids in both osteoblasts and osteoclasts. The effect of these inhibitors is characterised by a protection from glucocorticoid induced apoptosis in both osteoblasts and osteocytes, preventing a glucocorticoid suppression of bone formation. During chronic inflammatory diseases such as rheumatoid arthritis, the protective actions of 11β-HSD1 inhibition in response to exogenous therapeutic glucocorticoids in osteoblasts and osteocytes remains evident but is overshadowed by a resistance to the anti-inflammatory properties of exogenous glucocorticoids that results in aberrant osteoclast numbers and activation. Consequently, 11β-HSD1 inhibition results in rapid bone resorption and exacerbation of both local and systemic inflammatory bone loss.

11β-HSD1 are required to assess impact of therapeutic corticosteroids in this setting. Given the potent suppression of bone formation by therapeutic GCs, and the efficacy of transgenic deletion of 11β-HSD1 in preventing this, it could be predicted that inhibition of 11β-HSD1 in patients with chronic inflammation and receiving therapeutic GCs might have a similar protection from their anti-anabolic actions in osteoblasts and osteocytes (99). However, considering the exacerbation of inflammation in response to 11β-HSD1 deletion it may be that the inflammatory suppression of bone formation is increased and coupled with a shift towards increased pro-inflammatory factors by osteoblasts, such as RANKL, TNFα and IL-6 by osteoblasts that would favour osteoclast mediated bone resorption and bone loss (9, 26, 104, 105). Further considerations should include the lesser role of dysregulated bone formation in acute inflammatory bone loss where osteoclast mediated bone resorption has been shown to play a greater role (106–108). Consequently, it may be that any beneficial effects of 11β-HSD1 inhibition on bone formation in the context of inflammatory disease may be limited or realised over longer periods of GC administration (**Figure 3**).

The Role of 11β-HSD1 in Osteoclasts in Response to Therapeutic GCs in Inflammatory Disease

Increased bone resorption by osteoclasts is recognised as the primary driver of bone loss in many inflammatory diseases and this is rapidly suppressed in response to therapeutic GCs, however no clinical or *in vitro* studies have examined the role of 11β-HSD1 in this setting (106–108). Insights into this facet of GC action have instead come from a single study examining *in vivo* models of chronic inflammatory polyarthritis and therapeutic GC administration in animals with global and myeloid targeted transgenic deletion of 11β-HSD1 (9). In this study, suppression of osteoclast bone resorption by oral GCs was almost entirely abrogated in animals with global and myeloid

targeted deletion of 11β-HSD1, revealing a critical role for local GC activation by this enzyme in suppressing osteoclast numbers and activity in chronic inflammation. Whether these effects reflect a direct autocrine effect of GC activation by 11β-HSD1 within the osteoclast, or instead reflect wider changes in pro-inflammatory factors such as RANKL, TNFα and IL-6 that drive osteoclast driven bone resorption has yet to be determined (26, 104, 105). 11β-HSD1 has been shown to influence the RANKL/OPG ratio in murine models of inflammation and this could play a role in regulating inflammatory bone resorption (81). To validate these findings now requires more targeted Cre driven deletion of 11β-HSD1 in the osteoclast subset and *in vitro* experiments that can delineate inflammatory regulation and functional consequences of autocrine GC activation by 11β-HSD1 within osteoclasts. Despite the need for future work, it is clear that 11β-HSD1 is a critical mediator in suppressing bone resorption in response to GCs and mediating their rapid bone protective actions in this context.

Together, these studies reveal that in the context chronic inflammatory diseases, such as rheumatoid arthritis, whilst 11β-HSD1 inhibition prevents the anti-anabolic actions of therapeutic glucocorticoids in osteoblasts, they abrogate GC mediated suppression of osteoclast activity and inflammatory bone loss.

POTENTIAL ROLES FOR 11β-HSD1 IN BONE LINING CELLS AND ENDOTHELIUM

The endothelial cells that form the vascular structures of the skeletal system are increasingly seen as important in the processes of bone formation and maintenance. These vessels supply vital nutrients and signalling molecules to osteoblasts and osteocytes, which in turn act on endothelial cells to support further vascular development, hence the processes of angiogenesis and osteogenesis are said to be "coupled" (109). Osteoblasts and their progenitors are therefore found in close proximity to these osteogenesis-promoting endothelial cells, termed type H vessels due to their high expression of adhesion molecule CD31 and the endothelial sialomucin endomucin (110, 111). Osteoblasts and osteocytes support angiogenesis by producing vascular endothelial growth factor (VEGF) *via* the hypoxia-inducible factor α (HIFα) pathway (112, 113). VEGF stimulates blood vessel invasion by acting on endothelial cells, but also promotes migration and activation of osteoblasts, linking these complementary functions of angiogenesis and osteogenesis in formation or remodelling of bone (114, 115). Similarly, preosteoclasts secrete platelet-derived growth factor-BB (PDGF-BB) which promotes angiogenesis by recruiting endothelial and mesenchymal progenitors and inducing formation of type H vessels, this in turn stimulates bone formation and remodelling (116, 117). These processes are all attenuated by GC treatment, as seen in both *in vitro* and *in vivo* models (118–120). It is not known what role, if any, 11β-HSD1 plays in type H vessel cells. However, expression of 11β-HSD1 has been identified in vascular endothelial cells and found to inhibit angiogenesis by interfering with endothelial cell morphological changes required for tube formation (121–124).

Bone lining cells (BLCs) are derived from mature quiescent osteoblasts and thought to perform a number of functions in skeletal homeostasis [reviewed in detail by Wein (125)]. It is not known whether BLCs express 11β-HSD1 like other mesenchymal derived cells, if so it may perform a similar function as in osteoblasts under conditions of inflammation, ageing and GC excess (25). Treatment of mice with prednisolone was found to inhibit BLC activation and proliferation, including their conversion into new osteoblasts (126). However, despite their importance in bone growth and repair, much remains to be elucidated about the functions of BLCs and the importance of 11β-HSD1 metabolised GCs in this cell type.

FINAL CONCLUSIONS

The studies highlighted in this review reveal a complex role for 11β-HSD1 in bone remodelling, with some limited evidence for a role in normal physiology, but a much greater role in mediating the actions of GCs in conditions of exogenous and endogenous corticosteroid excess and inflammation. With the interest in therapeutic inhibitors of 11β-HSD1, these studies point to the potential for their application in conditions such as Cushing's disease, where they would be predicted to prevent the anti-anabolic action of GCs on bone to reduce the risks of osteoporosis and fracture. In contrast, their application in the context of inflammatory disease appears to be complicated by the risk of exacerbating inflammatory bone loss by osteoclasts. Therefore, in inflammatory disease the role of 11β-HSD1 appears to be protective, mediating the suppression of inflammatory factors that drive bone resorption and decrease osteoclast numbers and activity. Rather than inhibiting 11β-HSD1, approaches may instead benefit from targeting therapeutic GCs selectively to leukocyte and osteoclast populations to more effectively deliver their beneficial bone sparing actions in the context of inflammation. Whether the inflammatory induction of 11β-HSD1 within bone resorbing cells could be used to selectively facilitate metabolic targeting of GCs for intracellular activation within osteoclasts to suppress inflammatory bone resorption without driving off-target metabolic side effects has yet to be determined.

AUTHOR CONTRIBUTIONS

RH and MC devised topic. MC, RH, and CM jointly wrote manuscript and devised figures. All authors contributed to the article and approved the submitted version.

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Four-Year Teriparatide Followed by Denosumab *vs.* Continuous Denosumab in Glucocorticoid-Induced Osteoporosis Patients With Prior Bisphosphonate Treatment

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Objectives: In our previous 24-month study, we observed that teriparatide had some advantages over denosumab for bone mineral density (BMD) in glucocorticoid-induced osteoporosis (GIO) patients with prior bisphosphonate treatment. We conducted this extension study to investigate whether the advantage of teriparatide obtained in the first 2 years would be maintained after the switch to denosumab.

Materials and Methods: We switched patients who had completed 24-month daily teriparatide treatment to denosumab (switch group, n=18) and compared their BMD every 6 months up to 48 months with the group who continued to receive denosumab (denosumab group, n=16).

Results: At 48 months, the lumbar spine BMD was significantly increased from baseline in both groups (denosumab: 10.4 ± 8.7%, $p<0.001$; switch: 14.2 ± 6.8%, $p<0.001$). However, a significant increase in femoral neck BMD from baseline occurred only in the switch group (11.2 ± 14.6%, $p<0.05$); denosumab (4.1 ± 10.8%). The total hip BMD increased significantly from baseline in both groups (denosumab: 4.60 ± 7.4%, $p<0.05$; switch: 7.2 ± 6.9%, $p<0.01$). Femoral neck BMD was significantly increased in the switch *versus* the denosumab group ($p<0.05$).

Conclusion: In GIO patients with prior bisphosphonate treatment, the advantage of teriparatide may be maintained after the treatment period. A continuous increase in BMD can be expected with teriparatide followed by denosumab.

Keywords: bone mineral density, teriparatide, denosumab, bisphosphonate, glucocorticoid-induced osteoporosis

INTRODUCTION

Glucocorticoid-induced osteoporosis (GIO) is a common and serious adverse effect associated with glucocorticoid use. GIO is characterized by decreased bone formation due to the increased apoptosis of osteoblasts and osteocytes (1, 2). A fragility fracture occurs in 30%–50% of patients who undergo long-term glucocorticoid therapy, leading to worse life expectancy and quality of life (3, 4). The most commonly used drugs for GIO are bisphosphonates, and in several randomized controlled trials, the bisphosphonates alendronate, risedronate, and zoledronate were shown to increase the lumbar and femoral bone mineral density (BMD) of GIO patients (5–7). Alendronate and risedronate were also shown to significantly reduce the rate of vertebral fractures in patients with GIO (5, 6), and zoledronic acid was shown to increase the BMD in the lumbar spine and femur to a greater degree than risedronate (7). However, even after the administration of a bisphosphonate, the BMD of some patients does not improve. Although BMD reduction alone should not be considered a failure of treatment with bisphosphonates (8), BMD is an important predictor of fractures and is one of the indicators in considering whether GIO treatment should be changed. In GIO patients whose BMD does not improve after treatment with a bisphosphonate, there is limited evidence regarding which subsequent treatment can be recommended for increasing BMD.

Denosumab, which is a RANKL (receptor activator of nuclear factor kappa-B ligand) inhibitor, and teriparatide (i.e., recombinant human parathyroid hormone (1–34)), are drugs that are expected to increase the BMD of women with postmenopausal osteoporosis more effectively than bisphosphonates (9, 10). Denosumab and teriparatide have also been shown to be effective for GIO, and they were demonstrated to increase the lumbar spine BMD and hip BMD to a greater degree compared to bisphosphonates in several studies (11–13). In the Denosumab And Teriparatide Administration (DATA) extension study of patients with postmenopausal osteoporosis — which described excellent therapeutic effects of a combination of denosumab and teriparatide — the increases in the lumbar spine, femoral neck, and total hip BMD did not differ significantly between the denosumab-monotherapy group and the teriparatide-monotherapy group after 24 months of treatment (14).

However, our study of patients with GIO showed that, unlike the DATA extension study, denosumab and teriparatide did not have equivalent effects on BMD (15). In that study, we compared the effects of teriparatide and denosumab in GIO patients who achieved low T-scores (< −2.5) in the lumbar spine or femoral neck even after bisphosphonate treatment. We observed that at 24 months after patients were switched from a bisphosphonate to denosumab or daily teriparatide, the BMD in the lumbar spine increased significantly from baseline in both groups, and there was a significant increase in the femoral neck BMD only in the teriparatide group. We thus suspected that teriparatide might have some advantages over denosumab for treating GIO patients with prior bisphosphonate treatment. However, since the clinical use of teriparatide is limited to 24 months, GIO treatment must be modified after the completion of teriparatide therapy.

The later DATA-switch study of postmenopausal osteoporosis patients revealed that the transition from teriparatide to denosumab further increased the BMD increased by teriparatide (16). The efficacy of this sequential treatment has not been well studied in GIO. In the present 4-year study, we extended our 2-year study (15) and compared a treatment group that transitioned from teriparatide to denosumab with a treatment group that continued denosumab for 4 years. We investigated whether the teriparatide advantage gained in the first 2 years would be maintained in the subsequent 2 years.

SUBJECTS AND METHODS

Study Design

This study was conducted from 2014 to 2021 at Kindai University Hospital (Osaka, Japan). The original study (15) was a 24-month, prospective, open-label, non-randomized clinical trial. The present study's inclusion and exclusion criteria were the same as those of the original study. GIO patients being treated with glucocorticoids for connective tissue disease and low T-score BMD (< −2.5) in the lumbar spine or femoral neck after ≥2 years of bisphosphonate therapy were switched from the bisphosphonate to either denosumab or teriparatide.

Forty-seven patients were enrolled in the original study, and 20 of 24 patients who received denosumab and 21 of 23 patients who received teriparatide completed 2 years of treatment. In the present 2-year extension study, the patients who were treated with denosumab in the original study (n=20) received an additional 24 months of denosumab (60 mg subcutaneous injection, 1×/6 months). The patients who had received daily teriparatide (n=21) were switched to denosumab. In both groups, the patients also received elemental calcium or vitamin D during the administration of denosumab.

This study was conducted according to the principles expressed in the Declaration of Helsinki of 1983, and it was approved by the Research Ethics Committee of Kindai University of Medicine. Written informed consent to participate and have their data published was obtained from all patients.

Assessments

The demographic characteristics recorded at baseline included the patient's age, sex, body mass index (BMI), and daily dose of prednisolone (PSL). During the extended 2-year period, as in the original study, the patients were examined every 6 months. At months 30, 36, 42, and 48 from baseline, the BMD of each patient's lumbar spine (L1–L4) and femoral neck and total hip of the non-dominant leg were measured by dual-energy x-ray absorptiometry (Discovery A, Hologic, Marlborough, MA, USA). A marker of bone resorption, i.e., tartrate-resistant acid phosphatase 5b (TRACP5b), a marker of bone formation, i.e., procollagen type 1 N-terminal propeptide (P1NP), and albumin-corrected calcium were similarly assessed at months 30, 36, 42, and 48. The primary endpoint of this study was the percent change in BMD from the baseline of the original study to

48 months. The secondary endpoints were the percent changes in the bone turnover markers TRACP5b and P1NP every 6 months.

Safety

The treating physicians performed the physical examinations and laboratory tests (hematological, blood chemistry, and urinalysis). All adverse events were recorded.

Statistical Analyses

We used GraphPad Prism software (GraphPad Software, San Diego, CA) for all statistical analyses. The baseline characteristics of the denosumab and teriparatide groups were compared using the Mann-Whitney U-test (the ratio of females was tested using Fisher's exact test). Similarly, the changes in the BMD and bone turnover markers were compared between the two patient groups by the Mann-Whitney U-test. Within-group changes in the BMD and bone turnover markers were assessed by paired t-test. P-values<0.05 were considered significant.

RESULTS

Baseline Characteristics and Patient Disposition

Of the 20 patients treated with denosumab in the original study, 16 patients completed 48 months of denosumab treatment (the denosumab group). The reason for discontinuation in the other four patients were death (n=2), transfer to another hospital at the patient's request (n=1), and missing data (n=1). The cause of death in the two cases was exacerbation of the originally existing myelodysplastic syndrome in one case and newly developed lymphoma in the other.

Twenty-one patients who had been treated with teriparatide in the original 2-year study were switched to denosumab, and 18 of those patients completed a total of 48 months of treatment (the switch group). The reasons for discontinuation were hospital transfer at the patient's request (n=1), death due to cerebral infarction (n=1), and patient request (n=1). A final total of 34 patients was analyzed (denosumab group, n=16; switch group, n=18) (**Figure 1**).

The patients' underlying connective tissue diseases are listed in the **Supplementary Material**. The clinical characteristics of the patients at the baseline of the original study are summarized in **Table 1**. There were no significant between-group differences in age, sex, BMI, PSL dose, durations of PSL and bisphosphonate treatment, BMD, or the two bone turnover markers at baseline. No significant between-group difference was found in the daily average dose of PSL during the 48-month study period: denosumab group, 3.3 ± 2.2 mg/day; switch group, 2.9 ± 1.4 mg/day. One patient in the switch group was receiving etanercept, a tumor necrosis factor (TNF) inhibitor. No patient in either group received anti-interleukin-6 (IL-6) receptor antibody.

Changes in BMD

Figure 2 illustrates the percent changes in the BMD of the lumbar spine, femoral neck, and total hip over the 48-month treatment period. Seven patients dropped out of the present study, but the results up to 24 months were roughly similar to those in the original study. The 24-month results can be summarized as follows. A significant increase occurred in the lumbar spine and femoral neck BMD from baseline in the teriparatide-treated group (which is the

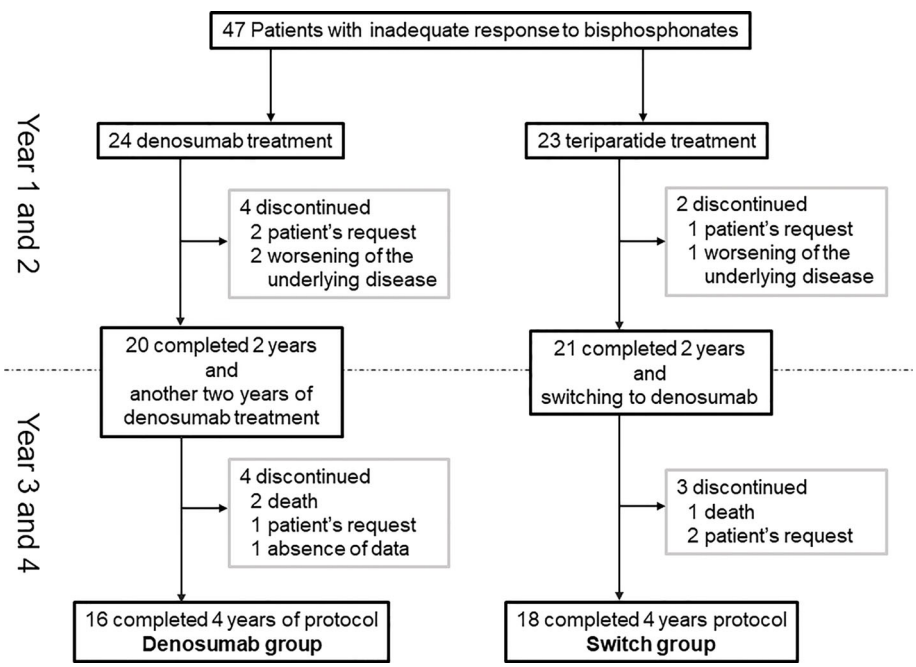

FIGURE 1 | Patient enrollment and disposition.

TABLE 1 | Clinical characteristics at baseline of the original study.

Characteristics	Denosumab group n = 16	Switch group n = 18	p-value
Age, years	65.8 ± 11.3	60.3 ± 12.4	0.11
Female, %	93.8	100	0.47
BMI, kg/m^2	20.9 ± 3.5	20.3 ± 3.0	0.56
Duration of predonisolone treatment, months	188.2 ± 106.4	201.0 ± 118.4	0.82
Dose of predonisolone at entry, mg	6.4 ± 5.1	5.0 ± 2.9	0.92
Duration of bisphosphonate treatment, months	143.2 ± 96.5	141.8 ± 79.4	0.88
BMD, g/cm2			
Lumbar spine	0.75 ± 0.12	0.74 ± 0.11	0.77
T score	-2.53 ± 1.12	-2.72 ± 1.20	0.53
Femoral neck	0.49 ± 0.08	0.50 ± 0.06	0.47
T score	-2.72 ± 0.66	-2.59 ± 0.52	0.39
Total hip	0.63 ± 0.09	0.64 ± 0.09	0.46
T score	-2.21 ± 0.70	-1.98 ± 0.86	0.46
Bone turnover markers			
Serum TRACP-5b, mU/dL	309.3 ± 116.8	253.0 ± 136.7	0.14
Serum P1NP, μg/L	32.7 ± 22.7	22.7 ± 15.7	0.13

Data are mean ± SD. BMI, body mass index; BMD, bone mineral density; TRACP-5b, tartrate-resistant acid phosphatase 5b; P1NP, procollagen type 1 N-terminal propeptide.

switch group in the present study), and a significant increase occurred in only the lumbar spine BMD in the denosumab group. At 12 months, the teriparatide-treated group showed a significant increase in the lumbar spine BMD and a tendency for a BMD increase in the femoral neck compared to the denosumab group.

At 48 months, the lumbar spine BMD had increased significantly from baseline in both groups (**Figure 2A**). The percent changes in the lumbar spine BMD from baseline to 48 months were as follows: denosumab group, 10.4 ± 8.7% ($p<0.001$); switch group, 14.2 ± 6.8% ($p<0.001$). At 48 months, there was no significant between-group difference in the lumbar spine BMD. In the femoral neck, the percent changes in BMD from baseline to 48 months were as follows: denosumab group, 4.1 ± 10.8% ($p=0.21$); switch group, 11.2 ± 14.6% ($p<0.05$) (**Figure 2B**). At 48 months, the BMD of the femoral neck was significantly increased from baseline only in the switch group, and the BMD was significantly increased in the switch group compared to the denosumab group ($p<0.05$). In the total hip, the percent changes in BMD from baseline to 48 months were: denosumab group, 4.60 ± 7.4% ($p<0.05$); switch group, 7.2 ± 6.9% ($p<0.01$) (**Figure 2C**). At 48 months, there was no significant between-group difference in the total hip BMD.

Compared to 24 months, the BMD in the denosumab group at 48 months was significantly increased in both the lumbar spine and total hip. In the switch group, compared to 24 months, the BMD at 48 months was significantly increased at all measurement sites. The percent changes in BMD from 24 to 48 months were not significantly different between the two treatment groups at any of the measurement sites.

As shown in the original study, a clinical vertebral fracture occurred in two patients in the denosumab group during the first 2 years. During the extended 2-year period, no new clinical fractures occurred in either patient group.

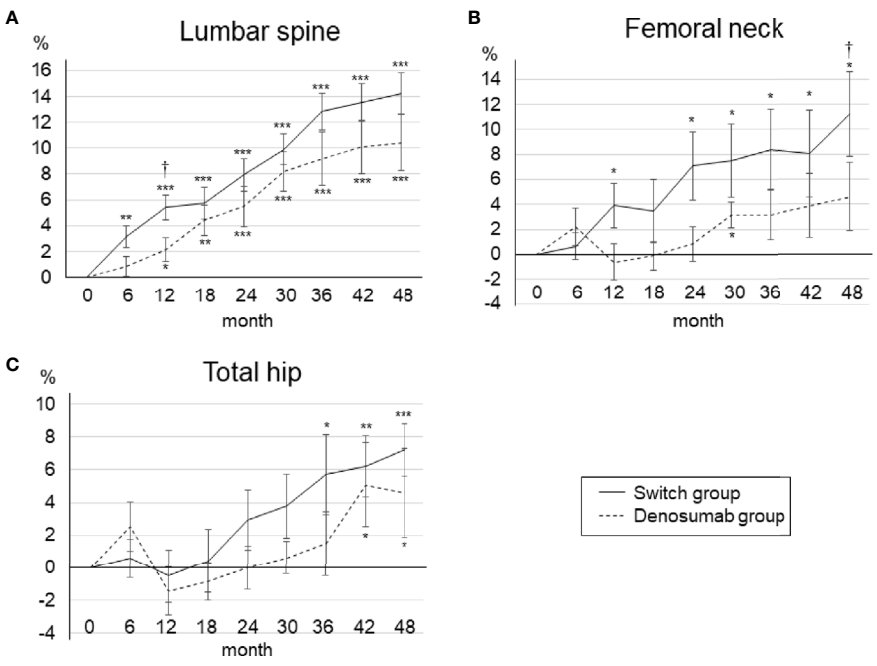

FIGURE 2 | Mean percent changes in BMD from baseline to 48 months in the lumbar spine **(A)**, femoral neck **(B)**, and total hip **(C)**. Error bars: SEM. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ *vs.* baseline. †$p < 0.05$, denosumab group *vs.* switch group.

Changes in the Bone Turnover Markers and Calcium

The changes in bone turnover markers are shown as a percentage change from baseline in **Figure 3**. In the denosumab group, the serum TRACP-5b levels were decreased the most at 6 months (−42.1 ± 6.2%) compared to baseline and significantly decreased until 30 months, and the serum P1NP levels were decreased the most at 6 months (−30.4 ± 8.3%) compared to baseline and significantly decreased until 12 months. In the switch group, both the serum TRACP-5b and serum P1NP levels increased the most at 6 months of teriparatide treatment compared to baseline (108.4 ± 25.1% and 491.6 ± 66.5%, respectively) and increased significantly until 24 months.

After the switch from teriparatide to denosumab, the serum TRACP5-b and serum P1NP levels decreased sharply at 30 months (−110.5 ± 26.9% and −12.3 ± 12.1%, respectively), and after 30 months there was no significant difference from baseline. During the first 24 months, the serum TRACP-5b and serum P1NP levels in the switch group were significantly increased compared to those of the denosumab group, but after 30 months, there was no significant difference between the two groups. There were no clinically meaningful changes in albumin-corrected calcium in the two groups (**Supplementary Material**).

Adverse Events

During the period of 24 to 48 months, one case each of ischemic enteritis, urinary tract infection, myocardial infarction, and ovarian tumor were reported in the denosumab group, and one case each of herpes zoster and angina pectoris were reported in the switch group. These adverse events were classified as unrelated to treatment by each patient's physician and the study investigators.

DISCUSSION

In the results of our 4-year study, treatment with teriparatide for 2 years followed by denosumab for 2 years significantly increased BMD from baseline in the lumbar spine, femoral neck, and total hip in GIO patients with prior bisphosphonate treatment. Continuous treatment with denosumab for 4 years also significantly increased BMD in lumbar spine and femoral neck, but the increase in femoral neck BMD was significantly greater with teriparatide followed by denosumab. Both denosumab and teriparatide are the effective agents to increase BMD in GIO patients. However, there are few reports investigating the effects of these drugs in patients with GIO who have previously been treated with bisphosphonates, and no study has compared the two drugs in such patients. Our original study compared the efficacy of both drugs in GIO patients with prior bisphosphonate treatment, and this extension study investigated effective long-term treatment strategies for GIO with these drugs.

There are only a few studies comparing the therapeutic effects of denosumab and teriparatide. In the DATA extension study, which was conducted in bisphosphonate-naïve women with postmenopausal osteoporosis, both the denosumab- and teriparatide-treated groups showed significant increases from baseline in BMD at the lumbar spine, femoral neck, and total hip, with no significant differences between the two groups (14). On the other hand, our earlier study demonstrated that teriparatide has some advantages over denosumab in GIO patients with prior bisphosphonate treatment (15). The discrepancy in the results may be due to the different backgrounds of the patients addressed in each study. Osteoporotic patients who have been treated with bisphosphonates have already had their bone turnover sufficiently suppressed, and it is possible that the therapeutic effect of denosumab (which suppresses bone turnover like bisphosphonates do) is restricted. In addition, since GIO is caused primarily by an inhibition of bone formation, we considered teriparatide, a bone-forming agent, appropriate for the treatment of GIO.

Denosumab and teriparatide are potent osteoporosis therapeutic agents that produce large increases in lumbar and femoral BMD values. However, the discontinuation of either of these drugs results in a rapid decline in BMD (17–19). Since the clinical use of teriparatide is limited to 24 months, an important issue must be

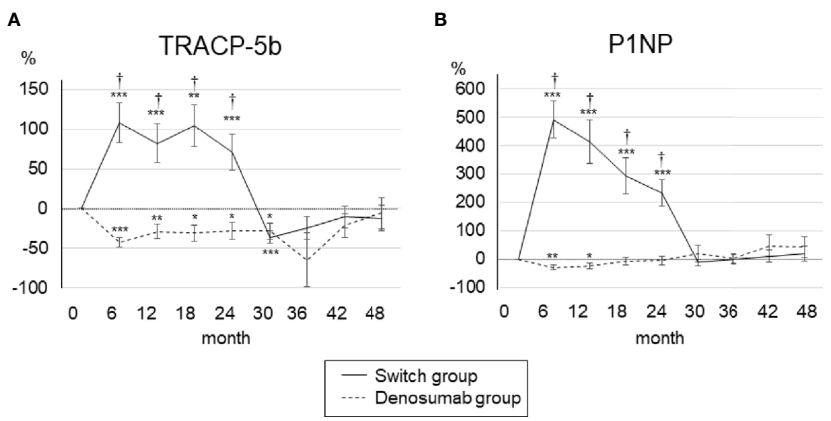

FIGURE 3 | Percent changes in serum TRACP-5b **(A)** and P1NP **(B)** from baseline to 48 months. Error bars: SEM. *p < 0.05, **p < 0.01, ***p < 0.001 *vs.* baseline. †p < 0.001, denosumab group *vs.* switch group. TRACP-5b, tartrate-resistant acid phosphatase 5b; P1NP, procollagen type 1 N-terminal propeptide.

addressed: how to maintain or further increase the BMD gain that was obtained during this period. The DATA-switch study of postmenopausal women with osteoporosis reported that the transition from teriparatide to denosumab showed a greater increase in BMD than the transition from denosumab to teriparatide (16). The order of the administration of denosumab and teriparatide may affect the outcome of increased BMD, but this has not been fully examined in GIO patients. As with postmenopausal osteoporosis, teriparatide followed by denosumab would lead to a favorable increase in the BMD of patients with GIO. In our results, femoral neck BMD at 48 months was significantly increased in the switch group compared to the denosumab group. This suggest that the BMD increase achieved with teriparatide may be greater with a subsequent denosumab administration, and that the advantage of teriparatide over denosumab may be maintained after the switch to denosumab.

The body's BMD depends on the balance between bone resorption and bone formation. In the switch group, both serum TRACP-5b, measured as a bone resorption marker, and serum P1NP, measured as a bone formation marker, were increased by teriparatide treatment, and these markers' values then decreased after the switch to denosumab. In the present denosumab group, both serum TRACP-5b and serum P1NP were suppressed, and the patients' serum levels of TRACP-5b decreased significantly compared to the baseline for a longer period than the serum P1NP. These changes in bone turnover markers may be related to the increase in BMD in both groups; however, changes in these markers alone may not be sufficient to explain the difference in the BMD results between the two groups. Switching from bisphosphonates to denosumab suppresses bone turnover markers in postmenopausal osteoporosis patients (20–22), but there are no long-term data over 4 years, and data in GIO patients are also insufficient. In our results, bone turnover markers in the denosumab group were reduced from baseline, but these suppressions were less than would be expected from previous reports. Although the exact cause of these discrepancies is unknown, there were differences in baseline characteristics of patients between our study and previous reports in that our study had a longer duration of treatment with bisphosphonates.

The dose of glucocorticoids used in inflammatory or autoimmune diseases depends on each disease and its severity. Strong immunosuppressive therapy for vasculitis and systemic lupus erythematosus requires high doses of glucocorticoids, whereas the use of ≤4 mg/day of PSL is often sufficient to improve symptoms in rheumatoid arthritis (23, 24). Because glucocorticoids increase the risk of BMD loss and fracture in a dose-related manner (25), differences in the underlying disease may affect the efficacy of therapeutic agents. Patients with various connective tissue diseases were included in the present study, but there was no significant difference in glucocorticoid dosage between the denosumab and switch groups.

In addition to long-term administration, the effects of glucocorticoids on bone metabolism are also observed in the short term. Even if administered for only a few days, high doses of glucocorticoids can affect bone metabolic markers and also cause increased serum intact parathyroid hormone (PTH) levels and urinary calcium excretion (26). In the present study, both the denosumab and teriparatide groups did not use more than 20 mg/day of PSL during the observation period, and there was no significant difference in the daily average dose of PSL between the groups. The usage of PSL in the two groups was thus considered to be similar.

This study was designed to evaluate patients with GIO, and patients were enrolled regardless of gender. Most of the patients who participated in this study were female, but one male was included in the denosumab group. The prevalence of osteoporosis is more common in women than in men. Women have a lower peak bone mass, smaller bone size, and tend to lose bone at a younger age than men (27). Excluding one male patient in the denosumab group did not significantly affect our results.

The major limitations of the present study are the lack of randomization and the small sample size. In the original study, the patients who chose the daily subcutaneous injection and were judged by their physician to be capable of self-injection were assigned to receive teriparatide, and the other patients were assigned to receive denosumab. Although there was no apparent difference in the baseline characteristics investigated between the two groups, it is possible that a larger number of patients in the denosumab group who were judged unable to perform self-injections by their physicians also contained patients with low physical activity. In postmenopausal women, exercise is effective for preventing lumbar spine BMD loss (28), and there is a report that the combination of teriparatide and whole-body vibration exercise resulted in a greater increase in lumbar spine BMD than teriparatide alone (29). Potential differences in physical activity and the exercise habits that might accompany it between the present denosumab and switch groups could have affected our results.

In addition, the present study's primary endpoint was the percent changes in BMD, not the incidence of fractures, and there were no regular radiographic examinations to identify fractures. It is not sufficient to determine the treatment effect solely by measuring the BMD without assessing the incidence of fractures; however, since BMD is an important predictor of fracture, we believe that the present evaluation of the changes in BMD is very meaningful.

In the final results of our study, the 4-year treatment with teriparatide followed by denosumab in GIO patients with prior bisphosphonate treatment resulted in a continuous and large increase in BMD in the lumbar spine and femur. Since glucocorticoid therapy for connective tissue diseases is long-term, continuous therapy for GIO is also necessary. It is desirable to judge the effects of a therapeutic drug for GIO by referring to changes in BMD and bone turnover markers; in addition, patients who are considered to have an inadequate response to bisphosphonates should be considered for a switch to an appropriate agent. Our findings are important for rational drug selection in the long-term continuum of drug therapy for GIO.

CONCLUSIONS

In our 4-year study, treatment with teriparatide followed by denosumab significantly increased lumbar and femoral BMD

values, with a greater increase in femoral neck BMD than treatment with continued denosumab. The advantage of teriparatide over denosumab in GIO patients who received bisphosphonate as a pretreatment may be maintained after the teriparatide treatment period, and treatment with teriparatide first and then with denosumab is expected to result in a continued BMD gain. Further studies with larger patient populations are needed to confirm the efficacy of this treatment strategy.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by The Research Ethics Committee of Kindai University of Medicine. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

YH and YN designed the study. YH and SO collected and analyzed the data. YH drafted the manuscript. YN, MS, KK, MF, and IM edited the manuscript. 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.2021.753185/full#supplementary-material

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Cortisol and Phosphate Homeostasis: Cushing's Syndrome Is Associated With Reversible Hypophosphatemia

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Objectives: The influence of hypercortisolism on phosphate homeostasis is relatively unknown. A few previous studies have reported on patients with Cushing's syndrome (CS) with hypophosphatemia in whom serum phosphate normalized after initiation of treatment for CS. We aimed to investigate the prevalence of hypophosphatemia in CS, the association between the degree of hypercortisolism and serum phosphate and the change in serum phosphate after remission of CS. We compared the prevalence of hypophosphatemia in CS with the prevalence in the population-based Rotterdam Study (RS).

Methods: Patients diagnosed with CS and treated at the Department of Endocrinology of Erasmus MC in the period of 2002-2020 were included and data was collected on age at diagnosis, sex, serum phosphate, calcium and potassium levels, kidney function and BMI. Using multivariate linear regression, we analyzed the association between 24h urinary free cortisol excretion (UFC) and serum phosphate. Changes in serum phosphate and covariates were tested with a repeated measurement ANOVA, using mean levels of laboratory values for the periods before remission, and 0-14 days and 15-180 days after remission.

Results: Hypophosphatemia before treatment was present in 16% of the 99 CS patients with data on serum phosphate, 24h UFC and covariates. In comparison, the prevalence of hypophosphatemia in RS was 2.0-4.2%. Linear regression showed a negative association between the level of UFC and serum phosphate at diagnosis, which remained significant after adjusting for covariates [β -0.002 (95%CI -0.004; -0.0004), p=0.021]. A subset of 24 patients had additional phosphate measurements at 0-14 days and 15-180 days after remission. In this subgroup, serum phosphate significantly increased from 1.03 ± 0.17 mmol/L prior to remission to 1.22 ± 0.25 mmol/L 15-180 days after remission ($p = 0.008$). BMI decreased after remission [-1.1 kg/m^2, (95%CI -2.09 to -0.07), p=0.037]. Other covariates did not show an equivalent change over time.

Conclusion: In this retrospective study, we found that 16% of patients with CS had hypophosphatemia. Moreover, serum phosphate was related to the level of cortisoluria and increased after remission of CS. Potential underlying mechanisms related to urinary phosphate excretion and possibly involving FGF23, BMI and parathyroid hormone levels should be further explored.

Keywords: Cushing's syndrome, cortisol, hypercortisolism, phosphate, hypophosphatemia, glucocorticoids

INTRODUCTION

Cushing's syndrome (CS) results from chronic exposure to either endogenous or exogenous excess of cortisol (1). A well-known complication of hypercortisolism in CS is glucocorticoid-induced osteoporosis (GOP) (2, 3). GOP is thought to be the result of a combination of decreased intestinal calcium absorption and renal calcium resorption, increased bone resorption, decreased bone formation and muscle wasting. Consequently, biochemical remission of Cushing's syndrome results in an increase in bone mineral density (3). Recently, it has been suggested that treatment with glucocorticoids could also affect phosphate homeostasis and even induce hypophosphatemia due to increased urinary phosphate excretion (4, 5). Among drugs that are associated with hypophosphatemia, glucocorticoids have been suggested to be among the most common pharmacological agents associated with profound hypophosphatemia in hospitalized patients (6). Similarly, some case reports have described hypophosphatemia in patients with CS (7–9). After treatment for CS, normalization of serum phosphate levels has been reported after two weeks and can take up to one year (7–9). Findling et al. reported seven patients with CS who went in remission after treatment. One year after remission, they reported a significant increase in tubular reabsorption of phosphate, a reduction in daily urinary calcium excretion, a decrease in immunoreactive parathyroid hormone (PTH) and a decrease in 1,25 dihydroxy vitamin D (1,25(OH)$_2$D) (9). Similar to glucocorticoid use, it has been hypothesized that hypercortisolism in CS can induce hypophosphatemia by increasing urinary phosphate excretion or by inhibiting intestinal phosphate absorption. This process may be mediated by Fibroblast Growth Factor 23 (FGF23) (8, 10, 11). Indeed, Delucchi et al. reported an association between sustained glucocorticoid treatment and increased intact FGF23 levels in pediatric renal transplant patients (12).

Phosphate is important for energy metabolism, cell signaling and oxygen transport. It is also a component of DNA and RNA, and it is critical for skeletal development and bone mineralization (13, 14). Most of phosphate in the human body is stored in bone, the remainder is localized in soft tissue (15). Phosphate deficiency can cause a variety of clinical problems such as muscle weakness, rickets in children and osteomalacia in adults (16). Phosphate homeostasis is regulated by several factors of which PTH, 1,25 dihydroxy vitamin D and FGF23 appear to be the most important (15, 17). Whereas knowledge of the role of phosphate and phosphate homeostasis is increasing, little is known about the relation between cortisol, and specifically Cushing's syndrome (CS) and phosphate homeostasis.

The prevalence of hypophosphatemia in CS is currently unknown and the changes in phosphate concentrations after treatment for CS have only been studied in very small patient groups. Moreover, the role of potential confounders of phosphate homeostasis, such as BMI and kidney function, have not been adequately explored yet. In this study, we aim to evaluate the prevalence of hypophosphatemia in CS, the association between the level of 24h urinary free cortisol excretion (UFC), as a marker of the degree of hypercortisolism, and serum phosphate concentrations; the role of potential confounders and the change in serum phosphate levels after remission of CS. We compare the prevalence of hypophosphatemia in CS to the prevalence in a population-based cohort study of males and females.

MATERIALS AND METHODS

Patients

This retrospective study included patients from the endocrinology department of the Erasmus University Medical Center, Rotterdam, the Netherlands, who were diagnosed with endogenous CS in the period 2002-2020. A diagnosis of CS was made based on three screening tests: late night salivary cortisol concentration, 24h UFC and the 1 mg overnight dexamethasone suppression test (1). In patients with adrenocorticotropin hormone (ACTH) dependent CS, a pituitary-dependent cause was differentiated from an ectopic cause by bilateral inferior petrosal sinus sampling in case of a non-visible adenoma on MRI or an adenoma less than 6 mm. In patients with ACTH-independent CS, CT or MRI was performed to image the adrenal glands. To study the prevalence of hypophosphatemia and the association between 24h UFC and serum phosphate concentration, we included patients for whom serum phosphate measurements were available that were taken after diagnosis and before remission. A total of 99 patients had complete data on serum phosphate, 24h UFC and covariates before remission and they were included to study the prevalence of hypophosphatemia and the association between UFC and serum phosphate. In addition, in the subset of this population in whom serum phosphate had also been measured after remission of CS, we studied the effect of treatment of CS on serum phosphate concentration. For 24 patients with serum phosphate measurements at time of diagnosis, serum phosphate measurements and covariates were available postoperatively and within 180 days after remission. Lastly, we determined the difference in serum phosphate concentration between the time of diagnosis and more than 180 days after remission. For this analysis

we included 45 patients with a serum phosphate measurement that was taken at time of diagnosis and a serum phosphate measurement taken more than 180 days, but less than 3 years, after remission, when the patient either used hydrocortisone at a physiological dosage or supplementation had stopped. We repeated this analysis in 30 patients with additional information on covariates.

We compared the prevalence of hypophosphatemia in CS to the prevalence in the Rotterdam Study (RS). RS is a population-based study of males and females aged 40 or more from the district Ommoord in Rotterdam, the Netherlands. The rationale and design have been described in more detail elsewhere (18). This study is ongoing since 1990 and is now composed of four cohorts, named RS-I, RS-II, RS-III and RS-IV (initiated in 1990, 2000, 2005 and 2017). The Rotterdam Study has been approved by the Medical Ethics Committee of the Erasmus MC (registration number MEC 02.1015) and by the Dutch Ministry of Health, Welfare and Sport (Population Screening Act WBO, license number 1071272-159521-PG). All participants provided written informed consent to participate in the study and to have their information obtained from treating physicians. A total of 5,182 participants from RS-I, 2,511 from RS-II and 3,435 from RS-III with information on serum phosphate concentration were included to study the prevalence of hypophosphatemia in RS.

Methods

Serum samples from patients were analyzed as part of standard care for CS, at the department of clinical chemistry of Erasmus MC. Prior to 2013, 24h UFC was measured with a chemiluminescence immunoassay using unextracted urine (Immulite XPi, Siemens AG, Munich, Germany). The upper limit of normal (ULN) of this assay was 850 nmol/24h. From 2013 onwards, UFC was measured using liquid chromatography/tandem mass spectrometry (LC/MSMS, Waters Xevo-TQ-S, Milford, MA). The ULN of this assay is 133 nmol/24h. Hypercortisolism was defined as cortisoluria higher than the ULN of 24 hour UFC. For the purpose of harmonisation for this study, the level of cortisoluria was defined as the times of ULN (xULN) of 24 hour UFC. Data on age, sex, cause of CS, level of cortisoluria at time of diagnosis, serum phosphate and Cushing related treatment were collected from the medical files. Furthermore, we collected data on serum creatinine, total calcium, potassium, body mass index (BMI), proton-pump inhibitors (PPI) use, thiazide and loop diuretics use, as these variables have been associated with phosphate homeostasis. BMI (kg/m^2) was estimated from weight and height measured at clinical presentation. To calculate the estimated glomerular filtration rate (eGFR), the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) equation was applied (19). Hypophosphatemia was defined as a serum phosphate concentration below 0.80 mmol/L (normal range: 0.80-1.40 mmol/L).

In the subset of the population with serum phosphate measurements after remission of CS, the treatment modalities leading up to remission varied. In this population, the date of remission was defined as follows: the date of biadrenalectomy or adrenalectomy; the date of removal of the ACTH producing tumor; the date of the transsphenoidal hypophysectomy resulting in remission and the date when cortisoluria was less than ULN in 24 hour urine in medically or radiologically treated patients.

Statistical Analysis

The associations between 24h UFC and serum phosphate were examined through linear regression models, with the serum phosphate measurement that was taken nearest to the date of diagnosis modeled as the dependent variable and xULN of 24 hour UFC modeled as the independent variable. Analyses were adjusted for serum potassium, eGFR, total calcium, BMI, smoking and use of loop diuretics, thiazide diuretics and PPIs.

To analyze the difference between mean serum phosphate before remission and several time periods after remission, we applied a repeated measures ANOVA. Measurements of serum phosphate are not part of standard care for CS (20). Therefore, it was expected that serum phosphate had been measured at different time points and there would be missing data. To study the change in serum phosphate postoperatively and after several months, mean serum phosphate levels were calculated for the periods before remission, 0-14 days after remission and 15-180 days after remission and a repeated measures ANOVA was performed. Normality was assessed using Shapiro-Wilk's test. Analyses were repeated after exclusion of any outliers in the data. Sphericity was tested with Mauchly's test of sphericity. In the models chosen for statistical analysis, it was not possible to adjust for covariates. Therefore, any change in total calcium, potassium, eGFR and BMI was studied by comparing the means before and after remission using the statistical approach as described above. Covariates that do not show a change after remission are considered to have little or no effect on any change in serum phosphate concentrations that may be observed.

To determine the change in serum phosphate concentration in patients with a serum phosphate measurement taken at the time of diagnosis and more than 180 days after remission, we applied a paired student T-test. For this analysis we included the serum phosphate measurement that was taken nearest to the date of diagnosis and the first serum phosphate measurement that was taken more than 180 days, but less than 3 years, after remission, when the patient either used hydrocortisone at a physiological dosage or supplementation had stopped. A hydrocortisone dosage of 10 milligram in the morning, 5 milligram in the afternoon and 5 milligram in the evening was classified as physiological. We chose a cut-off of 3 years because we consider this time period to be reasonably unaffected by change due to other factors such as ageing (21)

Lastly, as a sensitivity analysis, we tested differences in serum phosphate, cortisoluria, serum calcium, potassium, eGFR, BMI and diuretics and PPI use between patients with and without hypophosphatemia and between patients with ACTH producing pituitary adenomas and ectopic ACTH production using chi-square and Mann-Whitney U tests.

Results are presented as mean ± SD, except where otherwise indicated. A p-value less than 0.05 was considered statistically significant. All analyses were performed with IBM SPSS software, version 25 (SPSS, Chicago, IL) and R version 3.6.1 (Vienna, Austria). The medical ethical committee of the Erasmus MC approved this study.

RESULTS

The general characteristics of the study population (N=99) and of the subset of the population with serum phosphate measurements at 0-14 days and 15-180 days after remission (N=24) are depicted in **Table 1**. In the total population, 73.7% was female and the mean ± SD age at diagnosis was 46.4 ± 13.5 years. An ACTH producing pituitary adenoma was diagnosed in 74.7% of patients, ectopic ACTH production was diagnosed in 23.2% of patients and 2.0% had adrenal CS. In the subset of the population with measurements at 0-14 days and 15-180 days after remission (N=24), 67% was female and the mean age at diagnosis was 50.3 ± 12.8 years. Of these 24 patients, 62.5% was diagnosed with an ACTH producing pituitary adenoma and 37.5% was diagnosed with ectopic ACTH production.

Prevalence of Hypophosphatemia

In the total population of CS patients, mean serum phosphate at time of diagnosis was 1.01 mmol/L ± 0.21. 16% of these patients had hypophosphatemia. In RS-I of the Rotterdam Study (n=5,182), 61.4% was female, mean ± SD age was 70.3 ± 9.0, mean serum phosphate level was 1.19 mmol/L ± 0.20 and hypophosphatemia was present in 2.0% of the population. In RS-II (n=2,511), 54.6% was female, mean ± SD age was 64.7 ± 7.8, mean serum phosphate level was 1.08 mmol/L ± 0.16 and hypophosphatemia was present in 4.2% of the population. In RS-III (n=3,435), 56.4% of patients was female, mean ± SD age was 57.1 ± 6.8, mean serum phosphate level was 1.12 mmol/L ± 0.17 and hypophosphatemia was present in 2.9% of the population.

TABLE 1 | General characteristics of the study population at time of diagnosis.

	All	With 0-14 and 15-180 day measurements
N	99	24
Age at diagnosis, years	46.4 (13.5)	50.3 (12.8)
Female (%)	73 (73.7%)	16 (67%)
Phosphate, mmol/L	1.01 (0.21)	1.04 (0.19)
Hypophosphatemia (%)	16 (16.2%)	2 (8.3%)
Cortisoluria, xULN UFC median (min, max)	2.6 (0.5, 144.3)	3.9 (0.6, 89.7)
Calcium, mmol/L	2.31 (0.21)	2.27 (0.18)
Potassium, mmol/L	4.0 (0.6)	3.9 (0.7)
eGFR, mL/min/1.73m^2	97.7 (20.1)	100.6 (18.6)
BMI, kg/m^2	29.0 (6.7)	28.9 (7.5)
Thiazide diuretics use (%)	20 (20.2%)	4 (16.7%)
Loop diuretics use (%)	4 (4.0%)	3 (12.5%)
PPI use (%)	24 (23.3%)	5 (20.8%)
Current smoking (%)	22 (21.4%)	5 (20.8%)
Cause of hypercortisolism		
ACTH producing pituitary adenoma (%)	74 (74.7%)	15 (62.5%)
Ectopic ACTH production (%)	23 (23.2%)	9 (37.5%)
Adrenal CS (%)	2 (2.0%)	–
Treatment		
No remission (%)	8 (8.1%)	–
Hypofysectomy(%)	22 (22.2%)	4 (16.7%)
Medication(%)	28 (28.3%)	5 (20.8%)
Bilateral adrenalectomy(%)	28 (28.3%)	13 (54.2%)
Adrenalectomy (%)	2 (2.0%)	–
Carcinoid resection(%)	4 (4.0%)	–
Radiation therapy(%)	6 (6.1%)	1 (4.2%)
Unknown (%)	1 (1.0%)	–

Results are presented as mean (standard error) for continuous variables and count (percentages) for categorical variables, unless otherwise stated. BMI, body mass index; CS, Cushing's syndrome; eGFR, estimated glomerular filtration rate; PPI, protonpumpinhibitors; xULN UFC, the times of upper limit of normal of 24 hour urinary free cortisol.

Association Between the Level of Cortisoluria and Serum Phosphate at Time of Diagnosis

Linear regression analyses showed a significant inverse association between serum phosphate at time of diagnosis and xULN of 24h UFC [β (95% CI): β= -0.003 (-0.005 to -0.002), p<0.001], which remained significant after adjustment for serum total calcium, potassium, eGFR, BMI, smoking, use of loop diuretics, thiazide diuretics and PPIs [β (95% CI): β= -0.002 (-0.004 to -0.0004), p=0.021]. Additional adjustment for age and sex did not change results (data not shown).

Change in Serum Phosphate After Remission

In the group of 24 patients with serum phosphate measurements after remission, mean serum phosphate was 1.03 ± 0.17 before remission, 1.11 ± 0.30 mmol/L at 0-14 days and 1.22 ± 0.25 mmol/L at 15-180 days after remission **Figure 1** depicts the box and whisker plots with the medians and interquartile range for the different time points. In this group, 8% had hypophosphatemia at time of diagnosis. A repeated-measures ANOVA showed that the mean phosphate levels were statistically significantly different between the different time points before and after remission $F(2, 46) = 4{,}765$, $p = 0.013$. A *post hoc* test using Bonferroni correction showed a substantial increase in serum phosphate from 1.03 mmol/L prior to remission to 1.22 mmol/L 180 days after remission, a significant increase of 0.19 (95%CI 0.04 to 0.33) mmol/L, p = 0.008 (**Figure 2**). Analysis was repeated after exclusion of outliers of serum phosphate and yielded similar results.

Next, we determined the difference in serum phosphate concentration between the time of diagnosis and more than 180 days after remission. In this group of 45 patients, serum phosphate increased significantly from 1.02 ± 0.18 mmol/L at time of diagnosis to 1.12 ± 0.25 mmol/L at >180 days after remission, a significant increase of 0.09 mmol/L (95%CI 0.02 to 0.17, p=0.019). Results were similar when restricting the analysis to patients who also had eGFR, serum total calcium, potassium and BMI measured more than 180 days after remission: increase of 0.11 mmol/L (95%CI 0.001 to 0.21), p=0.051, N=30).

Changes in Covariates After Remission

In the group op 24 patients with phosphate measurements 0-14 days and 15-180 days after remission, no change was observed in eGFR after remission of CS. There was a slight increase in serum potassium concentration from 3.99 ± 0.45 mmol/L 0-14 days after remission to 4.33 ± 0.28 mmol/L 15-180 days after remission, a significant increase of 0.34 (95%CI, 0.07 to 0.61) mmol/L, p = 0.009. Moreover, serum total calcium increased from 2.14 ± 0.25 mmol/L at 0-14 days after remission (p=0.046)

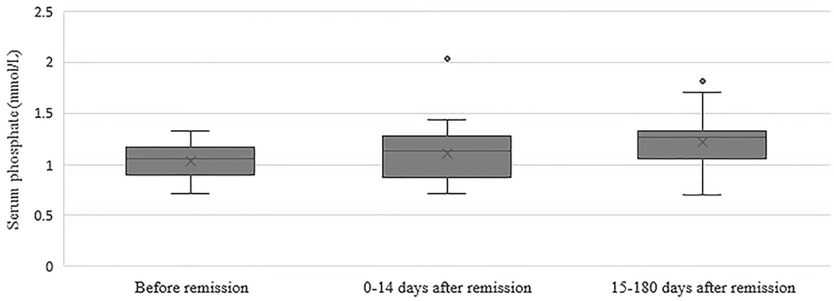

FIGURE 1 | Box and whisker plots illustrating serum phosphate concentrations before remission, 0-14 days after remission and 15-180 days after remission. Boxes include medians and interquartile range. Whiskers extend 1.5 times the interquartile range.

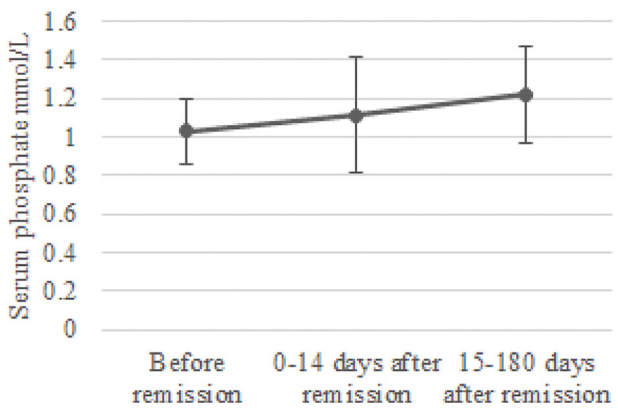

FIGURE 2 | Change in serum phosphate concentration after remission. Mean serum phosphate levels and standard deviation were calculated for the periods before remission, 0-14 days after remission and 15-180 days after remission.

to 2.28 ± 0.18 mmol/L at 15-180 days after remission (p=0.034). Lastly, we observed a significant decrease in BMI when comparing BMI at 15-180 days after remission with BMI before remission [paired t-test: -1.1 (95%CI -2.09 to -0.07), p=0.037] (**Figure 3**).

In the group of 30 patients with serum phosphate, total calcium, potassium, eGFR and BMI measurements taken at time of diagnosis and more than 180 days after remission, no change was observed in serum calcium. Interestingly, eGFR decreased from 98.83 ± 19.66 mL/min/1.73m^2 to 83.30 ± 24.39 mL/min/1.73m^2, a significant decrease of 15.53 mL/min/1.73m^2 (95%CI 8.07 to 22.97, p-value <0.001), while serum potassium increased from 4.11 ± 0.57 mmol/L to 4.46 ± 0.38 mmol/L, a significant increase of 0.35 (95%CI 0.05 to 0.64, p-value=0.022). BMI decreased from 30.2 ± 1.4 to 28.2 ± 1.5, a significant decrease of 2.0 (95%CI -3.0 to -0.9, p-value=0.001).

Lastly, differences between patients with hypophosphatemia and without hypophosphatemia and between patients with ACTH producing pituitary adenomas and ectopic ACTH production were tested using chi-square and Mann-Whitney U tests. Differences between patients with hypophosphatemia and without hypophosphatemia are depicted in **Table 2**. Here, xULN of 24h UFC was higher in patients with hypophosphatemia than in patients without hypophosphatemia (p=0.024). Patients with hypophosphatemia had lower serum calcium levels (p<0.001) and were more likely to have CS from ectopic ACTH production than patients without hypophosphatemia. Differences between patients with ACTH producing pituitary adenomas and with ectopic ACTH production are depicted in **Table 3**. Patients with CS from ectopic ACTH production were older (p=0.031), had lower phosphate concentration at time of diagnosis (p=0.004), were more likely to have hypophosphatemia (p=0.023), had higher xULN of 24h UFC (p<0.001) and had lower potassium concentrations (p<0.001) than patients with CS from ACTH producing pituitary adenomas.

DISCUSSION

In this study we investigated the prevalence of hypophosphatemia in CS, and the change in serum phosphate concentration and potential confounders of phosphate homeostasis after remission of CS. In addition, we explored the association between 24h UFC and serum phosphate before remission. Data from our study showed that hypophosphatemia was present in up to 16% of our patients with active CS. The prevalence of hypophosphatemia in these patients is four to six times higher than in participants from a population-based cohort study. We also found that serum phosphate increases after remission, which also suggests that hypercortisolism affects serum phosphate concentration. The level of cortisoluria found in hypophosphatemic patients and the inverse association between the 24h UFC level and serum phosphate concentration indicate modulatory effects of cortisol on phosphate homeostasis.

Our results indicate that hypercortisolism in CS affects serum phosphate even to the extent of causing hypophosphatemia. Hypophosphatemia can cause multiple symptoms such as fatigue and muscle weakness, which are complaints that are commonly reported by CS patients. Nearly 60% of patients with Cushing's syndrome have muscle weakness (22). Glucocorticoid

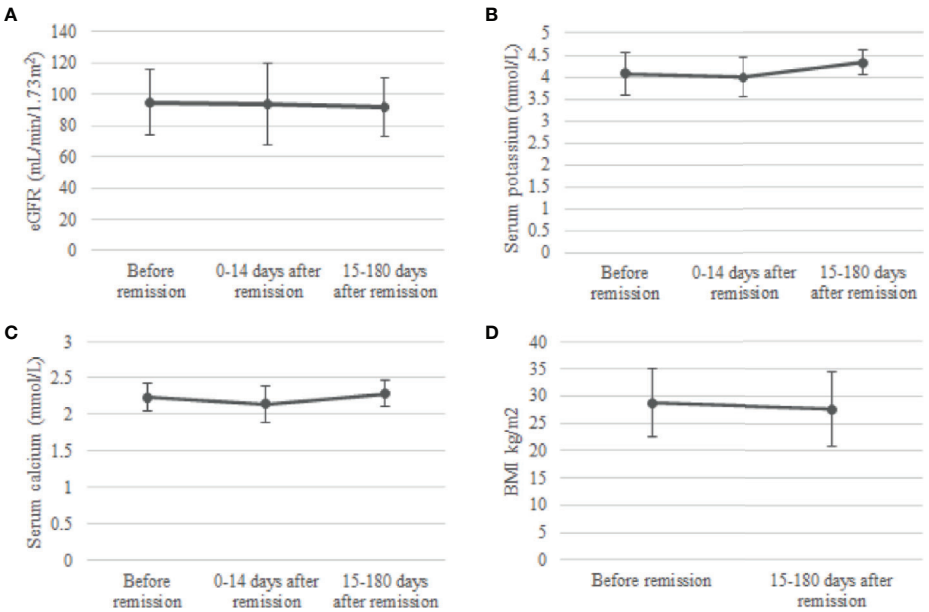

FIGURE 3 | Change in eGFR **(A)**, serum potassium **(B)**, serum calcium **(C)** and BMI **(D)** after remission. Means and standard deviations were calculated for the periods before remission, 0-14 days after remission and 15-180 days after remission. eGFR, estimated glomerular filtration rate.

TABLE 2 | Differences between patients with hypophosphatemia and with normal phosphate concentration before remission.

	Hypophosphatemia	Normal phosphate	*P*
N	16	83	
Age at diagnosis in years	49.9 (19.7)	46.5 (19.5)	0.849
Female (%)	13 (81.3%)	60 (72.3%)	0.550
Phosphate, mmol/L	0.68 (0.13)	1.06 (0.17)	<0.001
Cortisoluria, xULN UFC	5.6 (53.9)	2.6 (3.0)	0.022
Calcium, mmol/L	2.17 (0.37)	2.33 (0.21)	0.003
Potassium, mmol/L	4.0 (0.9)	4.1 (0.7)	0.362
eGFR, mL/min/1.73m²	101.7 (29.5)	100.1 (26.6)	0.680
BMI, kg/m²	26.8 (6.6)	27.6 (9.4)	0.356
Thiazide diuretics use (%)	0	20 (24.1%)	0.037
Loop diuretics use (%)	2 (12.5%)	2 (2.4%)	0.121
PPI use (%)	6 (37.5%)	17 (20.5%)	0.126
Current smoking (%)	3 (18.8%)	17 (20.5%)	0.590
Cause of hypercortisolism			
ACTH producing pituitary adenoma (%)	8 (50.0%)	66 (79.5%)	0.044
Ectopic ACTH production (%)	7 (43.8%)	16 (19.3%)	
Adrenal CS (%)	1 (6.3%)	1 (1.2%)	

Results are presented as median (interquartile range) for continuous variables and count (percentages) for categorical variables. ACTH, adrenocorticotropin hormone; BMI, body mass index; CS, Cushing's syndrome; eGFR, estimated glomerular filtration rate; PPI, protonpumpinhibitors; xULN UFC, the times of upper limit of normal of 24 hour urinary free cortisol.

TABLE 3 | Differences between patients with an ACTH producing pituitary adenoma and ectopic ACTH production before remission.

	ACTH producing pituitary adenoma	Ectopic ACTH production	*P*
N	74	23	
Age at diagnosis in years	44.6 (17.7)	54.6 (22.6)	0.031
Female (%)	55 (74.3%)	16 (69.6%)	0.788
Phosphate, mmol/L	1.06 (0.21)	0.92 (0.30)	0.004
Hypophosphatemia	8 (10.8%)	7 (30.4%)	0.042
Cortisoluria, xULN UFC	2.3 (2.1)	19.1 (42.1)	<0.001
Calcium, mmol/L	2.35 (0.20)	2.22 (0.24)	0.005
Potassium, mmol/L	4.1 (0.6)	3.7 (1.1)	<0.001
eGFR, mL/min/1.73m²	99.4 (27.9)	106.9 (20.9)	0.031
BMI, kg/m²	28.2 (9.0)	24.5 (4.7)	0.004
Thiazide diuretics use (%)	20 (27.0%)	0	0.003
Loop diuretics use (%)	3 (4.1%)	1 (4.3%)	1.0
PPI use (%)	16 (21.6%)	7 (30.4%)	0.408
Current smoking (%)	17 (23.0%)	3 (13.0%)	0.387

Results are presented as median (interquartile range) for continuous variables and count (percentages) for categorical variables. ACTH, adrenocorticotropin hormone; BMI, body mass index; eGFR, estimated glomerular filtration rate; PPI, protonpumpinhibitors; xULN UFC, the times of upper limit of normal of 24 hour urinary free cortisol.

induced myopathy is caused by an altered protein metabolism, resulting in muscle atrophy and muscle protein catabolism (22). In addition, it has been suggested that hypophosphatemia causes a decrease in muscle ATP synthesis (23). Consequently, hypophosphatemia may worsen muscle weakness in patients with CS and may also contribute to the development of glucocorticoid-induced low bone mineral density and fractures by causing osteomalacia. As can be expected, patients with CS based on ectopic ACTH production had higher 24h UFC levels than patients with CS due to ACTH producing pituitary adenomas (24), and were in turn more likely to develop hypophosphatemia.

Our findings are in line with and extend previous reported cases of hypophosphatemia in CS, in whom remission of CS resulted in normalization of serum phosphate (7, 8). Similarly, Findling et al. reported previously an increase in serum

phosphate concentration after treatment of ACTH-dependent CS in 7 patients. However, the pathophysiological mechanism(s) for these changes in serum phosphate concentrations is largely unknown. Previous studies have suggested that glucocorticoids may reduce intestinal absorption of phosphate and increase renal phosphate excretion (4, 10). Indeed, Findling et al. observed an increase in the tubular reabsorption rate of phosphate (TRP) after treatment for CS.

There are several potential hypothetical mechanisms that could explain the effect of glucocorticoids on serum phosphate concentration. These are summarized in **Figure 4**. One pathophysiological mechanism relates to FGF23, which is mainly expressed and secreted by osteocytes and osteoblasts (8, 10, 11). Expression of FGF23 is regulated by serum phosphate concentration. FGF23 regulates serum phosphate by e.g., increasing urinary phosphate excretion, but the role of glucocorticoids in FGF23 regulation is still unclear. Delucchi et al. reported an association between sustained glucocorticoid treatment and increased intact FGF23 levels in pediatric renal transplant patients (12). The same group reported an increase in bone FGF23 protein abundance and in FGF23 expression in MG53 cells, a human osteosarcoma cell line, when incubated with dexamethasone (12). In contrast, Feger et al. reported a downregulation of FGF23 transcription and protein synthesis in UMR106 rat osteoblast-like cells and MC3T3-E1 cells after incubation with dexamethasone or prednisolone. Similarly, injection of dexamethasone or prednisolone in mice lead to a decrease of serum C-terminal and intact FGF23 concentration and bone FGF23 mRNA expression, but, strikingly, also to increased renal phosphate excretion and decreased serum phosphate concentration, without affecting PTH (25). The authors state that their findings could be explained by the inhibitory effect of dexamethasone on membrane expression of sodium-dependent phosphate transporters in the kidney, resulting in increased renal phosphate excretion, as was previously reported (26). FGF23 is not routinely measured in patients with CS but Endo et al. reported a patient with hypophosphatemia due to ectopic ACTH production whose active FGF23 concentration was below the mean value previously found in healthy adults (11). We also recently observed normal C-terminal FGF23 levels in a patient who was diagnosed with hypophosphatemia and adrenal CS (unpublished observations). In this patient, serum phosphate concentration also recovered after adrenalectomy. These findings would support the hypothesis that the effect of glucocorticoids on serum phosphate concentration is independent of FGF23 and thus might be related to an effect of GCs on the sodium-dependent phosphate transporters. However there is clearly a need for larger studies on intact and C-terminal FGF23 before and after treatment of CS.

A second pathophysiological mechanism might relate to BMI. The majority of CS patients develop obesity (1, 27). Although the treatment of CS has been shown to lower BMI, patients treated for CS maintain a higher BMI than controls matched by sex and age (28, 29). Indeed, our study showed a decrease in BMI after treatment for CS. Previous literature has shown that BMI and serum phosphate levels are inversely associated (30, 31). Moreover, we recently observed evidence for a causal effect of BMI on serum phosphate using a Mendelian Randomisation approach (unpublished data). There are several theories on the

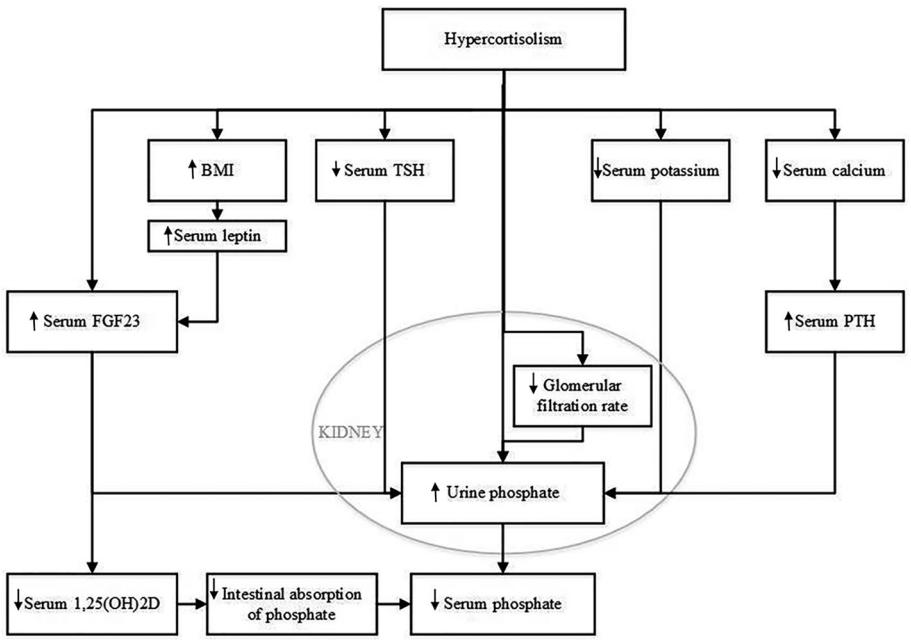

FIGURE 4 | Potential mechanisms that could explain the effect of hypercortisolism on serum phosphate concentration. BMI, body mass index; FGF23, Fibroblast Growth Factor 23; PTH, parathyroid hormone; TSH, thyroid-stimulating hormone; 1,25(OH)2D, 1,25 dihydroxy vitamin D.

pathophysiological mechanism behind this effect. A higher BMI is associated with lower 25-hydroxyvitamin D levels (32), which in turn could result in lower levels of $1,25(OH)_2D$ leading to impaired phosphate absorption from the intestine. FGF23 may also play a role in adiposity associated decreases in serum phosphate as adiposity has also been associated with FGF23. Leptin, which has been shown to function as a FGF23 secretagogue, is strongly related to adiposity (33–35). Put differently, the change in serum phosphate levels after treatment for CS may be, at least in part, due to the decrease in BMI.

A third potential mechanism may involve kidney function. An important consequence of chronic hypercortisolism is the increased risk for cardiovascular complications, including atherosclerotic vascular damage (28). In a case-control study in 18 patients, Haentjes et al. showed that patients with Cushing's disease have a decreased glomerular filtration rate compared to controls (36). Early stages of chronic kidney disease are associated with increased FGF23 levels and hyperphosphaturia (37). However, in these early stages of chronic kidney disease, serum phosphate levels are still maintained in the normal range. Hence, this would not explain why CS patients are more likely to develop hypophosphatemia. We did not observe a change in estimated glomerular filtration when we compared eGFR at time of diagnosis with 0-14 days and 15-180 days after remission. In contrast, we observed a decline in eGFR more than 180 days after remission.

A fourth possible pathophysiological mechanism that we considered involves serum potassium. Rat studies have shown that a potassium deficiency can result in phosphaturia (38). Similarly, in humans, potassium supplementation leads to a decrease in FGF23 and an increase in serum phosphate levels (39). Hypokalaemia can occur in any patient with CS (40). Due to hypercortisolism, the 11β-hydroxysteroid dehydrogenase type 2 enzyme, which converts cortisol into cortisone, can get saturated. Saturation of this enzyme results in activation of mineralocorticoid receptors, which results in increased renal excretion of potassium. Although we observed a slight increase in serum potassium concentration from 0-14 days after remission to 15-180 days after remission, we did not find a significant difference when comparing serum potassium before remission with serum potassium after remission.

A fifth potential pathophysiological mechanism involves serum calcium. Both serum calcium and serum phosphate levels are regulated by $1,25(OH)_2D$ and PTH. It has been postulated that glucocorticoids inhibit calcium absorption from the intestinal tract, but this effect remains controversial (41). In the case series of Findling et al, serum calcium did not change, but there was a reduction observed in urinary calcium excretion after treatment for CS. Interestingly, we observed a decrease in serum calcium levels at 0-14 days after remission compared to before remission. This decrease however was not seen for the period of 15-180 days after remission. In theory it is still possible that increased urinary calcium excretion combined with decreased intestinal absorption during active CS results in secondary hyperparathyroidism with an increase in urinary P excretion. Unfortunately, serum PTH levels were not measured in our patients because serum calcium was normal.

Other hypothetical mechanisms that could be considered include the role of hypothalamic-pituitary axes such as the hypothalamic-pituitary-thyroid axis. Thyroid-stimulating hormone and thyroid hormone can be influenced by glucocorticoid excess which may affect serum phosphate homeostasis (41–43). Most of our patients had ACTH dependent Cushing's syndrome. There is evidence that ACTH influences bone mass (44, 45), but a direct effect of ACTH on phosphate homeostasis remains to be elucidated.

This study has several limitations. A major limitation is the retrospective nature of the study and the considerable number of missing data. Because serum phosphate was not measured at set time points, we calculated mean serum phosphate levels. We can assume that this will negatively affect the variance of phosphate over time. To draw conclusions on the course of the phosphate levels over time we calculated several time points, including 0-14 days and 15-180 days after remission. It is not known at what time during the day the blood samples were drawn, which could affect serum phosphate levels (46). Finally, serum FGF23, $1,25(OH)_2D$, PTH nor urinary phosphate concentrations were available to us.

In conclusion, we showed that hypophosphatemia can occur in up to 16% of patients with CS, that serum phosphate concentration is related to the degree of hypercortisolism and that remission of CS results in an increase in serum phosphate. Effects were stronger in patients with CS due to ectopic ACTH production. These results suggest that hypercortisolism in CS affects phosphate homeostasis. We postulate that hypophosphatemia in CS patients may contribute to fatigue, muscle weakness and impaired bone quality. Therefore, the effect of hypercortisolism on FGF23 and urinary phosphate excretion should be further evaluated in a prospective setting and all patients with CS should be evaluated for hypophosphatemia, especially when it concerns CS from ectopic ACTH production.

DATA AVAILABILITY STATEMENT

The datasets presented in this article are not readily available because of restrictions based on privacy regulations. Requests to access the datasets should be directed to CZ, m.c.zillikens@erasmusmc.nl.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Medical Ethics Committee of the Erasmus MC. Written informed consent for participation was not required for the retrospective study in accordance with the national legislation and the institutional requirements. All participants from the Rotterdam Study provided written informed consent to participate in the study and to obtain information from their treating physicians.

AUTHOR CONTRIBUTIONS

AB, AvB, RF and CZ designed the research. AB conducted the research, performed the statistical analysis, and wrote the paper.

RF provided the essential databases. AB and CZ have primary responsibility for final content. All authors contributed to the article and approved the submitted version.

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The Use of Herbal Medicines for the Prevention of Glucocorticoid-Induced Osteoporosis

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Glucocorticoids are drugs that are widely used to suppress inflammation and the activation of the immune system. However, the prolonged use or at high doses of glucocorticoid can result in adverse side effects including osteoporosis, bone loss, and an increased risk of fracture. A number of compounds derived from natural plant sources have been reported to exert anti-inflammatory activity by interacting with the glucocorticoid receptor (GR), likely owing to their chemical similarity to glucocorticoids, or by regulating GR, without a concomitant risk of treatment-related side effects such as osteoporosis. Other herbal compounds can counteract the pathogenic processes underlying glucocorticoid-induced osteoporosis (GIOP) by regulating homeostatic bone metabolic processes. Herein, we systematically searched the PubMed, Embase, and Cochrane library databases to identify articles discussing such compounds published as of May 01, 2021. Compounds reported to exert anti-inflammatory glucocorticoid-like activity without inducing GIOP include escin, ginsenosides, and glycyrrhizic acid, while compounds reported to alleviate GIOP by improving osteoblast function or modulating steroid hormone synthesis include tanshinol and icariin.

Keywords: glucocorticoid-induced osteoporosis, herb medicine, escin, ginsenoside, glycyrrhizic acid, icariin

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INTRODUCTION

Glucocorticoids are drugs that modulate a diverse array of signaling pathways, modifying cognitive signaling, exerting immunosuppressive and anti-inflammatory activity, and preserving normal organ homeostasis and function (1). Since their first clinical deployment in the 1950s, glucocorticoids have been widely adopted and are the most commonly utilized immunosuppressive drug class in the world (2). The prolonged use of glucocorticoids, however, particularly at higher doses, can result in a variety of adverse side effects including arterial hypertension, Cushing's syndrome, type 2 diabetes mellitus, osteoporosis, and increased susceptibility to infection (3).

Endogenous glucocorticoids regulate key processes including calcium homeostasis in the intestines and kidneys, bone development, and mesenchymal cell differentiation at physiological concentrations. By stimulating mature osteoblasts to increase canonical Wnt protein production, glucocorticoids can promote the activation of β-catenin signaling in mesenchymal progenitor cells such that they differentiate into osteoblasts rather than chondrocytes or adipocytes, thus favoring osteogenesis. In osteoblasts, Wnt signaling also leads to the expression of osteoprotegerin (OPG),

which suppresses osteoclastogenesis to maintain bone homeostasis (4). At very high doses, however, glucocorticoids can negatively impact bone integrity through a range of mechanisms, with GIOP having first been described in individuals with Cushing's disease expressing excess endogenous glucocorticoid levels (5).

Owing to their potent anti-inflammatory and immunomodulatory activity, glucocorticoids are widely used in clinical contexts. However, their prolonged use can lead to adverse outcomes including glucocorticoid-induced osteoporosis (GIOP), which is the most common secondary cause of osteoporosis and an important iatrogenic risk to patients in many contexts (6). Such osteoporosis has been reported in patients with chronic inflammatory diseases including inflammatory bowel disease, chronic obstructive pulmonary disease, and systemic lupus erythematosus (SLE) (7). Most SLE patients undergo chronic glucocorticoid treatment, and one Dutch study with a 6-year follow-up period detected a dose-dependent association between the use of glucocorticoids and lumbar spine bone loss (8). Similarly, a cohort study of individuals between the ages of 18 and 64 undergoing glucocorticoid treatment for a range of disorders found that higher doses, longer treatment durations, and continuous use were associated with the highest fracture risk (9). Sustained treatment with prednisone (10 mg/d) for over 90 days was associated with 7- and 17-fold increases in the risk of hip and vertebral fractures (9).

Glucocorticoids can modulate bone biology *via* a number of different mechanisms (**Figure 1**), suppressing osteogenesis and promoting the apoptotic death of osteoblasts and osteocytes (10). Additionally, these drugs can increase the number of osteoclasts and enhance their function, resulting in an overall increase in osteoclast lifespan (11).

Osteoblast signaling pathways that can be directly impacted by glucocorticoid exposure include the peroxisome proliferator-activated receptor γ2 (PPARγ2) (12), CCAAT/enhancer-binding protein-α (C/EBPα) (13), adipocyte protein 2 (aP2) (14), and canonical WNT signaling pathways (15). Glucocorticoids promote PPARγ2, C/EBPα, and aP2 upregulation, leading precursor cells to preferentially differentiate into adipocytes instead of osteoblasts, thereby leading to a decrease in overall osteoblast numbers (12–15). Glucocorticoids also increase the expression of inhibitory molecules including sclerostin in the WNT-β-catenin signaling pathway while simultaneously inhibiting the expression of WNT16 in a dose- and time-dependent fashion, further contributing to reduced osteoblastogenesis and bone loss (16, 17).

The receptor activator of nuclear factor-κB ligand (RANKL)-osteoprotegerin (OPG) pathway is also amenable to modulation by glucocorticoids, which increase RANKL production and suppress OPG *mRNA* expression (18–20). Glucocorticoids can also enhance Notch signaling in osteoblasts and osteocytes, leading to increased Notch target gene expression including

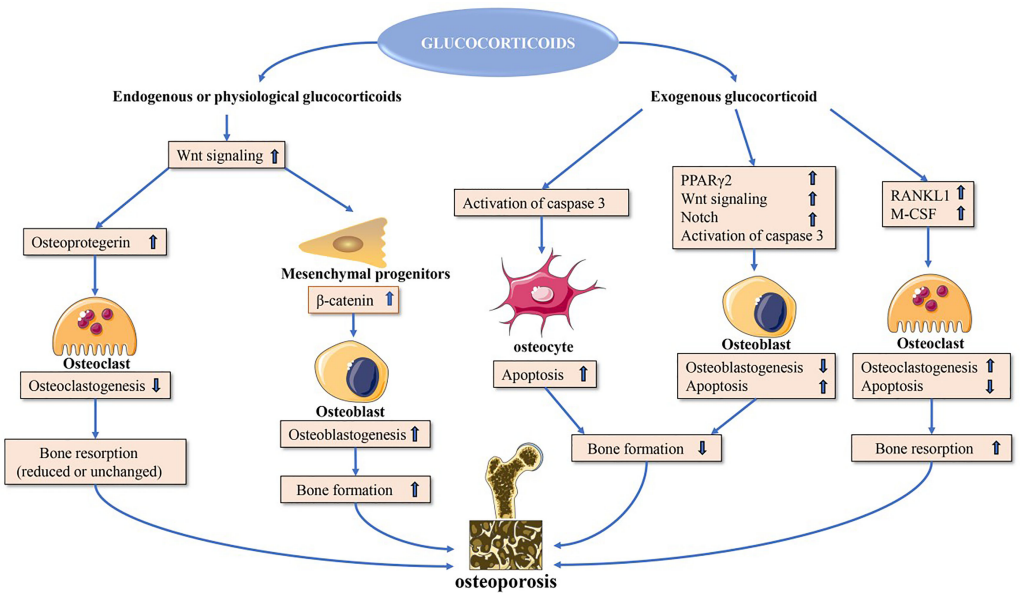

FIGURE 1 | Direct glucocorticoid effects on bone. Endogenous or physiological glucocorticoids stimulate mature osteoblasts to produce canonical Wnt proteins, which activate the β-catenin signaling cascade in mesenchymal progenitor cells and promote them to differentiate towards osteoblasts. These actions favor bone formation. Additionally, Wnt signaling in osteoblasts and osteocytes promotes osteoprotegerin expression, which in turn inhibits osteoclast formation resulting in decreased or unchanged bone resorption. Exogenous glucocorticoids negatively affect osteoblast and osteocyte function. In osteoblasts and osteocytes, increased PPARγ2 and Notch target gene expression and decreased wnt signaling contribute to decreased osteoblastogenesis, and activation of caspase 3 results in increased osteoblast and osteocyte apoptosis. Glucocorticoids induce upregulation of expression of RANKL and M-CSF, which leads to increased osteoclastogenesis and osteoclast lifespan. PPARγ2, peroxisome proliferator-activated receptor-γ2; RANKL, receptor activator of nuclear factor-κB ligand; M-CSF, macrophage colony-stimulating fact.

hairy and enhancer of split (Hes) and Hes-related with YRPW motif (Hey), which are repressive transcription factors that have the potential to mediate the impairment of osteoblast functionality and consequent reductions in osteogenesis (21, 22).

Glucocorticoid-induced apoptosis is linked to the enhanced activity of effector proteins including caspase 3, 7, and 8 downstream of the pro-apoptotic Bim and Fas/FasL death receptor pathways (10). Glucocorticoids can also stabilize GSK-3β activity to induce osteoblast apoptosis.

Glucocorticoids can impact osteoblasts to increase the RANKL : OPG ratio, thereby promoting osteoclast differentiation and maturation such that the overall rate of bone resorption increases. This effect can be further exacerbated by the ability of glucocorticoid treatment to induce the production of macrophage colony stimulating factor (M-CSF), which is released from osteoblasts and enhances the differentiation and activity of osteoclasts (23). The long-term impact of glucocorticoids on osteoclast function, however, is less certain, with multiple reports indicating that these compounds can interfere with the osteoclast cytoskeleton such that the activity of these cells may be increasingly impaired even as their longevity increases (24–26).

THE IMPACT OF HERBAL MEDICINES ON GLUCOCORTICOID- INDUCED OSTEOPOROSIS

Many studies have shown that herbal medicines can significantly increase bone density and improve clinical findings in GIOP patients, thus serving as novel tools for the treatment and/or prevention of this debilitating glucocorticoid-related complication (27–29).

Herbal Medicines Exert Glucocorticoid-Like Anti-Inflammatory Activity Without Inducing GIOP

A range of herbal compounds have been suggested to mediate anti-inflammatory activity by signaling through the glucocorticoid receptor, likely owing to their structural similarity to glucocorticoids. Notably, these compounds seem to be able to mediate these effects without a significant risk of negative glucocorticoid-related side effects such as GIOP.

Escin

Escin is a natural mixture of triterpene saponins extracted from the seeds of Aesculus chinensis Bge. or Aesculus wilsonii Rehd. Escin has been reported to exhibit pharmacological effects similar to those associated with glucocorticoid administration. For example, oral escin (5 and 10 mg/kg, p.o.) intake has been found to suppress carrageenan-induced paw edema and to inhibit prostaglandin E2 (PGE2) production (30). Notably, when compared with glucocorticoids, escin (2 mg/kg, i.v.) has been shown not to induce thymic or splenic immune cell apoptosis in mice, nor does it promote the enhanced secretion of endogenous corticosterone (31). Zhang et al. found that the sustained administration of escin (0.45 and 0.9 mg/kg for a period of 10 days, i.v.) in the context of post-surgical bone fracture healing has no adverse impact on wound or bone healing processes (32). There is also evidence that glucocorticoids and escin (2 mg/kg, i.v.) exhibit synergistic anti-inflammatory activity when administered *in vitro* and *in vivo* at low doses, suggesting at least partial overlap in the pharmacological pathways impacted by these compounds (33). Combination glucocorticoid and escin(5 and 10 mg/kg for a period of 16 days, i.g.) treatment can significantly decrease synovial inflammatory infiltration, synovial hyperplasia, and bone erosion in a rat model of adjuvant-induced arthritis (AIA) rats while reversing some of the adverse effects of glucocorticoid treatment alone such as reductions in boy weight and increases in the spleen index relative to untreated rats (34). Administering escin (10 mg/kg for a period of 14 days, p.o.) together with a low dose of dexamethasone (Dex) has been shown to markedly suppress paw swelling, joint pathology, arthritic index scores, and immune organ pathology in an animal model, all while reducing the necessary Dex dose and thus decreasing the rate of adverse effects associated with Dex administration (35). The anti-edema and anti-inflammatory properties of escin may be attributable to its ability to bind to the glucocorticoid receptor (GR), consistent with glucocorticoid-like activity (36). Escin (1.8 and 3.6 mg/kg, i.v.) may additionally augment the antioxidant capacity of tissue in the context of lipopolysaccharide (LPS)-induced acute lung injury (ALI) and endotoxin-induced liver injury by suppressing the production of inflammatory compounds including NO, TNF-α, and IL-1β while simultaneously promoting GR upregulation in the liver and lungs (37, 38).

Ginsenosides

Ginsenosides are the primary active ingredients isolated from ginseng, and they have been reported to exhibit anti-inflammatory activity *in vitro* and *in vivo* owing to their structural similarity to steroid hormones. Compound K is a ginsenoside that has, *in vitro*, been shown to suppress TNF-α-induced fibroblast-like synoviocyte (FLS) migration, proliferation, and secretion, consistent with joint-protective activity (80 mg/kg for a period of 14 days, i.g.) (39). In a rat model of myocardial infarction (MI), ginsenoside Rg3 (30 mg/kg for a period of 7 days, i.g.) reduces inflammation *via* the inhibition of the NF-κB pathway (40). Combining the ginsenosides Rh1 (20 mg/kg, i.p.) and Rg2 (20 mg/kg, i.p.) can suppress LPS-induced tissue damage and inflammation by interfering with the ability of LPS to bind to and trigger the activation of TLR4 (41). Ginsenoside Rb1 (10 and 20 mg/kg, i.p.) markedly alleviates LPS- or cantharidin-induced acute kidney injury, LPS-induced septicemia, and dimethyl benzene-induced ear edema in mice (42). Ginsenoside treatment is also not associated with any significant adverse reactions. In mice overexpressing TNF-α, ginsenoside Rg1 (20 mg/kg, i.g.) can prevent bone erosion, inhibit synovial inflammation, and reduce serum levels of both IL-6 and TNF-α, and treatment for 12 weeks with ginsenoside Rg1 was not associated with any liver or kidney damage (43). Ginsenoside Rd (10 mg/kg, i.p.) can suppress ischemia-induced microglial activation and inhibit

proinflammatory cytokine production while inducing fewer severe side effects as compared to glucocorticoids (44). Importantly, these ginsenosides can also work in synergy with glucocorticoids to inhibit inflammation. For example, combining corticosterone with low concentrations of Rg1 can suppress the LPS-induced production of NO and TNF-α by RAW264.7 macrophages while simultaneously promoting GR upregulation (45). Ginsenosides can also shape cellular responses in a GR-mediated manner, as in the case of Rg1 (12.5 mg/kg, i.p.), which suppresses LPS-induced NF-κB nuclear translocation and inflammatory cytokine production in a GR-dependent fashion. Notably, Rg1 (20 mg/kg for a period of 21 days, i.g.) has no adverse impact on murine osteoblast differentiation or proliferation (46). In a murine collagen-induced arthritis (CIA) model system, the ginsenoside Rh1 (10 mg/kg for a period of 10 days, i.p.) was also able to augment the anti-inflammatory activity of Dex by enhancing GR expression and binding without inducing hyperglycemia (47). Ginsenoside CK (112 mg/kg for a period of 24 days, i.g.) can also activate GR to suppress β-arrestin2 expression, thereby inhibiting inflammation (48).

Glycyrrhizic Acid and Glycyrrhetinic Acid

Glycyrrhizic acid (also known as glycyrrhizin) is the primary glycoside derivative of licorice, and it has been ascribed a range of anti-inflammatory activities. By suppressing signaling through the Smad3 and MAPK pathways, for example, glycyrrhizin (30 and 100 mg/kg for a period of 28 days, i.g.) has been shown to reduce the severity of bleomycin-induced inflammation and pulmonary fibrosis in mice (49). Glycyrrhizin (10 mg/kg for once every day in the first 3 weeks following by given once every 3 days until the twelfth week, intra-articular knee injection) treatment can also alleviate inflammation and the degeneration of cartilage tissue in a rat model of osteoarthritis *via* regulating the TLR4/NF-κB and HMGB1 pathways (50). *In vivo*, glycyrrhizic acid undergoes hydrolysis to yield glycyrrhetinic acid, which is structurally similar to steroid hormones such that it is able to exert a range of biological effects including glucocorticoid-like anti-inflammatory activity through interactions with steroid hormone receptors and metabolic enzymes. For example, in a murine ALI model system, glycyrrhetinic acid (10, 20 and 40 mg/kg for a period of 7 days, i.g.) was able to reduce injury severity by suppressing NLRP3 inflammasome activation through the ROS-PI3K/AKT pathway (51). Glycyrrhetinic acid (40 μM) may also be hepatoprotective in the context of chronic liver inflammation, functioning by suppressing the phosphorylation of IκBα phosphorylation and the nuclear translocation of p65 so as to reduce iNOS expression, thus alleviating inflammation (52). Glycyrrhetinic acid and glycyrrhizic acid can interact with GR as ligands, modulating glucocorticoid resistance can also prevent inflammation by disrupting the GR-HSP90 (53, 54). As a relatively weak glucocorticoid-like drug, glycyrrhizic acid can enhance the effects of glucocorticoids while antagonizing the adverse effects associated with high-dose glucocorticoid treatment. Licorice (75 mg/kg for a period of 5 days) can also suppress 11 beta-hydroxysteroid dehydrogenase mRNA expression while potentiating glucocorticoid activity (55). Therefore, glycyrrhizic acid and glycyrrhetinic acid seem to be able to exert glucocorticoid-like anti-inflammatory activity without a significant risk of negative glucocorticoid-related side effects such as GIOP.

Herbal Medicines Capable of Inhibiting or Treating GIOP

Icariin

Icariin is the main active ingredient of epimedium, which is a natural compound that has been increasingly studied in the context of osteoporosis treatment and prevention, as it has been shown to simultaneously suppress bone resorption and expedite bone formation (56). Icariin (125 mg/kg for a period of 14 days, i.g.) can promote primary osteoblast maturation and associated bone remodeling, inducing osteoblast mineralization and the expression of key markers of terminal differentiation such as alkaline phosphatase (ALP) and type I collagen (57–60). Icariin (0.1 μM) also exhibits robust anti-apoptotic activity, promoting BMSC proliferation and osteogenic differentiation *via* Wnt/β-catenin pathway activation (61).

With respect to the symptoms of GIOP, icariin (5 μM for a period of 48 h) can enhance trabecular bone density in the context of glucocorticoid exposure, promoting osteogenic differentiation *via* the suppression of Notch signaling (62). Through the enhancement of autophagic activity, icariin (50 mg/kg for a period of 30 days, i.p.) can reduce OVX-induced bone loss in animal model systems (63), in addition to disrupting the Dex-induced apoptotic death of osteocytes (58). Icariin can also activate the ERK and ER pathways to control bone homeostasis, promoting OPG expression and Wnt pathway activation. Inhibiting osteoclastogenesis is at least partially responsible for the anti-osteoporotic activity of icariin and compounds derived therefrom. The levels of the osteoclast differentiation marker tartrate-resistant acid phosphatase (TRAP) are reduced in a dose-dependent manner when osteoclast precursor cells are treated with icariin (10 nM, every 3 days) (64). Icariin (10 μM) is also able to directly suppress RANKL-induced hemopoietic cell differentiation into osteoclasts (65). In addition to regulating osteoclastogenesis, icariin (50 and 100 μM) can arrest cell cycle progression in osteoclast precursors, thereby inducing their apoptotic death (66). It can further reverse deleterious Dex-induced trabecular phenotypes while stimulating bone remodeling, increasing bone calcium, OCN, and FGF-23 levels while reducing the levels of bone resorption markers including CTX and TRAP-5b. Indeed, in GIOP model mice, icariin (100 mg/kg for a period of 6 or 12 weeks, p.o.) treatment has been shown to protect against bone degeneration, hypercalciuria, and hypocalcemia (67). As such, icariin may be a valuable tool for use in the induction of bone regeneration owing to its potent osteogenic bioactivity.

Many clinical studies have shown that Chinese medicine containing epimedium has achieved good clinical effects in the treatment of GIOP patients. Hugu Capsules (comprised of epimedium, polygonum multiflorum, rehmannia glutinosa and other traditional chinese medicines) can significantly increase the bone mass of 51 patients with GIOP, improve bone turnover, and relieve pain (68). Through observation of 50 GIOP patients, Wu et al. found that taking Xianling Gubao capsule while using

glucocorticoids treatment can increase the BMD of the patient's lumbar spine and proximal femur, thereby reducing the incidence of osteoporotic fractures and having fewer adverse reactions (69). Through clinical observation of 66 patients with GIOP, Shi et al. found that Bugu Capsules (including epimedium) can significantly reduce the impact of OP caused by glucocorticoids, reduce blood calcium, parathyroid hormone levels, and increase bone density (70).

Tanshinones

Tanshinone IIA, extracted from Salvia miltiorrhiza Bunge, is a perennial herbal plant widely used as a folk remedy in Asian countries. Several studies have proved that Tanshinone IIA possesses many biological activities, such as anti-inflammatory, free-radical scavenging abilities, antioxidant properties, liver protection, and anti-cancer properties. Tanshinones are compounds that can also simultaneously inhibit osteoclastogenesis and bone resorption while promoting more robust bone formation with concomitant osteoblastogenesis. These tanshinones (2, 5 μg/ml) suppress osteoclast development through the disruption of RANKL-mediated NF-κB, MAPK, Akt, and M-CSF/c-Src signaling pathway activation (71, 72). Tanshinone IIA, for example, can inhibit osteoclastogenesis through the inhibition of RANKL-induced c-Fos and NFAT c1 (71), with Tanshinone IIA (20 μg/mL for a period of 30 min) pretreatment reportedly reducing the fusion, actin ring formation, and resorptive activity of osteoclasts in a co-culture system containing M-CSF and RANKL-treated calvarial osteoblasts and BMCs (73). Mechanistically, Tanshinone IIA (10 μg/mL) can function as a selective COX-2 inhibitor to suppress PGE2 and to thereby modulate OPG and RANKL expression (74), all of which are related to osteoclast function. Tanshinones (1 μM for a period of 24 h) can also disrupt the apoptotic death of osteoblasts and consequent osteoporosis observed upon glucocorticoid treatment by inactivating Nox4 (75). In osteoporosis model mice, Tanshinone IIA (10 mg/kg for a period of 6 weeks, p.o.) was able to decrease the incidence of fractures and severe osteopenia while augmenting bone strength, mineral levels, and collagen in the bone matrix (76). Tanshinone (10 mg/kg for a period of 21 days, i.v.) was also able to upregulate phosphoglycerate dehydrogenase and to thereby suppress OVX-induced osteoporosis and BMSC senescence (77). Tanshinone can alleviate the adverse effects of Dex treatment and consequence cellular injuries such as caspase-9-dependent apoptosis, increased cytosolic cytochrome c and Nox levels, and increased ROS generation (75). Current preclinical evidence suggests that these Tanshinones

TABLE 1 | Herbal medicines capable of inhibiting or treating GIOP.

Origin	Main components	*In vitro*		*In vivo*		Mechanism of bone protection
		Cells	Dosage	Animal	Dosage and administration route	
Celastrus genus of the Celastraceae family	Celastrol (78)	–	–	Male C57BL/6J mice	1 mg/kg, per day for 12 weeks, i.m.	Activating Wnt signaling pathway
Daphne odora var. *marginatai*	Daphnetin (81)	MC3T3-E1 cells	20 μM for 48 h	Male SD rats	i.m., i.p.	
Herba Cistanches	Echinacoside (82)	MC3T3-E1 cells	10 mg/l for 48h	–	–	Induction of osteoblast apoptosis
Ginkgo Biloba	Ginkgo biloba extract (83)	–	–	Female Wistar rats	28, 56 mg/kg, per day for 20 days or 30 days, i.g.	
Red Ginseng	Red Ginseng (79)	MC3T3-E1 cells	250, 500, 1000 μg/mL for 48h	–	–	
Cnidium monnieri (L.) Cusson	Osthole (80)	–	–	Female SD rats	10, 20 mg/kg, per day for 8 weeks, i.m.	Regulating TGF-β/Smad signaling
Rhizoma gastrodiae	Gastrodin (84)	MC3T3-E1 cells	1, 5 μM for 48 h	Female SD rats	1, 5 mg/kg, per day for 60 days, i.g.	Upregulating expression of BMP
Myrica rubra Sieb. et Zucc.	Myricetin (85)	MC3T3-E1 cells	20 μM	Male SD rats	2.5 mg/kg,once every other day for a period of 5 weeks, i.p.	
Curcuma longa	Curcumin (86)	–	–	Male C57BL/6J mice	200 mg/kg per day for 12 weeks, i.g.	Inhibiting the activity of RANKL/RANK signaling
Chansu	Gamabufotalin (87)	BMMs	100, 150 nM for 3-5 days	–	–	
Piper sarmentosum Roxb.	Piper sarmentosum (88)	–	–	Male SD rats	125 mg/kg	Inhibiting the activity of 11β-HSD1
Achyranthes bidentata Bl.	β-ecdysone (89)	BMSC	10^{-7} M for 8 h	Male Swiss-Webster mice	0.5 mg/kg	Inhibiting the autophagy produced by osteoclasts
Pueraria Lobata	Total Flavones of Pueraria Lobata (90)	–	–	Female SD rats	100, 200 mg/kg, per day for 12 weeks, i.g.	Promoting bone matrix formation
Pueraria pseudo-hirsuta TANG et WANG	Chilk extracts (91)	–	–	Female wistar rats	200mg/kg, per day for 6 months, i.g	Decreasing sex hormone levels
Lycium chinense Miller	Lycium barbarum polysaccharide (92)	–	–	Wistar rats	2.6 g/kg, per day for 12 weeks, i.g.	Regulating calcium and phosphorus metabolism
Salvia miltiorrhiza Bunge	Salvianolic acid B (93)	–	–	Male SD rats	40, 80 mg/kg, per day for 12 weeks, p.o.	Regulating lipid metabolism balance

preserve skeletal integrity primarily by suppressing bone resorption and osteoclast formation, underscoring their potential value for the treatment of GIOP.

Many other herbal medicines have also been found to reduce GIOP incidence or severity through a range of mechanisms. For example, celastrol can suppress GIOP incidence in rats by modulating the Wnt and PI3K/AKT signaling pathways (78), while KRG can induce the apoptotic death of osteoblasts, highlighting its potential therapeutic utility as a tool to delay the onset of osteoporosis (79). Osthole has been shown to prevent Dex-induced osteoporosis in female rats, potentially by normalizing hormone and cytokine homeostasis through increases in TGF-β1 production (80) (**Table 1**).

CONCLUSION

Much like other hormone molecules, glucocorticoids can exert a range of effects on tissues and organs when employed at physiological and pharmacological doses. While awareness of osteoporosis and other risks associated with prolonged or high doses glucocorticoid use is growing, GIOP remains underdiagnosed and inadequately treated. Herbal medicines characterized to date have been shown to treat GIOP through two primary mechanisms, with some exerting glucocorticoid-like activity without a risk of adverse reactions, and the others treating GIOP through mechanisms including the regulation of Wnt signaling pathway, the induction of osteoblast apoptosis, and the inhibition of RANKL/RANK signaling.

However, Further clinical studies of these herbal medicines are needed to demonstrate prevention properties in GIOP patients. For example, sodium aescinate has been widely used in clinic to treat traumatic and inflammatory edema, etc. A randomized, parallel, controlled clinical trial can be conducted to evaluate the anti-inflammatory efficacy combined with glucocorticoids, as well as the side effects, GIOP.

AUTHOR CONTRIBUTIONS

LZ and FF conceived the idea. XL and TY drafted the manuscript. LZ, TW, and FF supervised the process and contributed to editing. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

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REVIEW
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When to Start and Stop Bone-Protecting Medication for Preventing Glucocorticoid-Induced Osteoporosis

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Glucocorticoid-induced osteoporosis (GIOP) leads to fractures in up to 40% of patients with chronic glucocorticoid (GC) therapy when left untreated. GCs rapidly increase fracture risk, and thus many patients with anticipated chronic GC exposures should start anti-osteoporosis pharmacotherapy to prevent fractures. In addition to low awareness of the need for anti-osteoporosis therapy among clinicians treating patients with GCs, a major barrier to prevention of fractures from GIOP is a lack of clear guideline recommendations on when to start and stop anti-osteoporosis treatment in patients with GC use. The aim of this narrative review is to summarize current evidence and provide considerations for the duration of anti-osteoporosis treatment in patients taking GCs based on pre-clinical, clinical, epidemiologic, and pharmacologic evidence. We review the pathophysiology of GIOP, outline current guideline recommendations on initiating and stopping anti-osteoporosis therapy for GIOP, and present considerations for the duration of anti-osteoporosis treatment based on existing evidence. In each section, we illustrate major points through a patient case example. Finally, we conclude with proposed areas for future research and emerging areas of interest related to GIOP clinical management.

Keywords: glucocorticoid-induced osteoporosis, glucocorticoids, bone fractures, bone density, anti-resorptive treatment, bone density conservation agents, bisphosphonates, teriparatide

INTRODUCTION

Glucocorticoids (GCs) are potent immunosuppressive and anti-inflammatory medications with a host of beneficial and negative effects (1). GCs are commonly prescribed to reduce inflammation and to suppress the immune system for a broad spectrum of indications, including chronic lung disease, inflammatory arthritis, connective tissue disease, and organ transplantation. As such, GC

use is prevalent globally: at any time, 1.0 to 4.6% of UK and US adults (up to 13.7 million persons, collectively) are taking oral GCs, and 27% to 65% of these patients will receive long-term (≥3 months) GC treatment (2–6). GCs are essential medications, but chronic GC use has detrimental effects, including metabolic disorders [e.g., type 2 diabetes mellitus (7)]; impaired wound healing (8); increased risk of infection (9); and glucocorticoid-induced osteoporosis (GIOP), the most common cause of secondary osteoporosis (10). GCs effects on bone health are potent, and they increase fracture risk independently of other risk factors like low bone mineral density (BMD) (11). Untreated GIOP can lead to debilitating fractures that cause morbidity, with reduced quality-of-life, mortality, and healthcare costs (12–14). Up to 40% of patients who have GC use longer than 3 months will experience a vertebral fracture (15). Thus, anti-osteoporosis treatment is indicated for patients on long-term GC therapy to preserve bone health and reduce fracture risk. Many pharmacologic therapies for primary osteoporosis, like antiresorptive treatments and teriparatide, have evidence of anti-fracture benefits in patients with GIOP (16–18).

Unfortunately, GIOP is underdiagnosed and undertreated (6, 19–23). In one population-based US study from 2006, only half of all postmenopausal women with long-term GC use received anti-osteoporosis treatment, and this proportion decreased to 5% for women less than 50 years of age and men (6). A more recent investigation found only 42% of US patients with chronic conditions warranting glucocorticoid exposure received any osteoporosis monitoring or treatment (24). Lack of awareness of the fracture risk caused by GC use limits appropriate initiation of anti-osteoporosis therapy (25, 26). In addition, an urgent focus on management of the condition for which GCs are prescribed (e.g., active rheumatoid arthritis [RA]), which may include a plethora of tests and examinations to assist in diagnosis and symptom improvement, may also contribute to poor anti-osteoporosis treatment levels. Fortunately, some interventions have shown to substantially improve uptake of therapy to prevent GIOP and fractures: a recent educational program in the UK improved the proportion of patients on chronic GCs who were indicated for therapy from 25 to 92% (27). Other educational interventions have improved treatment, yet to a lesser degree (28). However, even among clinicians aware of the risk of GIOP, appropriate treatment is largely hindered by a lack of clear evidence and recommendations regarding populations that are indicated for GIOP therapy and when to start and stop treatment to prevent GC-induced fractures.

The aim of this narrative review is to summarize current evidence and provide considerations for the initiation and discontinuation of anti-osteoporosis therapy for patients taking systemic GCs based on pre-clinical, clinical, epidemiologic, and pharmacologic evidence. Inhaled GCs or GC replacement (i.e., Addison's disease) are not considered in this review. We first provide an overview of GIOP pathophysiology, review current guideline recommendations for anti-osteoporosis therapy initiation among patients with long-term GC use, and present considerations regarding the discontinuation of anti-osteoporosis treatment for GIOP. We use a mock patient case to illustrate the key points and clinical debates that exist throughout the review. We then conclude with proposed areas for future research and emerging topics of interest related to GIOP clinical management. The main points of this article are presented graphically in **Figure 1**.

PATHOPHYSIOLOGY AND EPIDEMIOLOGY OF GIOP

Patient Case: Part 1

Your patient is a 45-year-old Caucasian pre-menopausal female (pronouns: she/her) living in Canada who has been recently diagnosed with systemic lupus erythematosus (SLE) with musculoskeletal and mucocutaneous involvement and a SLE Disease Activity Index (SLEDAI) of 6, indicating moderate disease activity (29). You initiate hydroxychloroquine (HCQ) therapy and a tapering course of prednisone 15 mg per day, reduced by 2.5 mg weekly down to 2.5 mg prednisone per day. A 6-week follow-up visit is scheduled. Your patient has no other chronic conditions or prior fractures, does not smoke, consumes 2 alcoholic drinks per week, and has a BMI of 23.

Should you initiate anti-osteoporosis therapy for this patient at her initial appointment? Do you need additional investigations (e.g., dual-energy X-ray absorptiometry [DXA] scan)?

GIOP Pathophysiology

GCs increase fracture risk through a variety of mechanisms. Like the pathogenesis of primary osteoporosis, GCs induce an imbalance in the bone remodeling cycle governed by osteoclasts and osteoblasts that break down and build bone, respectively (30). Bone loss from GC exposure occurs in two major time periods: a rapid initial phase where approximately 2-9% of the BMD is lost within the first six months depending on GC dose, with a steady reduction in BMD of about 0.5% to 2% annually during continued treatment (11, 31–35). GCs decrease BMD in trabecular bone, mainly in the vertebrae and femoral neck, to a greater extent than in other types of bone (11, 36). Initially, GCs appear to induce a transient excess of bone resorption. GCs extend the lifespan of osteoclasts through upregulation of receptor activator nuclear factor kappa-B ligand (RANKL) while suppressing osteoprotegerin (OPG) in osteogenic cells and suppression of apoptosis signals (37, 38). Thereafter, GCs cause their most profound negative effects on bone formation and quality (39) by reducing pro-osteogenic gene expression and suppressing osteoblast differentiation and proliferation, inducing apoptosis in osteoblasts and osteocytes, and increasing osteocyte autophagy (40). GCs also disrupt the function of bone marrow stromal cells (41), preventing their subsequent maturation to osteoblasts and osteocytes (42). Comprehensive reviews with further details of *in vitro* and *in vivo* studies of glucocorticoid effects on bone cell function have been recently published (39, 43).

GCs appear to increase fracture risk beyond their effects on bone turnover and BMD. The independence of fracture risk from BMD changes is even more profound for GIOP than primary

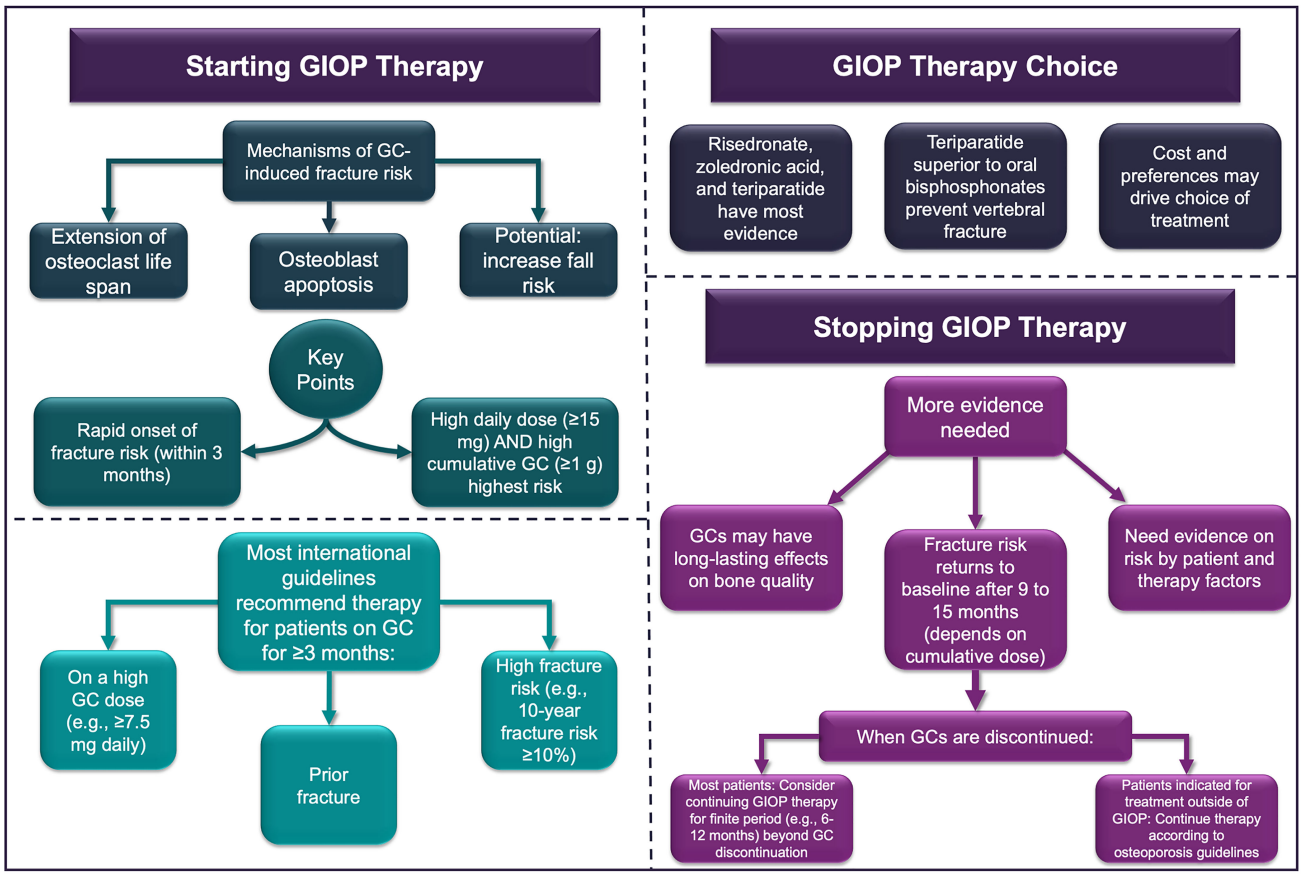

FIGURE 1 | Graphical representation of the main points presented in this article.

osteoporosis. Multiple randomized controlled trials (RCTs) have demonstrated that patients taking GCs had a significantly higher vertebral fracture risk compared to similar patients with primary osteoporosis and the same BMD values (31, 32, 44, 45). A meta-analysis of epidemiologic studies showed that the BMD changes seen at the spine and hip among GC users would correlate to an expected relative risk of vertebral and hip fracture of 1.48 and 1.41, respectively, in patients with primary osteoporosis (45); however, the observed relative risks for vertebral and hip fracture were 2.40-3.05 and 1.54-2.34, respectively, depending on the patient's cumulative GC exposure (11). To account for the poorer correlation between BMD and fracture risk in GIOP, 10-year fracture probabilities from the fracture risk assessment (FRAX) tool are often increased by a factor of 1.15 to 1.20 for patients currently exposed to 7.5 or more milligrams (mg) of prednisone equivalents per day (prednisone equivalent daily dose; henceforth, DD) (46). The disparity between the predicted and observed fracture risk in patients taking GCs compared to primary osteoporosis is often attributed to GC effects on bone quality, though mechanisms for this effect are unclear. The extension of osteoclast cell lifespans by GCs may impair osteoclast functioning long-term (47), reducing rates of bone turnover and potentially resulting in lower bone quality. GCs also affect the mineralization of bone by reducing expression of bone matrix proteins, and GC effects on osteoblasts likely reduce bone quality as well (30).

Fracture Risk Associated With GC Use: Effects of Daily and Cumulative Dose

GC use strongly increases fracture risk, with highest observed effects on vertebral fractures (48). Compared to matched controls, patients on any dose of long-term GC therapy have an average 3-times higher risk of vertebral fracture and a 2-times higher risk of hip fractures (11). As in primary osteoporosis, vertebral fractures can be asymptomatic and not come to clinical attention. Up to 40% of patients taking chronic GCs have an asymptomatic vertebral fracture, and 14% have two or more asymptomatic fractures (15).

Dose-dependent effects of GCs on bone are also well-established. A large observational UK cohort study found that hip fracture risk was 2.21-times higher among those taking 7.5 mg or more per day versus GC users taking less than 2.5 mg per day (11). This dose-

dependent relationship was even more profound for vertebral fracture, where the risk among those taking 7.5 mg or more per day was 2.83-times higher compared to those taking less than 2.5 mg per day. Even low-dose chronic GC therapy (<2.5 mg per day DD) is associated with a 1.5-times increased vertebral fracture risk compared to no use (11). Low doses do not appear to affect hip fracture risk (relative rate 0.99 [95% CI 0.85 to 1.20]) (11). A more recent study confirmed that, after confounder adjustment, GC doses below 7.5 mg per day DD independently increased the risk of clinical vertebral fractures (HR 1.59 [95% CI 1.11-2.29]) with strong dose-response effects, but no association with overall osteoporotic fracture risk was found (49).

Cumulative GC exposure also appears to affect fracture risk independently of the DD. A case control study of Danish data found that, compared to never-users, a high DD (≥15 mg) and high cumulative dose (≥1 gram prednisone equivalent cumulative dose [CD]) were independently associated with hip fractures (adjusted odds ratio [$OR_{adjusted}$] for DD ≥15 mg: 1.64 [95% CI 1.54 -1.74]; $OR_{adjusted}$ for CD ≥1 gram: 2.50 2.19-2.85)] and clinical vertebral fractures ($OR_{adjusted}$ for DD ≥15 mg: 3.75 [95% CI 2.97 to 4.77]); $OR_{adjusted}$ for CD ≥1 gram: 2.57 [95% CI 2.30 to 2.87]) (50). Those with both high DD and high CD were at greatest risk (DD ≥15 mg and CD ≥1 gram: $OR_{adjusted}$ for clinical vertebral fracture 4.36 [95% CI 3.32-5.72] and hip fracture $OR_{adjusted}$ 2.94 [95% CI 2.52-3.43]) (50). Another study found that intermittent high-dose GC without high cumulative exposure (≥15 mg/day DD but <1 gram total CD) was associated with a modest increased risk in vertebral fracture and no other fracture risk, but the risk of all types of fracture increased dramatically if the patient had cumulative GC exposures ≥1 gram (51). Similarly, another population-based Danish case-control study illustrated that among patients with COPD, intermittent high dose GC use (≥15 mg DD) was only associated with osteoporotic fracture risk when the CD exceeded 1 gram (52). In a population-based US study, a subgroup of patients less than 50 years of age only experienced a higher risk of fracture after receiving a CD of 1350 mg or higher (24). Conversely, in the CPRD study, the association between CD and fracture was nullified after accounting for the DD, age, and other potential confounders (11). However, a high DD and a CD greater than 5 grams was associated with a profound increase in all fracture risk compared to prior periods of no exposure (vertebral fractures: RR 14.42 [95% CI 8.29 to 25.08]; hip fractures: RR 3.13 [95% CI 1.49 to 6.59]) (11).

GCs may also indirectly increase fracture risk through other mechanisms. GCs induce muscle atrophy by reducing protein synthesis (30). Decreased muscle strength and insufficient balance can thus lead to falls and increase impact of a fall, particularly in older adults (53–55). GCs also impair gastrointestinal and renal reabsorption of calcium and may result in hypocalcemia and subsequent disruption of the bone turnover cycle (56), though evidence on whether these changes have clinical impacts remains controversial (57). Finally, some diseases that GCs are used to manage and treat (e.g., RA) have detrimental effects on bone from chronic inflammation (58, 59). It is still uncertain whether there is a tolerable dose of GCs for those with severe conditions that may prevent disease-induced bone loss while avoiding increasing risk through the aforementioned mechanisms either through low doses, intermittent use, or concurrent use of anti-osteoporosis therapies (59). For example, two prospective studies in SLE patients showed that a DD of less than 7.5 mg was not associated with bone loss in these patients (60, 61); however, these low GC doses have been consistently demonstrated to increase vertebral fracture risk in other populations, so this topic remains controversial (11, 49).

Patient Case: Part 1 Response

At her initial appointment, anti-osteoporosis treatment is not indicated for MP, and no DXA scan is needed at this stage. First, the target prednisone DD (2.5 mg) and estimated CD after 6 weeks (367.5 mg) are below thresholds where fracture risk increases in patients younger than 50 years of age (7.5 mg DD per day and 1-1.350 g total CD) (11, 24, 49). Next, as GC treatment is planned to be used as bridging therapy until HCQ is anticipated to take effect, we can anticipate that MP will receive fewer than 3 months of GC exposure and therefore is at lower risk of fracture (11). Finally, outside of her GC exposure, due to young age and no other major fracture risk factors (e.g., no recent fragility fracture or prior vertebral fractures), she has an overall low fracture risk (FRAX score estimating a 4.5% risk of sustaining a major osteoporotic fracture over the next 10 years).

STARTING TREATMENT TO PREVENT GIOP

Patient Case: Part 2

At her 6-week follow-up appointment, your patient reports that her joint symptoms have worsened. She has ongoing mouth ulcers and she reports pleuritic chest pain. In addition, she is found to have proteinuria, low complement levels, and elevated double-stranded DNA antibody levels resulting in a high SLEDAI score (16). You decide to initiate azathioprine as adjunct treatment to HCQ and increase her prednisone dose to 40 mg daily for 1 month with subsequent reduction to a maintenance dose of 10 mg daily thereafter until her symptoms are better managed and disease score reduced. The patient also undergoes a DXA scan, and her femoral neck BMD T-score is -1.6. Her updated calculated FRAX score suggests a 6.1% probability of sustaining a major osteoporotic fracture in the next 10 years.

Should you initiate anti-osteoporosis therapy at this visit?

GIOP-induced Fracture Risk at Treatment Onset

Evidence suggests that bone loss and fracture risk increases rapidly following GC initiation (62). A meta-analysis of ten observational studies showed that the largest decrease in BMD occurs in the first three months of GC treatment among first-time users, regardless of daily dose (11). When considering fracture risk, RCTs have shown an elevated vertebral fracture risk in the first year after GC therapy initiation (21–25), while population-based studies have found that fracture risk occurs within three to six months after initiation (8, 20). One observational study demonstrated a heightened fracture risk in the first 30 days after GC initiation among adult patients less

than 65 years of age using a self-controlled case series design (62). The exact onset of fracture risk may also differ between patients with varying baseline fracture risk.

While fracture risk remains heightened in users of GCs throughout treatment, both fracture risk and the rate of bone loss appear to stabilize after the first six to 12 months of exposure, even among those receiving high GC doses (2, 11). This pattern of risk may be due to the biphasic effects of GCs on bone. The rapid increase in fracture risk in the first months of GC exposure likely results from the initial rapid increase in bone resorption due to enhanced osteoclast activity that results in a negative uncoupling between bone formation and bone resorption phases (63). This early phase is paralleled by a second, more progressive phase where bone formation is hampered (40, 64). Longitudinal gene expression profile studies have indeed shown an early induction of genes related to osteoclast function followed by a long-lasting suppression of genes related to osteoblasts (65). Thus, the sustained, but stable, fracture risk after the initial period likely results from the long-term effects of glucocorticoids on osteoblast proliferation and function.

Current Treatments for GIOP

Current therapies approved for the treatment of glucocorticoid-induced osteoporosis in most jurisdictions include oral bisphosphonates (21–23); intravenous bisphosphonates (24), primarily zoledronic acid; denosumab (66–68); and anabolic agents (18), primarily teriparatide (44, 69). Most therapies were approved to treat GIOP primarily on the basis of BMD bridging studies (70), with larger trials suggesting anti-fracture effectiveness thereafter (16, 17, 69). Oral bisphosphonates have been shown in multiple trials and population-based studies to be associated with a significantly reduced fracture risk in GC users (e.g., HR 0.58 [95% CI 0.51 to 0.66] for vertebral fractures and HR 0.71 [95% CI 0.57 to 0.89] for nonvertebral fractures) (17). Zoledronic acid is superior to risedronate in BMD benefits (34). Teriparatide has robust evidence for its anti-fracture benefits (44), and appears to be even more effective in GIOP than primary osteoporosis in preventing vertebral fractures (69), perhaps due to GC effects on bone formation (59). Teriparatide also has evidence that it is more effective than alendronate, zoledronic acid, and risedronate in increasing BMD and preventing vertebral fractures (16, 44, 71). Denosumab, a monoclonal antibody with anti-resorptive effects, has superior effects on BMD over 24 months as compared to risedronate (67), though fracture rates and adverse effects were not statistically different between denosumab and risedronate. A comprehensive summary of evidence of the effectiveness of therapies for GIOP on fractures, BMD, and bone turnover has been recently published (18).

Clinical Guideline Recommendations for Starting Treatment to Prevent GIOP: Who to Treat and Which Therapy

Current international clinical guidelines differ in their assessment of who is indicated for anti-GIOP therapy (the American College of Rheumatology [ACR, 2017] (72); the International Osteoporosis Foundation and European Calcified Tissue Society [IOF-ECTS, 2012] (73); Royal College of Physicians, National Osteoporosis Society, and Bone and Tooth Society [RCP, 2002] (74); and the UK National Osteoporosis Guideline Group [NOGG, 2017]) (75). A summary of recommendations from international clinical guidelines is presented in **Table 1**, with the ACR 2017 fracture risk criteria available in **Table 2**. Generally, all recommend that patients initiating ≥3 months of any dose of GC therapy and who have experienced a prior fragility fracture should start anti-osteoporosis therapy, regardless of age or GC dose. Similarly, for those without a prior fracture, fracture risk assessments that account for clinical risk factors (e.g., age, sex, GC dose) or estimate a GC-adjusted fracture probability (such as that derived from FRAX) are recommended to determine whether treatment is indicated. However, thresholds for treatment are heterogenous between guidelines, potentially from a lack of evidence examining the anti-fracture benefits of GIOP treatment among patients with varying fracture risk factors. Similarly, the guidelines differ in how patients are categorized as high versus low fracture risk. Nevertheless, all recommend starting therapy as soon as possible for those who are indicated based on evidence from pharmacologic and observational studies; however, only the ACR guidelines explicitly suggest undertaking a BMD measurement within three to six months of initiation as part of fracture risk assessment (72).

Despite general agreement that anti-osteoporosis medications should be initiated in patients at high fracture risk, the current guidelines do not consistently recommend certain therapies over others for GIOP patients. Only the ACR guideline explicitly recommends initiating oral bisphosphonates (in addition to calcium and vitamin D supplementation) over other anti-osteoporosis treatments (72). The IOF-ECTS and NOGG guidelines suggest that oral bisphosphonates can be considered for first-line therapy in GIOP patients (73, 75). The RCP guidelines do not suggest or recommend a certain therapy for GIOP (74).

Oral bisphosphonates are justified in the ACR guidelines as the preferred first-line therapy for the prevention of fractures in GIOP due to their robust effectiveness, oral formulation, low cost, well-characterized safety profile in immunosuppressed patients, and lack of evidence showing that other therapies have superior anti-fracture (not BMD) effectiveness (72). Zoledronic acid, denosumab, and teriparatide are recommended by ACR as second-line treatment if oral bisphosphonates are not effective or not tolerated (72). However, there is increasing clinical trial and observational evidence that teriparatide is superior to oral bisphosphonates in preventing vertebral fractures in GC-naïve and GIOP patients with severe spinal osteoporosis (44, 76, 77). Additionally, considering the pathophysiology of GIOP is driven by effects on osteoblasts, teriparatide is a particularly attractive treatment option as it stimulates bone formation (77). We therefore suggest that teriparatide may be considered as first-line therapy in patients at high risk of vertebral fractures (e.g., with a recent

TABLE 1 | Summary of guideline recommendations on anti-osteoporosis treatment for adults with glucocorticoid use.

Guideline	Populations to be treated with an anti-osteoporosis therapy	Treatment start	Treatment duration
2017 American College of Rheumatology Guideline for the Prevention and Treatment of Glucocorticoid-Induced Osteoporosis (72) (ACR 2017)	Adults aged ≥40 years at moderate risk** of fracture[C] Adults aged ≥40 years at high risk** of fracture[A] Adults age <40 years at moderate or high risk** of fracture[C] Special populations[C]: • Women of childbearing age at moderate to high fracture risk who do not plan to become pregnant within the period of OP treatment • Adults aged ≥30 years receiving very-high dose GCs (initial dose prednisone ≥30 mg/day and cumulative dose > 5 g in 1 year) • Adults with organ transplant, eGFR ≥30 ml/min and no evidence of metabolic bone disease who continue treatment with GCs	Fracture risk screening and potential treatment initiation as soon as possible, but max within 6 months of GC initiation for all patients with anticipated long-term GC treatment (≥3 mo)[X] Repeat fracture risk assessment every 12 months[X] Recommended first-line therapy: Oral bisphosphonates[C]	Adults ≥40 years continuing GC treatment: continue treatment as long as GCs used, then re-assess. Adults ≥40 years stopping GC treatment: • Low fracture risk: discontinue osteoporosis medication but continue calcium and vitamin D[C] • Moderate or high fracture risk: "complete" treatment with OP medication like for general osteoporosis[A]
UK clinical guideline for the prevention and treatment of osteoporosis, National Osteoporosis Guideline Group, (NOGG, 2017) (75)	Postmenopausal women taking GCs who are: over ≥70 years of age, have had a previous fragility fracture, or are taking ≥7.5 mg prednisolone/day or equivalent[C] Bone protective therapy may be appropriate in some men and premenopausal women on GC therapy who have a previous fracture or are taking ≥7.5 mg/day prednisolone equivalent[C]	Start therapy immediately for indicated patients[C] Suggested first-line therapy: Alendronate or risedronate[X]	Continue treatment as long as GC use continues, can consider stopping if GC withdrawn[C]
International Osteoporosis Foundation and the European Calcified Tissue Society 2012 (IOF-ECTS 2012) (73)	Postmenopausal women and men aged ≥50 years committed or exposed to ≥3 months oral GCs: • ≥70 years of age, or • Prior fragility fracture, or • taking ≥7.5 mg/day prednisone equivalent, or • BMD T-score -1.5 or above country-specific GC-adjusted FRAX intervention threshold*[X] Premenopausal women and men <50 years committed or exposed to ≥3 months oral GCs who have had a prior fragility fracture[X] • Also consider treatment if taking ≥7.5 mg/day prednisone equivalent[X]	Start therapy at the onset of GC treatment[X] Suggested first-line therapy: Bisphosphonates or teriparatide (choice of treatment mainly influenced by cost and tolerability)[X]	Consider withdrawal of therapy with reassessment of fracture risk, preferably with a BMD measurement[X]
Royal College of Physicians, National Osteoporosis Society, and Bone and Tooth Society 2002 (74)	Consider treating patients with anticipated GC use≥3 months [C]: AND • >65 years, or • prior fragility fracture, or • BMD T score ≤-1.5[C]	Start at initiation of GC therapy[X] No suggested first-line therapy.	Not specified

MOF, major osteoporotic fracture (clinical vertebral, hip, wrist, or humerus).
[A]recommendation based on randomized trial evidence.
[C]evidence based on expert opinion, pharmacologic/preclinical evidence, or first principles.
[X]recommendation not graded, evidence not assessed, or good practice recommendation only.
**thresholds derived locally due to limitations of the algorithm.*
***See **Table 2** for definitions of high/moderate/and low fracture risk per the ACR 2017 guidelines.*

vertebral fracture or very low vertebral BMD). Teriparatide therapy is limited to 24 months in many jurisdictions and should be followed up with antiresorptive treatment (78). We also acknowledge that the use of teriparatide may be limited by cost, its required daily injections and its contraindications such as a prior history of skeletal malignancy or radiotherapy (79). Denosumab may not be a preferred first-line option for GIOP, as discontinuation is associated with a rapid loss of effectiveness

TABLE 2 | Fracture risk assessments in the 2017 American College of Rheumatology guideline for the prevention and treatment of glucocorticoid-induced osteoporosis.

High risk	Moderate risk	Low risk
Adults ≥40 years: • Prior osteoporotic fracture, or • Hip or spine BMD T score ≤-2.5 in men age ≥50 years or postmenopausal women, OR • FRAX (GC-adjusted*) 10-year risk of MOF ≥20%, or • FRAX (GC-adjusted*) 10-year risk of hip fracture ≥3% • Adults <40 years: • Prior osteoporotic fracture	Adults ≥40 years: • FRAX (GC-adjusted*) 10-year risk of MOF 10-19%, OR • FRAX (GC-adjusted*) 10-year risk of hip fracture >1% but <3% • Adults <40 years: • Hip or spine BMD T score < -3 or rapid bone loss (≥10% at the hip or spine over 1 year) AND • Continuing GC treatment at ≥7.5 mg prednisone/day for ≥6 months	Low risk: Adults ≥40 years: • FRAX (GC-adjusted*) 10-year risk of MOF < 10%, OR • FRAX (GC-adjusted*) 10-year risk of hip fracture ≤1% • Adults <40 years: • None of the above risk factors other than GC treatment

MOF, major osteoporotic fracture (clinical vertebral, hip, wrist, or humerus).
**If GC treatment >7.5 mg/day prednisone or equivalent, increase major osteoporotic risk by 1.15 (15%) and hip fracture risk by 1.2 (20%).*

and there is some limited data to suggest that fracture risk might be transiently increased (80–82). Anti-osteoporosis therapy is often stopped after GCs are discontinued, as discussed in the following section. Choice of treatment will likely also be influenced by clinical characteristics like menopausal status and renal dysfunction as well as insurance reimbursement and formulary policies, geographic location, costs, and patient preferences.

Patient Case: Part 2 Response

Your patient is at high fracture risk and, despite having no prior fractures, we recommend that she be started on antiresorptive therapy at this visit. Our recommendation to treat is based on data that show independently increased risk of fractures for patients less than 50 years of age associated with: a) CD greater than 1350 mg (patient's estimated cumulative dose at this timepoint: greater than 2700 mg) (24); b) a DD higher than 7.5 mg (11, 24) per day and the excess risk added when a patient has CD > 1 g *and* >15 mg DD per day (50); c) projected use longer than 3 months that can increase fracture risk even with low doses (11, 49); and d) a reduced BMD, which is also an important factor in determining her fracture risk (83). This recommendation is also in line with current recommendations from select guidelines: treatment is indicated due to GC exposure ≥ 30 mg prednisone per ACR guidelines (72). According to RCP guidelines (74), treatment is indicated because of projected therapy duration of more than 3 months with a DD of more than 7.5 mg/day in context of a BMD T-score which is less than -1.5. Current IOF-ECTS and NOGG guidelines, however, would not recommend starting treatment in this case (73, 75). MP would be indicated according to the IOF-ECTS guidelines if she had a prior fragility fracture *or* was older than 50 years of age and postmenopausal. Similarly, she would be indicated by the NOGG guidelines if she were postmenopausal, but these guidelines suggest that some premenopausal women taking ≥7.5 mg DD may be indicated for anti-osteoporosis therapy without further elaboration. Finally, we recommended using antiresorptive therapy (bisphosphonates) rather than teriparatide as first-line therapy, as your patient does not have severe spinal osteoporosis and thus antiresorptive therapy may be sufficient to prevent fractures. We would recommend an oral bisphosphonate as initial treatment due to the evidence of effectiveness for reducing fracture risk in GIOP (16), particularly with risedronate therapy; low cost, as highlighted by ACR recommendations (72); and ability for the clinician to stop treatment abruptly once bone protecting treatment is no longer required. However, the choice of bisphosphonate may be driven by cost, formulary restrictions, and patient/prescriber preferences.

STOPPING TREATMENT TO PREVENT GIOP

Patient Case: Part 3

Nine months after disease onset, your patient's joint symptoms have improved, mouth ulcers and pleuritic chest pain have resolved, and blood tests and urinalysis have normalized, indicating low SLE disease activity. The patient currently takes 7.5 mg prednisone each day, and you begin a tapering regimen for her prednisone over the next 12 weeks and continue HCQ and azathioprine therapy. She responds well to the tapering regimen and is found to have adequate adrenal reserve on 2.5 mg prednisone daily. Twelve months after her disease onset, the patient is able to stop prednisone altogether, and her disease remains well-controlled with combination treatment with HCQ and azathioprine.

Can you discontinue antiresorptive therapy? If yes, when should therapy be stopped?

Fracture Risk Following GC Discontinuation

It is widely accepted that GIOP is to some extent reversible. Pre-clinical evidence suggests this reversibility comes from a rapid recovery of osteoblasts after GCs are discontinued. For example, in patients with Cushing's disease, osteoblast activity as well as bone mineralization dramatically renews to baseline levels within 6 months after cure (84). GIOP's effects on bone quality may endure beyond this period, however; patients with a median duration of remission from Cushing's disease of 6 years showed similar BMD values as age- and sex-matched controls but had altered bone material properties (85). This impact on bone quality may come from longstanding impact of GCs on osteoclasts and osteocytes, which are less studied than osteoblasts (43).

While understanding recovery of preclinical markers helps to attest to the reversibility of GIOP, the duration of excess fracture risk

after GC therapy is stopped determines the appropriate duration of anti-osteoporosis therapy. Although select studies have shown that prior GC use is associated with increased fracture risk (86) and FRAX includes previous use of GCs as a fracture risk factor (46), multiple large population-based studies have independently shown that fracture risk decreases rapidly after GC exposure is stopped (24, 51, 87). A 2018 US study of over 289,000 patients with GC use and chronic conditions (RA, asthma, chronic obstructive pulmonary disease, inflammatory bowel disease, multiple sclerosis, lupus, or sarcoidosis) found the first major decrease in fracture risk after 60 to 182 days off therapy (adjusted HR [versus current use] 0.73, a 27% decreased relative risk), with only marginal further decreases in risk after longer periods off-therapy (35% decreased relative risk after more than 365 days off therapy) (24). Similar trends were observed in a cohort of US patients with RA (87). An earlier study on intermittent glucocorticoid use examined the relationships between time since discontinuation, daily dose, cumulative dose and various types of fracture (51). Among those with >15 mg DD per day, a rapid decrease in the risk of all fractures was seen in the 3 months after discontinuation, with the most profound decreases observed for vertebral fracture risk in this period (51). No elevated risk was seen beyond 12 months after discontinuation of GC therapy. Those with less than 15 mg per day DD had no excess risk beyond 9 months after their last dose. When examining cumulative exposures, fracture risk returned to baseline levels after 6 months in those with less than 1 gram total CD, while those with ≥1 gram did not have a reduction to baseline levels until 15 months later. Conversely, a Danish population-based case-control study did not observe this dramatic decrease in vertebral fracture risk after discontinuation; when compared to never-users, distant past users (>1 year) still had an elevated risk of vertebral fracture ($OR_{adjusted}$ 1.23 [1.16-1.30]) but no apparent risk of hip fracture ($OR_{adjusted}$ 0.97 [95% CI 0.93-1.01]) (50). Notably, most studies have not examined whether fracture risk after GC discontinuation differs by age, sex, or other fracture risk factors.

Due to evidence of the reversibility of GC effects on bone, all current treatment guidelines suggest that anti-osteoporosis therapy can be stopped after GC is discontinued (**Table 1**). However, none recommend specific timing to stop therapy based on empiric evidence. In addition, most recognize that the role of BMD monitoring post-GC use has not been established. The highest quality evidence for lower fracture risk patients stopping anti-osteoporosis therapy after stopping GC treatment comes from observational studies (11, 24, 50, 51), though we note that some were published after the guidelines were released. Consequently, most of the guideline recommendations are based on expert opinion or *in vitro* studies as, to date, there are no trials examining fracture risk with varying durations of osteoporosis treatment after GC discontinuation. A recent review on the pharmacology of GIOP and GCs recommends continuing therapy for six to 12 months after discontinuation of GCs (43). The only recommendation that is strong with high quality evidence in the guidelines is the ACR 2017 recommendation to continue anti-osteoporosis treatment if the patient is indicated per primary osteoporosis guidelines after GC therapy, which is based on RCT evidence in primary osteoporosis (72).

Based on the available evidence, including preclinical data, stopping anti-osteoporosis treatment immediately at the time of GC discontinuation may not be ideal. However, additional real-world evidence on fracture risk following both GC and anti-osteoporosis medication is critical. In addition, estimating the exact end time of glucocorticoid exposure is difficult using many research data sources given tapering regimens and as needed doses that are not captured through claims data, so observational studies on the effects of discontinuing GCs and continuing anti-resorptive therapy beyond GC discontinuation often have methodological limitations (50). Nevertheless, based on epidemiologic studies of the duration of elevated fracture risk for up to 15 months after stopping GCs in some patients (24, 50, 51), clinicians might choose to continue therapy for another three to six months for lower cumulative exposures (e.g., less than 1 gram CD), with longer periods (e.g., six to eighteen months after GC discontinuation) for greater cumulative exposures.

Patient Case: Part 3 Response

Based on existing clinical and preclinical evidence, since your patient is at low fracture risk aside from GC treatment, we recommend that antiresorptive therapy can be stopped after GC therapy is discontinued. We may consider continuing antiresorptive therapy for six months after discontinuation of GC therapy. The ACR, IOF-ECTS, and NOGG guidelines all recommend (ACR) or suggest (IOF-ECTS, NOGG) stopping anti-osteoporosis therapy once GC therapy is stopped (72, 73, 75). The RCP guidelines do not comment on discontinuing therapy once GCs are stopped (74). As stated in **Table 1**, no international guideline recommends a specific timing on stopping anti-osteoporosis therapy.

Our recommendation to stop anti-osteoporosis treatment and our suggestion to stop 6 months after last GC exposure are limited based on available evidence, particularly the effects of stopping among patients with high CD but are less than 50 years of age. First, evidence has shown that patients of similar age to MP (average age of 47 years) but who had cumulative exposures of 675 mg or less (substantially lower CD than MP) had a substantial decrease in fracture risk after 60 to 180 days of stopping GC therapy (24). Patients with CD greater than 1 gram, but who had an average age of 64 years also had a decrease in fracture risk starting at 3 months after last GC dose, but risk remained elevated from never users until 15 months after stopping GCs (51).

FUTURE RESEARCH

While there is abundant evidence that anti-resorptive and anabolic treatments help to prevent fractures in GIOP, there are clear gaps in knowledge regarding the timing of treatment, particularly when to discontinue in patients with few other fracture risk factors. Studies of the effects of discontinuation of GCs on fracture risk also have methodological limitations; future research could validate algorithms to ascertain true timing discontinuation of GC therapy to improve exposure measurement in fracture effects studies.

In addition, most studies supporting the effectiveness were underpowered to study effects in subgroups with additional fracture risk factors (e.g., low body mass index, recent fragility fracture). Future studies in these subgroups are particularly important to determine the benefits of GIOP treatment in patients with different baseline fracture risk. In addition, some GCs may have bone-sparing effects (88) by controlling inflammation and providing better disease control (89). An RCT is underway to examine fracture and BMD outcomes among patients with RA randomized to low dose GC or placebo added to standard RA treatment that will provide evidence on this knowledge gap (90). The utility of microindentation measurements to assess and predict fracture risk, both while exposed to GC therapy and after discontinuation (85) is another future area of study. Finally, recent preclinical evidence suggests that GC-induced fracture risk might result in part from the disturbance in circadian rhythm (91), yet studies in humans are needed.

CONCLUSION

Patients on long-term GC therapy should be assessed for fracture risk and potentially initiated on treatment to prevent GIOP. Most guidelines recommend initiating anti-osteoporosis therapy immediately for those on high-dose GC therapy, with a prior fracture, or at high fracture risk according to guideline-specific categories, though evidence shows other groups are also at risk of GC-induced fractures. Recommendations on stopping therapy with GC discontinuation are less clear. Though anti-osteoporosis therapy can be stopped in patients at low fracture risk after GC therapy is discontinued, it may be appropriate to continue therapy beyond GCs for a finite time (e.g., 6-12 months) due to a residual, dose-dependent fracture risk after stopping GC therapy. In particular, patients stopping after high cumulative GC exposure may benefit from extended treatment. Clinical trials comparing the relative anti-fracture benefits of varying lengths of treatment after GC discontinuation are critical to form strong recommendations on duration of treatment for GIOP.

AUTHOR CONTRIBUTIONS

KH drafted and revised this manuscript and provided final approval of this manuscript. EW contributed to conception, design, and interpretation of the work; revised this manuscript; and provided final approval of this manuscript. UB revised this manuscript; and provided final approval of this manuscript. BH contributed to conception, design, and interpretation of the work; revised this manuscript; and provided final approval of this manuscript. AB revised this manuscript; and provided final approval of this manuscript. All authors contributed to the article and approved the submitted version.

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Distinct Glucocorticoid Receptor Actions in Bone Homeostasis and Bone Diseases

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Glucocorticoids (GCs) are steroid hormones that respond to stress and the circadian rhythm. Pharmacological GCs are widely used to treat autoimmune and chronic inflammatory diseases despite their adverse effects on bone after long-term therapy. GCs regulate bone homeostasis in a cell-type specific manner, affecting osteoblasts, osteoclasts, and osteocytes. Endogenous physiological and exogenous/excessive GCs act *via* nuclear receptors, mainly *via* the GC receptor (GR). Endogenous GCs have anabolic effects on bone mass regulation, while excessive or exogenous GCs can cause detrimental effects on bone. GC-induced osteoporosis (GIO) is a common adverse effect after GC therapy, which increases the risk of fractures. Exogenous GC treatment impairs osteoblastogenesis, survival of the osteoblasts/osteocytes and prolongs the longevity of osteoclasts. Under normal physiological conditions, endogenous GCs are regulated by the circadian rhythm and circadian genes display oscillatory rhythmicity in bone cells. However, exogenous GCs treatment disturbs the circadian rhythm. Recent evidence suggests that the disturbed circadian rhythm by continuous exogenous GCs treatment can in itself hamper bone integrity. GC signaling is also important for fracture healing and rheumatoid arthritis, where crosstalk among several cell types including macrophages and stromal cells is indispensable. This review summarizes the complexity of GC actions *via* GR in bone cells at cellular and molecular levels, including the effect on circadian rhythmicity, and outlines new therapeutic possibilities for the treatment of their adverse effects.

Keywords: glucocorticoid receptor, transgenic mice, osteoporosis, osteoblast, osteoclast

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INTRODUCTION

Glucocorticoids (GCs) are steroid hormones that respond to stress and the circadian rhythm. Endogenous GCs are released by the adrenal glands upon activation of the hypothalamic-pituitary-adrenal (HPA) axis. Excessive or insufficient levels of endogenous GCs, Cushing's syndrome or Addison's disease, respectively, result in low bone mass and increased fracture risk (1–5). Due to their anti-inflammatory potential, exogenous GCs like dexamethasone, prednisolone, and many others are synthesized for pharmacological applications. Since the late 1940s they are widely used to treat autoimmune and chronic inflammatory diseases, recently they have been also utilized for

Covid-19 treatment (6–8). However, long-term GC therapy can cause severe adverse effects in bone such as osteoporosis, and 30-50% of those patients experience fractures (9, 10).

Once GCs enter their target cell, they become activated by the 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1) or deactivated by 11β-HSD2 (11, 12). After that initial step, the activated GCs bind to the glucocorticoid receptor (GR), a member of the nuclear receptor superfamily. GR is ubiquitously expressed and acts as a monomer, homodimer or even a tetramer (7, 13). The ligand-bound GR translocates into the nucleus and induces transactivation or transrepression of target genes in several ways (7): 1) direct binding of GR homodimers or oligomers to DNA associated GC-response elements (GRE), 2) direct binding of GR monomers to GRE, 3) tethering as a GR monomer to other DNA-bound inflammatory transcription factors such as NF-κB, AP-1, IRF-3 or Stat3.

Despite this common mechanism of GCs *via* GR, endogenous and exogenous GCs act distinctively in bone and are dependent on pathophysiological environments. Thus, it is necessary to understand the role of GCs in bone cells and their mechanism of action in several bone diseases. This review summarizes the status of current studies on cellular and molecular, endogenous and exogenous GC actions *via* the GR in bone cells. Additionally, it describes the effect of circadian rhythmicity in GC actions, and outlines new therapeutic possibilities for the treatment of their adverse effects.

ENDOGENOUS GC ACTION IN BONE HOMEOSTASIS

Endogenous GCs directly regulate bone homeostasis *via* the GR in a cell-type specific manner.

Several animal models have proved that GC signaling in osteoblast-lineage cells is critical to maintain bone mass. The effect of inactivated GC signaling in mature osteoblasts and osteocytes was investigated by overexpression of 11β-HSD2, the responsible enzyme for GC inactivation. A 2.3 kb or 3.6 kb fragment of *Col1a1* promoter-driven overexpression of 11β-HSD2 (Col2.3-HSD2 or Col3.6-HSD2) reduced cortical and trabecular bone mass in mice, which suggests the importance of GC signaling in osteoblast-lineage cells to regulate bone mass (14–16). Interestingly, another mouse model blocking GC action in osteoblast-lineage cells by osteocalcin promoter-driven overexpression of 11β-HSD2 (OG2-11β-HSD2) did not show any alteration in the bone under normal physiological conditions (17). These discrepancies among different mouse models could be explained by determining the specific stages of osteoblast-lineage cells or investigating cell-type specific conditional knock-out mouse models. Notably, GR deficiency in mice using cre overexpression under the control of early committed osteoblast progenitor markers (Runx2 or Osx1) resulted in decreased bone mass (18, 19). Taken together, endogenous GC signaling in osteoblast-lineage cells is essential in bone mass regulation. However, osteocyte-specific endogenous GC action remains inexplicit.

Osteoclasts, another key cell type for bone mass regulation, are not affected by endogenous GC signaling. Osteoclastogenesis and bone formation were normal in mice with the GR deleted in osteoclast progenitor cells ($GR^{LysMCre}$) (18). Osteoclast-specific overexpression of 11β-HSD2 using the tartrate-resistant acid phosphatase (TRAP) promoter (TRAP-HSD2) did not alter bone mass in mice (20). Collectively, endogenous GC signaling does not affect osteoclastogenesis under normal physiological conditions.

However, GCs have a profound effect on bone loss that is induced by a model of microgravity- the hindlimb unloading (HU), a model that was developed for simulating the environment of astronauts during space voyages. In this HU model, rodents showed an elevated endogenous corticosterone level (21), which led to a decreased bone mass due to decreased osteoblastogenesis, and increased apoptosis of osteoblasts and osteocytes (22). However, blocking of GC signaling in mature osteoblasts and osteocytes using Col2.3-HSD2 transgenic mice did not alter cortical bone mass in the HU model (22). Osteoclastogenesis and bone resorption were enhanced during HU due to enhanced receptor activator of nuclear factor-κB ligand (RANKL) production in osteocytes (22). This outlines the importance of endogenous GC signaling in mature osteoblasts and osteocytes, in response to mechanical loading.

EXCESSIVE EXOGENOUS GC ACTION IN BONE AND GC-INDUCED OSTEOPOROSIS

Long-term GC therapy is the most common cause of secondary osteoporosis, which leads to an increased risk of fractures (23, 24). In patients, exogenous GCs with doses higher than 2.5 mg for more than 3 months are shown to weaken bone quality (25). There is also clear evidence that exogenous GCs inhibit osteogenesis (6, 10). Bone marrow stromal cells (BMSCs) isolated from patients with corticosteroid-induced osteonecrosis showed impaired osteogenesis (26). Similarly, BMSCs isolated from a rat GIO model displayed decreased proliferation and osteogenic differentiation (27). Application of exogenous GCs *in vivo* suppressed proliferation and differentiation of osteoblasts and induced apoptosis of osteoblasts and osteocytes, resulting in a low bone mass (17, 18). This side effect could partially be rescued by leukemia inhibitory factor (LIF) treatment that activated Stat3, Mapk/Erk, and Akt signaling in GC-treated cells (28). Despite long-term exposure to high dose GCs, osteoblast lineage-specific GR deficient mice ($GR^{Runx2cre}$) displayed normal bone formation and unaltered osteoblast and osteocyte numbers (18). This is corroborated by studies with GC inactivation in mature osteoblasts and osteocytes, using mice overexpressed 11β-HSD2 under the osteocalcin gene 2 (OG2) promoter (OG2-11β-HSD2). In these mice, GC-mediated increased apoptosis of osteoblasts and osteocytes is abrogated as well (17). These studies show that exogenous GC treatment leading to GC excess impairs osteoblastogenesis, the survival of osteoblasts, and osteocytes.

GCs affect the cross-talk among bone cells. Exposure to high doses of GCs results in an increased amount of RANKL secreted by

osteoblasts and osteocytes. In turn, this increases the RANKL to osteoprotegerin (OPG) ratio and enhances bone resorption by osteoclasts (29–31).

Excessive GCs can also directly affect osteoclastogenesis (20, 32). During the initial phase of the therapy, GCs increase bone resorption by promoting osteoclast proliferation, osteoclast differentiation, and prolonging their life span (20, 33–35). However, the effect of long-term GC exposure on osteoclasts is still not entirely resolved. A few studies reported that long-term GC excess rather reduces osteoclast activity due to disrupted cytoskeleton of the osteoclasts (35, 36). However, several other studies addressed osteoclast apoptosis after long-term GC exposure (32, 34, 35, 37). Some studies showed that GCs reduce osteoclast apoptosis (34, 35), while others reported that GCs do not affect osteoclast apoptosis at all (32, 37). Collectively, pharmacological GCs affect osteoclastogenesis and bone resorption either directly, or *via* increased RANKL secretion from osteoblasts/osteocytes.

GCs IN SKELETAL STEM CELLS

Skeletal stem cells are essential for bone development, growth, and maintenance (38). During the last decade, skeletal stem cell markers have been identified in humans and rodents (38–41). To date, however, the role of GCs in these cells has not yet been extensively explored. Earlier, a study demonstrated that GR deletion in mesenchymal tissues using Dermo1-Cre induces postnatal lethality due to defects in the lung and intestines (42). GR silencing on human BMSCs showed an inhibited osteogenic differentiation *in vitro* (43). These studies imply that GC signaling *via* the GR plays a key role in mesenchymal stem cells (MSCs) differentiation towards osteoblasts.

It is also known that GIO is clinically described by decreased bone mass along with increased marrow adiposity (24), indicating that GR regulates the balance between osteoblastogenesis and adipogenesis of MSCs (44). High GC doses (1 μM Dexamethasone) increased adipogenesis of human BMSCs regulated by c-Jun signaling (43). Other studies suggested that GCs induce adipogenic regulators. Adipogenesis was promoted in cortisol (1 μM) treated mouse bone marrow-derived stromal cell line ST-2, by increasing expression of Peroxisome proliferator-activated receptor-gamma2 (PPAR-γ2) and CCAAT/enhancer-binding protein (C/EBP) transcription factors that are the adipocyte master regulator (45). Similarly, C/EBPalpha expression was increased in bone of dexamethasone-treated mice (50 mg/kg daily for 5 weeks) as well as in primary BMSCs isolated from those mice (46). Dexamethasone treatment in rat BMSCs also increased PPAR-γ expression in a dose-dependent manner, whereas a PPAR-γ knockdown promoted osteogenesis (47). This GC-induced PPAR-γ expression increases Secreted frizzled-related protein 5 (SFRP5) expression which inhibits the Wnt/β-catenin pathway and thus suppresses osteogenesis (47).

Taken together, endogenous GCs promote osteoblastogenesis of MSCs, whereas exogenous or excessive GCs regulate the balance between osteoblastogenesis and adipogenesis of MSCs. Further studies are necessary to investigate the role of GCs in the fate decision of skeletal stem cells *in vivo*.

CIRCADIAN RHYTHMICITY AND GCs

Endogenous GCs are released under the control of circadian rhythms, that are modulated by the central circadian clock in the suprachiasmatic nucleus (SCN) of the hypothalamus (48). The daily rhythmicity of plasma GC levels modulates physiological processes in many peripheral tissues including bone (48, 49).

Indeed, diurnal rhythm appears in some bone metabolic markers such as the bone resorption marker C-terminal cross-linked telopeptide of type I collagen (CTX), osteocyte function marker fibroblast growth factor 23 (FGF23), and turnover marker serum osteocalcin (50–53). Other bone markers such as sclerostin, procollagen type 1 N-terminal propeptide (P1NP), OPG, or soluble RANKL serum levels did not display rhythmicity (50, 52). However, the 24-hour serum profiles of men displayed that bone formation marker P1NP was significantly reduced after a long-term (3 weeks) disruption of the circadian rhythm despite no alteration of CTX level (54). In mice, disrupted circadian rhythm by weekly alternating light-dark cycles (10 or 15 weeks) led to a reduced level of both P1NP and CTX, implicating a decreased bone turnover due to disrupted circadian rhythm (55). This is likely due to the altered expression level of circadian locomotor output cycles kaput (*Clock*) genes that regulate the circadian rhythm in bone cells (55). Unexpectedly, unlike with the P1NP level, osteoblast surface increased in these mice (55). Together with decreased osteoclast surface, trabecular bone mass was increased in these mice despite altered *Clock* gene expression in the bone due to disrupted circadian rhythm (55). Nevertheless, this study indicated the importance of circadian rhythm in bone health. Investigations considering different ages and duration of circadian rhythm disruption would provide further insights into the effects of circadian rhythm in bone.

Furthermore, genetic deletion of *Clock* genes in mice leads to altered bone phenotypes (56–61). Under normal physiological conditions, brain and muscle aryl hydrocarbon receptor nuclear translocator-like protein 1 (*Bmal1*) and period 1 (*Per1*) genes are expressed with oscillatory rhythmicity in bone (58, 61). *Bmal1* knock-out mice and mice with *Bmal1* deletion in Osx+ osteoblast precursors and their progeny showed a decreased bone mass with increased bone resorption, suggesting that *Bmal1* regulates bone homeostasis by controlling osteoblast-mediated bone resorption (58). An osteoclast-specific *Bmal1* knock-out mouse showed an increased bone mass due to reduced osteoclast differentiation, indicating *Bmal1* also regulates osteoclast-mediated bone resorption (57). The *Clock* gene that forms heterodimers with *Bmal1* or *Bmal2* regulates bone formation *via* protein disulfide isomerase family A member 3 (*Pdia3*), shown by reduced bone formation and increased apoptosis of osteoblasts in *Clock* knock-out mice (56). On the other hand, physical stress-induced GC signaling induces only the *Per1* gene in mouse liver, heart, lung, and stomach by binding the GR to the

GRE in the *Per1* promoter (62). However, it is not yet known if the GR directly binds to *Bmal1*, *Clock*, and *Per1* promoters to modulate their actions in bone cells.

Conversely, a single injection of synthetic corticosteroids can reset the circadian time in the periphery such as the liver, kidney, and heart by modulating circadian gene expression (63–65). Short-term dexamethasone treatment (2 hours) synchronizes circadian gene expression in osteoblast and osteoclasts *in vitro* (66, 67). Upon GC treatment, this circadian rhythm was also observed in cultured osteoblasts of Per1::luciferase transgenic mice (58). A single injection of dexamethasone could restore the circadian rhythm of osteoclast-related genes such as cathepsin K (*Ctsk*) in adrenalectomized mice (66). *Per2* knock-out mice could not restore the GC-induced bone loss despite a bisphosphonate (Zoledronic acid) treatment, although *Per2* knock-out osteoblasts showed an increased proliferation capacity (68).

However, constant GC exposure by inserting slow-release corticosterone pellets led to a shutdown of the endogenous HPA axis due to negative feedback, and thus to a flattening of GC-mediated circadian rhythm mediated gene expression (69). This resulted in bone loss not only by the excessive effects of GCs but also due to disrupted circadian gene expression, increased circulating bone resorption marker, and decreased bone formation (69).

Taken together, daily endogenous GC rhythm is important for bone homeostasis. A single treatment with exogenous GCs can regulate circadian gene expression, whereas disrupted circadian rhythm by continuous GC exposure contributes in addition to direct GC effects on osteoporosis.

INFLUENCE OF GCs ON BONE FRACTURE HEALING

It is well known that patients undergoing long-term GC medication are at a significantly increased risk for bone fractures (23, 70). Even though steroid use has not been found to be a major risk factor for non-union fracture healing in clinical studies (71), preclinical studies indicate that GCs also influence the complex fracture healing process (6, 72). This applies not only to GC therapy but also to endogenous GCs which control many physiological processes and, as stress hormones, are released upon a bone fracture. It can be anticipated that endogenous as well and exogenous or excessive GCs influence all stages of bone fracture healing, which necessitates a finely tuned interaction between multiple cell types, including immune, bone, and stromal cells which are all crucially regulated by GCs (6, 72). A fracture leads to the disruption of bone, blood vessels, soft tissues, and the release of danger-associated molecular patterns (DAMPS). These quickly trigger an innate immune response to contain the damage, and clear the wound site from tissue debris and pathogens (73–75). The initial response involves the activation of the complement system, the release of inflammatory chemokines and cytokines from local immune, endothelial and mesenchymal cells, as well as the recruitment and activation of further immune cells, mainly neutrophils, monocytes, and macrophages. Later, lymphocytes are also recruited to the fracture site and initiate an adaptive immune response. The inflammatory phase is regarded to promote the recruitment, proliferation, and differentiation of mesenchymal and endothelial precursor cells, which are essential for subsequent healing processes. This process comprises of the formation of a soft callus with fibrous and cartilaginous tissue, which is then continuously transformed into the bone by endochondral ossification. Finally, the hard callus is remodeled until the original bone structure is restored (73–75).

So far, only a few studies have addressed the role of endogenous GCs during fracture healing by using mouse models with impaired GC signaling (76–79). Fracture healing was significantly impaired when the endogenous GC action was globally eliminated by using mice with an inducible GR knock-out ($GR^{gtROSACreERT2}$) (78). In these mice, the early systemic and local immune responses upon fracture were significantly increased. During callus formation, cartilage-to-bone transformation was disturbed, confirmed by persisting cartilage and reduced bony bridging of the fragments in $GR^{gtROSACreERT2}$ mice. This study suggests a crucial role of endogenous GCs in all stages of fracture healing. Several studies showed the role of GC signaling in distinct cell types during bone regeneration. When GC signaling was disrupted in osteoblasts using Col2.3-11ß-HSD2 mice (76), intramembranous bone formation was not affected, whereas GR deletion in chondroblasts using $GR^{Col2CreERT2}$ mice resulted in impaired endochondral bone healing, by increasing the cartilaginous fraction of the fracture callus (77). To investigate whether GR dimerization (which is regarded to be essential for the anti-inflammatory effects of GCs) is important for fracture healing, Hachemi et al used mice with a defective GR dimerization ability (GR^{dim}) (79). Impaired GR dimerization had no significant effect on the healing process in a model of isolated femur fracture (79). However, in a model of compromised fracture healing, induced by hyperinflammation in a combined model of fracture and thoracic trauma, impaired GR dimerization in GR^{dim} mice reduced inflammation and abolished the deleterious effects of posttraumatic hyperinflammation on fracture healing (79). In summary, these studies demonstrate that endogenous GCs promote fracture healing by controlling the immune response and by stimulating cartilage-to-bone transition.

In contrast to endogenous GCs, exogenously applied GCs can provoke negative effects on the fracture healing process as demonstrated in pre-clinical investigations in different species, including rabbits (80, 81), rats (82), and mice (83, 84). Consistently, these studies report impaired cartilage-to-bone transformation, reduced quality and structure of the newly formed bone, and poor biomechanical properties of the fracture callus. However, these studies are mostly descriptive and the molecular and cellular reasons for the delayed bone healing under long-term GC therapy are still not fully understood.

GCs ACTION IN RHEUMATOID ARTHRITIS

Although GCs are used to ameliorate the symptoms of rheumatoid arthritis (RA) since the 1950s, there are still surprises concerning the

mode of action of GCs, their activating enzymes 11β-HSD1 and the GR requirement in distinct cell types. In RA and osteoarthritis, GCs are still in frequent use, in combination with other treatment regimens (85). Preclinical animal models for the GC modulating enzyme 11β-HSD1/2 and the GR in distinct cell types revealed distinct requirements of GC function in different cells depending on the model. First of all, the attenuation of complete GR dimerization by a knock-in of a point mutation into the second zinc finger demonstrates that an intact function of the GR allows gene regulation beyond the suppression of cytokines in different RA models (86, 87). Accordingly, global inhibition of the GC activating 11β-HSD1 abrogated the therapeutic response towards corticosterone by reduction of inflammatory symptoms in mice (88).

However, the definition of critical cell types for mediating GC action present in RA varied in distinct animal models. In antigen-induced arthritis, GR in T cells (presumably in Th17 cells) was critical to confer anti-inflammatory effects, since mice lacking the GR in T cells were completely resistant to the dexamethasone-mediated reduction of joint swelling (86). In serum transfer-induced arthritis, however, there was the surprising discovery that global GR deletion in hematopoietic cells by hematopoietic stem cell transfer into irradiated wild-type mice did not abrogate the therapeutic effects of dexamethasone (87). Vice versa GR global knock-out mice and mice with attenuated GR dimerization failed to respond to dexamethasone, even when their hematopoietic system was reconstituted by GR wild-type cells (87). These mice could not induce anti-inflammatory macrophages in the joint which are critical to resolve inflammation in RA (87). Elimination of the GR in fibroblasts (Col1a2CreERT2) attenuated the therapeutic response, strongly suggesting that GCs affect the fibroblast like-synovial cell (FLS) – macrophage crosstalk *via* the GR (87). Intriguingly, Hardy and colleagues showed that GC production in myeloid cells might be necessary for re-activating GC function (88). Thus, cellular cross-talk targeted by systemic and locally produced GCs seems to underly the therapeutic actions of GCs, which need to be further elucidated. Given that FLS exists in pro-inflammatory and anti-inflammatory subsets (89, 90) and interstitial/lining macrophages are existing with different fates in arthritis (91), this raises the complexity and fine-tuning of GR cross-talk.

CONCLUSIONS AND PERSPECTIVES

GCs are frequently used drugs in clinics despite their detrimental effects on bone after long-term use. They act in cell-type specific manner, and *via* cellular crosstalk mechanisms, which are still partially unknown. Currently, some drugs are applied to treat GIO by either inhibiting osteoclast activity (Bisphosphonates and Denosumab) or stimulating osteoblast activity (Teriparatide) (92). However, the utilization of drugs to treat unwanted effects caused by other drugs is not ideal for patients. Thus, it is of utmost importance to develop new therapies with a cell-type specific delivery of GCs, and/or targeting downstream molecules to avoid or minimize the detrimental effects. Further understanding of the controversial effects of endogenous and exogenous/excessive GCs on the bone that are both anabolic and catabolic will help to develop therapeutic concepts (**Figures 1A, B**). Daily GC rhythm should be considered during GC therapy. Chronotherapy when administering GCs could help to increase therapeutic efficacy, and reduce detrimental effects, although further investigations are required considering that the drug half-life and bioavailability can be inflexible (93). In addition, preclinical models considering factors such as physical stress, aging, and diseases can be introduced to investigate diverse clinical settings. Advanced technologies such as single-cell RNA sequencing and lineage-

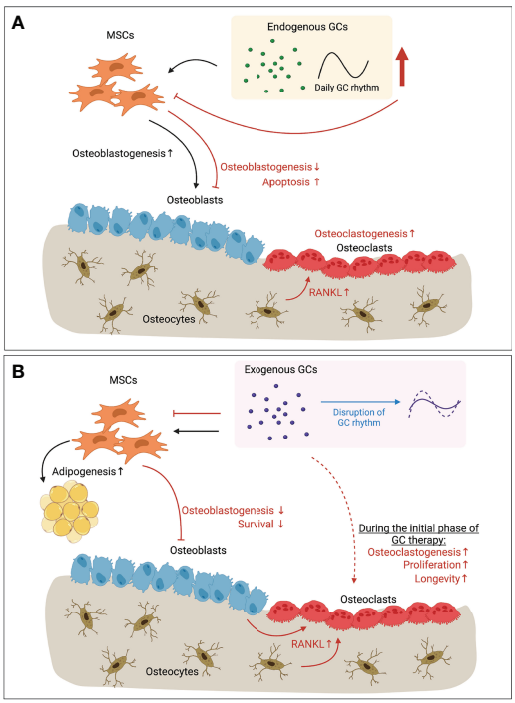

FIGURE 1 | Paradoxical effects of GCs in bone. **(A)** Endogenous GCs regulated by circadian rhythm (and expressing daily GC rhythm accordingly) have anabolic effects on osteoblastogenesis (black arrows). When endogenous GC level is increased upon stress (e.g. mechanical unloading), however, bone mass is decreased due to inhibited osteoblastogenesis, increased apoptosis of osteoblasts and osteocytes, and enhanced osteoclastogenesis due to the increased RANKL secreted by apoptotic osteocytes (red arrows). **(B)** Long-term exogenous GC therapy inhibits osteoblastogenesis and survival of osteoblasts (red arrows). Increased RANKL secretion by osteoblasts and osteocytes let enhance bone resorption by osteoclasts (red arrows). Direct action of exogenous GCs on osteoclasts has showed with increased osteoclastogenesis, increased proliferation and longevity of osteoclasts during the initial phase of GC therapy (dotted red arrow). However, direct effects of long-term GC therapy on osteoclasts still remain elusive. Exogenous GCs also regulate the balance between osteoblastogenesis and adipogenesis of MSCs that is one of feature of GIO (black arrows). On the other hand, continuous exogenous GC therapy can flatten the endogenous GCs rhythm (blue arrow), resulting in disrupted circadian gene expression and levels of circulating bone turnover markers. Together, Long-term GC therapy leads to bone loss by its direct action on bone cells, and/or via disrupting GC rhythm. GC, Glucocorticoid; RANKL, Receptor activator of nuclear factor-κB ligand; MSC, Mesenchymal stem cell; GIO, GC-induced osteoporosis. This illustration was created with BioRender.com.

tracing animal models will allow us to map the alteration of specific cell types present in bone in response to GCs. It will be also helpful to determine dynamic spatial profile and crosstalk among bone cells in clinically relevant models such as fracture healing and RA. Further studies are needed to understand how GC rhythm affects such disease models. These actions in combination will ultimately broaden our scope to approach innovative therapies.

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The Impact of High Dose Glucocorticoids on Bone Health and Fracture Risk in Systemic Vasculitides

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Systemic vasculitides are a range of conditions characterized by inflammation of blood vessels which may manifest as single organ or life-threatening multisystem disease. The treatment of systemic vasculitis varies depending on the specific disease but historically has involved initial treatment with high dose glucocorticoids alone or in conjunction with other immunosuppressive agents. Prolonged glucocorticoid treatment is frequently required as maintenance treatment. Patients with small and large vessel vasculitis are at increased risk of fracture. Osteoporosis may occur due to intrinsic factors such as chronic inflammation, impaired renal function and to a large extent due to pharmacological therapy with high dose glucocorticoid or combination treatments. This review will outline the known mechanism of bone loss in vasculitis and will summarize factors attributing to fracture risk in different types of vasculitis. Osteoporosis treatment with specific consideration for patients with vasculitis will be discussed. The use of glucocorticoid sparing immunosuppressive agents in the treatment of systemic vasculitis is a significant area of ongoing research. Adjunctive treatments are used to reduce cumulative doses of glucocorticoids and therefore may significantly decrease the associated fracture risk in patients with vasculitis. Lastly, we will highlight the many unknowns in the relation between systemic vasculitis, its treatment and bone health and will outline key research priorities for this field.

Keywords: vasculitis, osteoporosis, glucococorticoids, bone, fracture risk, fractures, large vessel vasculitis, AAV

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INTRODUCTION

Systemic vasculitides frequently present as acute inflammation of various sized blood vessels which can lead to stenosis and aneurysm of the aorta and its branches in large vessel vasculitis (LVV) or necrosis of arterioles, capillaries and venules in small vessel vasculitis (SVV). Untreated large and small vessel vasculitis can lead to rapid organ damage and consequent threat to life. Hence many conditions require strong immunosuppression most commonly with a prolonged course of high dose Glucocorticoids (GC). Long-term sequelae are frequently a result of acute and chronic inflammation, failure to suppress

inflammatory activity or secondary to immunosuppression, in particular GC (1, 2). Osteoporosis and increased fracture risk are known comorbidities of prolonged and high cumulative GC doses (3, 4). It is unclear how much the disease process and the inflammation itself contribute to accelerated bone loss or if the increased fracture risk is mainly a result of the negative impact of GC on bone health and muscle strength. This narrative review will explore the mechanism for rapid bone loss and increased fracture risk in vasculitis, summarize current fracture data in various vasculitis subgroups and outline recent developments which can prevent or mitigate this issue.

MECHANISM OF BONE LOSS AND INCREASED FRACTURE RISK IN VASCULITIS

Bone undergoes continuous remodeling and restructuring to maintain its strength and function. In healthy individuals, a precisely coordinated process of bone resorption through osteoclasts and bone formation by osteoblasts allows the repair of damaged bone and replacement of old bone with newly formed mineralized osteoid. Disruption of this remodeling cycle and an increase in bone resorption and/or suppression of bone forming activity leads to systemic bone loss and osteoporosis (5). The most important factors influencing bone turnover in systemic vasculitis are shown in **Figure 1** and discussed in detail below.

Chronic Inflammation in Vasculitis

In large and small vessel vasculitis the inflammation of vessels is frequently widespread with multisystem involvement and patients usually present with signs of pronounced systemic inflammation (1, 6). The impact of acute or chronic vasculitis on bone physiology is poorly studied. Most data about the interplay between inflammation and bone derives from more common chronic inflammatory conditions such as rheumatoid arthritis (7), spondyloarthritides (8), or connective tissue diseases such as systemic lupus erythematosus (SLE) (9, 10).

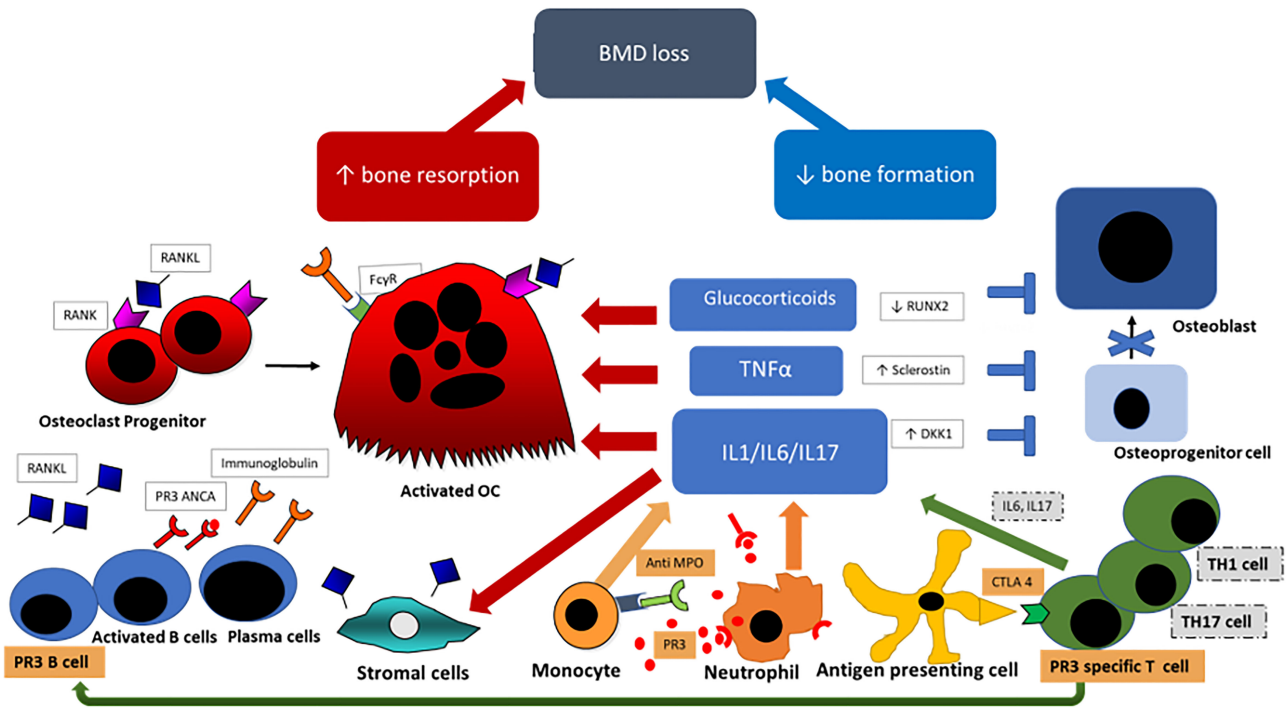

FIGURE 1 | Pathogenesis of bone loss in vasculitis; Anti-neutrophil cytoplasmic antibody (ANCA)-associated vasculitis specific cells and antibodies are highlighted in orange. Primed neutrophils express PR3 [proteinase 3] or MPO [myeloperoxidase] which bind ANCAs and trigger further neutrophil activation and through CD4+ T-lymphocytes stimulation further ANCA production by B-lymphocytes. Key cells and cytokines in the pathogenesis of large vessel vasculitis {LVV} are highlighted in gray. Dendritic cells in the adventitia trigger the inflammatory cascade by activation of T-lymphocytes, predominantly T helper 1 (Th1) and Th17 cells, and express interferon and IL17. Primed neutrophils and Th cells promote proinflammatory cytokine production (Interleukin-6 (IL6), IL1 and Tumour Necrosis Factor (TNF)-alpha) which stimulates osteoclastogenesis through increased RANKL production by stromal cells and through direct osteoclast stimulation. Inflammatory cytokines also inhibit the formation of osteoblasts by increased DKK1 and Sclerostin expression. Glucocorticoids suppress osteoblastogenesis by RUNX2 suppresion and stimulates osteoclast proliferation and longevity. BMD, bond mineral density; RANK4, receptor activator of nuclear factor kappa-B (ligand); PR3, proteinase 3; ANCA, anti-neutrophil cytoplasmic antibody; FcγR, Fc gamma receptor; OC, osteoclast; TNFα, tumuor necrosis factor alpha; IL, interleukin; MPO, myeloperoxidase; RUNX2, runt-related transcription factor 2; DKK1, Dickkopf WNT Signaling Pathway Inhibitor 1; CTLA 4, cytotoxic T-lymphocytes antigen 4; TH1/TH17, T-helper type 1/type 17 cell.

Inflammatory arthritides and vasculitides have a number of common pathways leading to chronic inflammation with key inflammatory cytokines and cells, supported by the fact that these conditions frequently share some immunosuppressive therapies (11–14). However vasculitides in particular anti-neutrophil cytoplasmic antibody (ANCA)-associated vasculitis (AAV) frequently present with an acute systemic inflammation which can affect multiple organs including kidney, lungs and peripheral nerves, and requires rapid potent immunosuppression including high dose GC in order to prevent severe organ damage and death (15). In contrast, inflammatory arthritides frequently present in an insidious way with polyarthritis as the main manifestation which can be treated initially with mild to moderate immunosuppression and if necessary with subsequent escalation of therapy (16).

A) ANCA Associated Vasculitis

Microscopic polyangiitis (MPA) and granulomatosis with polyangiitis (GPA) are ANCA associated vasculitides. AAV are characterized by small-to-medium size blood vessel inflammation and the presence of circulating ANCA antibodies which recognize proteinase 3 (PR3) or myeloperoxidase (MPO). Most GPA patients have ANCA with a cytoplasmic pattern (c-ANCA) that are specific for PR3 whereas in MPA patients ANCA with a perinuclear pattern (p-ANCA) with MPO specificity are frequently found. In AAV, an initial trigger such as infection causes T helper cells to stimulate macrophages, in turn activating neutrophils and leading to formation of neutrophil extracellular traps (NETs) (17–19). The complement system and altered T-lymphocyte homeostasis lead to priming of neutrophils (18, 20, 21). NET degradation is impaired, causing prolonged exposure to NET contents which disrupts tolerance to antigens including PR3 and MPO, leading to ANCA production (19). PR3 and MPO may be expressed on primed neutrophils which bind ANCAs and trigger further excessive neutrophil activation, and both neutrophils and CD4+ T-lymphocytes stimulate further ANCA production by B-lymphocytes, setting up a vicious cycle resulting in proinflammatory cytokine production [Interleukin-6 (IL6), IL8 and Tumour Necrosis Factor (TNF)-alpha (1, 22)] and endothelial damage *via* reactive oxygen species, lytic enzymes and NET components such as histones and matrix metalloproteinases (MMPs) (12, 19, 23–26). The pathogenicity of various immune complexes including PR3 ANCA can be modulated by posttranslational modifications such as glycosylation of immunoglobulins. Genetic associations support a predisposition to AAV or to disease relapse. Examples include patients more commonly expressing specific human leucocyte antigen (HLA) polymorphisms such as HLA-DPB4 or less commonly expressing functional immunoregulatory T-cell receptors such as the cytotoxic T lymphocyte antigen 4 (CTLA) and program death 1 (PD1) (27–29).

B) Large Vessel Vasculitis

LVV is characterized by inflammation of the artery wall with predominant CD4+ T-lymphocytes and macrophages which can undergo granulomatous organization in the form of giant cells. In LVV activated dendritic cells in the adventitia can trigger an inflammatory cascade with activation of T-lymphocytes, predominantly T helper 1 (Th1) and Th17 cells, and express interferon and IL17 (30). Dendritic cells drive the inflammatory process and IL1, IL6 and IL21 are highly expressed in giant cell arteritis (GCA) (31, 32).

Chronic Inflammation and Bone Turnover

Proinflammatory cytokines and their interaction with T- and B-cells propagate chronic inflammation which in turn promotes the differentiation of myeloid cells into macrophages and osteoclasts. The differentiation from multinucleated precursor cells into mature bone resorbing osteoclasts requires the interaction of two crucial cytokines: Macrophage colony-stimulating factor (M-CSF) and Receptor activator of nuclear factor kappa-B ligand (RANKL) (33). Osteoprotegerin (OPG) is a decoy receptor to RANKL and an important regulator of osteoclastogenesis. Mechanisms such as binding of anti-MPO to monocytes or phagocytosis of PR3 expressing neutrophils stimulate the release of inflammatory cytokines including IL1β, IL6, IL8 and TNFα (22, 34). Pro-inflammatory cytokines, particularly IL6 and TNFα, have also been shown to suppress bone formation. Overexpression of TNFα can inhibit osteoblast differentiation either directly through inhibition of Runt-related transcription factor 2 (Runx2) or *via* increased Dickkopf 1 expression which is an important regulator of the Wnt pathway (35–37).

A) Large Vessel Vasculitis- Inflammatory Cytokines

The crucial importance of IL6 in the pathogenesis of LVV was confirmed by the success of the introduction of IL6–inhibitors as corticosteroid sparing agents (7, 38). Inflammatory cytokines such as IL1, IL6, IL17 and TNFα can upregulate RANKL production by osteoblasts, T-cells and stromal cells and promote differentiation of osteoclast precursor cells (39) or stimulate osteoclast activity by RANKL independent mechanisms (40, 41). Murine and *in vitro* models have also demonstrated IL6 mediated suppression of osteoblast differentiation which can have a direct impact on skeletal development (42, 43).

B) ANCA Associated Vasculitis - the Role of B cells

The clinical success of B-cell depletion in AAV in suppressing disease activity and assuring long term remission provides strong evidence for the important role of B-cells in AAV pathophysiology (44–46).

B cell and bone cell development are closely interlinked.

Stromal cell derived cytokines including RANKL, Osteoprotegerin (OPG) and IL7 are important regulators of osteoclast maturation and differentiation and are also important factors for the development of B cells (47). In murine studies RANK knock out not only resulted in an increased bone mass phenotype (osteopetrosis) but also in impaired lymphocyte development (48).

B cells also produce cytokines which regulate bone cells, in particular RANKL which promotes osteoclastogenesis. Ovariectomy in mice not only causes bone loss through estrogen deficiency and osteoclastic bone resorption but also

due to proliferation of RANKL expressing B cells leading to further acceleration of bone resorption (49). In ovariectomized mice lacking B-cells bone loss is attenuated (50).

In particular, activated B cells in the context of chronic inflammation promote bone loss through increased RANKL production and other inflammatory cytokines that promote bone resorption. In addition B cells and in particular plasma cells may influence bone homeostasis through the production of immunoglobulins. In Rheumatoid Arthritis for example immunoglobulins have been shown to directly interact with bone cells, specifically with osteoclasts (51, 52), either *via* the Fcγ receptor on the osteoclast surface (51, 53) or indirectly through blocking OPG (52, 54).

B cell depletion therapy therefore may have a beneficial impact on bone and may prevent accelerated bone loss in chronic inflammatory conditions. To date only a small study of 45 patients with RA who received B cell inhibitor treatment (Rituximab) was performed. After one year of treatment no substantial improvement in BMD was found compared to baseline bone density (55). However this study was likely underpowered and the time frame was too short to detect a significant BMD change. Further studies and particular clinical trials are required to establish the impact of B cell depletion on bone.

Glucocorticoid Induced Osteoporosis (GIOP) Pathophysiology

GC remain a cornerstone of treatment for most vasculitides and the mainstay of treatment in LVV (56, 57).

The impact of corticosteroids on bone turnover is complex; the most profound effect seems to be on bone formation. Weinstein et al. (58) have shown that chronic GC treatment in mice decreases proliferation of osteoblast precursors and stimulates osteoblast and osteocyte apoptosis, which together leads to a reduction of bone formation. These findings were confirmed on biopsies of patients with GIOP (59). Long-term GC exposure increases expression of the transcription factor peroxisome proliferator-activated receptor (PPAR)γ2 which promotes the differentiation of mesenchymal cells to adipocytes as opposed to osteoblasts. At the same time Runx2, a pivotal transcription factor for osteoblastogenesis, is repressed by GC. GC treatment also has a significant impact on bone resorption. Corticosteroids suppress OPG production (60) which leads to an increase in RANKL/OPG ratio and subsequent stimulation of osteoclast proliferation (59, 60). GC also prolong the lifespan of osteoclasts, further contributing to the imbalance of bone formation and resorption in favour of resorption and hence to net bone loss (58, 61). Therefore, long-term corticosteroid use leads to bone loss and fatty transformation of bone marrow (59, 62, 63).

Extra-skeletal actions of GC on organs such as muscles, kidney and the endocrine system contribute to accelerated bone loss and increased fracture risk. GC decrease calcium absorption in the gastrointestinal tract (64) and decrease the production of sex steroids such as Luteinising hormone (LH), Follicle stimulating hormone (FSH) or Testosterone and Growth hormone (GH) that puts a halt on bone turnover (65). Steroid associated muscle loss (sarcopenia) leads to reduced skeletal loading and postural instability, which is an important risk factor for falls (66).

Other Medications

Parenteral or oral Cyclophosphamide is frequently used in organ- or life-threatening vasculitis (67, 68). The use of Cyclophosphamide is associated with a number of potential serious side effects including premature ovarian failure characterized by a sharp drop of oestrogens causing early menopause and accelerated bone loss (69). Recently Miyano et al, (70) showed that in an AAV group who sustained fractures, Proton Pump Inhibitor (PPI) users had a higher risk of fractures than histamine-3 receptor antagonist users. Of interest, Abtahi et al. (71) demonstrated in a cohort of patients with rheumatoid arthritis a synergistic effect of GC and PPI in increasing fracture risk. These findings may be of particular importance in patients with GCA and LVV who at disease onset are frequently treated with a combination of high dose GC and PPI.

Organ Involvement

Acute and chronic renal failure can occur as a consequence of an acute flare of small to medium sized vessel vasculitis (3). Patients with Chronic Kidney Disease (CKD) are at increased risk of osteoporotic fractures (72–74). The mortality associated to fractures increases with worsening renal function (6) and the risk of hip fracture in a population with End Stage Renal Disease (ESRD) is approximately two to four times higher than in the general population (72, 73). The reasons for disturbed bone metabolism in CKD are manifold. Beside accelerated bone loss causing osteoporosis, additional metabolic disorders such as secondary hyperparathyroidism, phosphate retention, elevated fibroblast growth factor -23 (FGF 23), sclerostin overproduction and chronic metabolic acidosis can have a detrimental impact on bone quality. Metabolic bone disorders can result in renal osteodystrophy, adynamic bone disease, osteitis fibrosa or osteomalacia. Additionally, secondary factors such as vitamin D deficiency may increase fracture risk even further (75, 76)

Peripheral neuropathy is one of the frequent long-term sequelae of AAV. A pooled analysis of multiple therapeutic trials showed that 14% of microscopic polyangiitis (MPA) and 22% of granulomatosis with polyangiitis (GPA) patients were found to have developed peripheral neuropathy in long-term outcomes analysis (3). Peripheral neuropathy can lead to gait disorders and increased falls risk (77) which strongly increases fracture risk (78), likely by bone mineral density (BMD) independent mechanism (79). Visual and hearing loss can occur both in LVV and SVV (3, 80) which again substantially increases falls (81) and subsequent fracture risk (82, 83).

Relative Immobilisation

Clinical manifestations of systemic vasculitis such as mononeuritis multiplex, stroke, blindness or severe arthritis can lead to relative immobility (84–86). A prolonged period of decreased physical activity and chronic inflammation leads to

bone loss in addition to an accumulation of visceral fat and sarcopenia (87–89). Recently sarcopenia, measured by reduced hand grip strength, and associated with the type of vasculitis, severity and high C-reactive protein (CRP), seemed to predict increased fracture risk (90). This is in line with previous studies which have shown that change of body composition in form of muscle loss and addition of visceral fat associated with glucocorticoid use increase the risk of osteoporosis and the risk of sustaining fragility fractures (91, 92).

In summary fracture risk in patients with systemic vasculitides is a composite score of BMD-related and BMD-independent risk factors as shown in **Figure 2**. In order to modify fracture risk many factors, for instance suppression of inflammation, minimizing GC use and avoiding prolonged immobility, should be considered.

OSTEOPOROSIS AND FRACTURE RISK IN DIFFERENT VASCULITIS SUBGROUPS

Giant Cell Arteritis (GCA)/Polymyalgia Rheumatica (PMR)

GCA is the most common primary systemic vasculitis with incidence reported between 1.1 and 43.6 cases per 100,000 in populations aged over 50 years, with significant variation noted geographically (93). PMR is an inflammatory disorder characterized by bilateral upper limb and hip girdle pain and stiffness, with incidence rates of 41 to 112 cases per 100,000 (94–97) among patients over 50 years. GC remain the mainstay of treatment for GCA and PMR. In cohorts of GCA patients, median starting Prednisolone dose was 20-50 mg/day and cumulative doses at 52 weeks were 4000-4800 mg (57). In PMR initial treatment of Prednisolone 15–25 mg is generally followed by a slow taper over 1–2 years (98, 99). Cumulative doses of 3.2 g to 5.4 g are reported (100–102). Treatment beyond 2 years is common, with up to 60% of patients remaining on GC at this point (103).

High rates of osteoporosis are seen in patients with GCA and PMR. Reported prevalence of osteoporosis in GCA varies from 6.25% to as high as 85% (104, 105). The risk of osteoporosis increases over time following diagnosis of GCA and PMR, with the rate of increase highest in the 6 months following diagnosis (105–107). **Table 1** summarises available studies (4, 57, 104, 105, 107–117) on bone health in LVV.

Higher rates of fractures are seen in both GCA and PMR compared to controls (4, 108) with hazard ratio for fracture 1.63 in PMR and 1.67 in GCA compared to controls. Prospective studies of GCA and PMR patients reveal fragility fracture incidence of 11-14% within 1 to 2 years of diagnosis (109, 110). Rates of fracture correlate with increased cumulative GC doses (4). Evidence from claims data suggests that higher cumulative doses of GC lead to higher complications and increased risk of osteoporosis and fracture, with hazard ratio (HR) for bone-related adverse events increasing 5% for every 1 g increase in cumulative dose of Prednisolone-equivalent GC (111). Similar findings have been established in cohort studies for cumulative doses over 10 g or duration over 2 years (112, 118).

There is some evidence that a lower dose of 5 mg Prednisolone daily can lead to reduced BMD (119), but rates

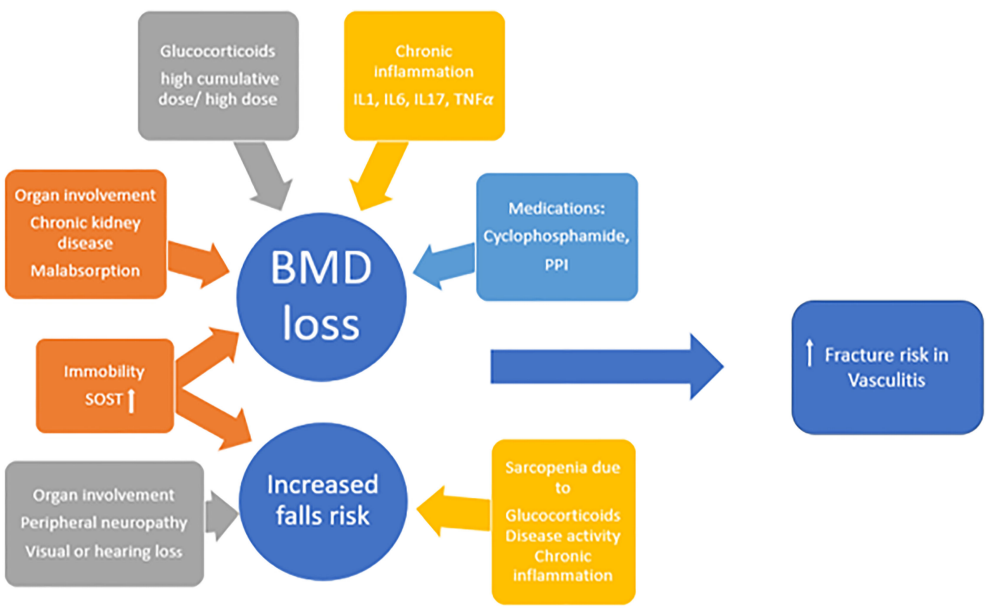

FIGURE 2 | The multifactorial aetiology of increased fracture risk in vasculitides; *IL* interleukin, *TNF* tumour necrosis factor, *PPI* proton pump inhibitor, *SOST* sclerostin, *BMD* bone mineral density.

TABLE 1 | Summary of studies on osteoporosis and fracture risk in Giant Cell Arteritis (GCA) and Polymyalgia Rheumatica (PMR).

First author	Year	Study population	Age	Details	Level of evidence	Outcome measures	Results
Healey (109)	1996	25 GCA or PMR patients in treatment group 23 GCA or PMR patients in placebo group	71.6	RCT of GC-treated GCA or PMR patients receiving calcium, vitamin D and calcitonin, or receiving calcium, vitamin D and placebo	1b	- Change in BMD at lumbar spine after 2 years - New vertebral fractures at 2 years	- Mean change in lumbar BMD -0.1% intervention group), -0.2% (placebo) - Vertebral fracture incidence 11% and 14% - Higher cumulative GC dose associated with greater loss in BMD
Kermani (107)	2017	204 GCA patients	71.3	Prospective cohort of GCA patients	2b	- Damage items as per Vasculitis Damage Index and LVV Index of Damage	- 22 (10.8%) developed osteoporosis
Petri (104)	2015	4671 GCA patients	N/A	Retrospective cohort of GCA patients (n=4671)	2b	- Incidence of GCA - Cumulative GC dose - Comorbidities associated with GCA	- RR 2.9 for developing osteoporosis after diagnosis of GCA
Mohammad (113)	2017	768 GCA patients 3072 controls	76.1	Retrospective cohort of GCA patients	2b	- Occurrence of osteoporosis or fragility fracture	- RR 2.81 for incident osteoporosis - RR 1.56 for incident fracture
Broder (111)	2016	2497 GCA patients	71	Retrospective cohort of GCA patients	2b	- GC-related adverse events including osteoporosis and fragility fracture	- For every 1g increase in cumulative GC dose, HR 1.05 for osteoporosis and 1.04 for fracture - Osteoporosis rate 0.099 events per person year - Fracture rate 0.066 events per person year
Gale (57)	2018	8777 GCA patients	73	Two retrospective cohorts of GCA patients	2b	- GC cumulative dose - GC-related adverse events - Association of adverse event risk with GC use greater than 52 weeks	- OR of osteoporosis for every 1g increase in cumulative GC dose 1.03-1.06 - OR for fracture for every 1g increase in cumulative GC dose 1.02-1.09 - Risk of osteoporosis for every 1g increase in cumulative GC dose 3-3.4% - Risk of fracture for every 1g increase in cumulative GC dose 1-1.9%
Hatz (105)	1992	47 GCA or PMR patients	N/A	Prospective cohort of GCA and PMR patients	2b	- Side effects attributed to GC at 6 months	- 7 (15.0%) developed osteoporosis within 6 months
Andersson (114)	1990	26 GCA patients	78	Retrospective cohort of GCA patients	2b	- BMD at heel X-ray signs of osteoporosis	- 69% of female patients developed severe spinal osteoporosis after 5 years
Mazzantini (115)	2012	222 PMR patients	71	Retrospective cohort of PMR patients treated with low-dose GC	2b	- Fragility fractures - Osteoporosis	- 55 (24.8%) developed osteoporosis - 31 (14.0%) sustained fragility fractures - GC duration and cumulative dose were significantly associated with osteoporosis and fragility fractures
Sokhal (110)	2021	652 PMR patients	72.4	Prospective cohort of PMR patients	2b	- Fragility fractures at 12 and 24 months	- 72 (11.0%) sustained fragility fracture within 12 months of diagnosis - 60 (9.2%) sustained fragility fracture 12-24 months after diagnosis
Mateo (112)	1993	28 GCA patients 28 PMR patients 48 controls	N/A	Case-control study of patients with GCA, PMR and controls	3b	- BMD at lumbar spine and femoral neck	- Age and cumulative GC dose significant predictors of femoral BMD in men - Age and weight, but not cumulative GC dose, were significant predictors of femoral

(Continued)

TABLE 1 | Continued

First author	Year	Study population	Age	Details	Level of evidence	Outcome measures	Results
Wilson (108)	2017	5011 GCA patients 5011 controls	72.9	Retrospective case-control study of GCA patients versus control	3b	- Incidence of osteoporosis or fracture	BMD in women - GCA patients had lower BMD - IRR for osteoporosis 2.4 in GCA patients - IRR for fracture 1.4 in GCA patients
Paskins (4)	2018	2673 GCA patients 12,136 PMR patients 59,236 controls	71.9	Retrospective case-control study of GCA patients PMR patients	3b	- Time to fracture	- Fracture incidence rate per 10,000 person years 148 for PMR and 147 for GCA - HR for fracture 1.63 for PMR and 1.67 for GCA
Wilson (116)	2017	5011 GCA patients	72.9	Nested case-control studies of GC doses in GCA	3b	- Risk of osteoporosis or fracture associated with increasing GC dose	- 511 (10.2%) developed osteoporosis, mean time to developing osteoporosis 3 years - 408 (8.1%) developed fracture, mean time to fracture 3.2 years - Increased risk of osteoporosis with increasing cumulative GC dose
Haugeberg (117)	2000	GCA or PMR patients - 26 currently treated - 28 previously treated - 30 newly diagnosed	71	Cross-sectional study of BMD in currently treated, previously treated and newly diagnosed GCA or PMR patients	3b	- BMD at radius, spine, hip	- No significant difference in BMD between groups

GC, glucocorticoid; BMD, bone mineral density; RCT, randomized controlled trial; IRR, incidence rate ratio; OR, odds ratio; RR, relative risk; LVV, large vessel vasculitis; HR, hazard ratio.

of BMD loss and fracture risk are generally shown to correlate with doses over 10 mg daily of Prednisolone (4, 112).

Few studies have established the risk of osteoporosis attributable to the disease process itself in LVV. Much of the work describing higher rates of osteoporosis in LVV is unable to definitively establish a causative link with GC therapy (120, 121). Rates of osteopenia and osteoporosis are higher in relapsing than newly diagnosed patients with GCA (122), which may relate to higher cumulative doses of GC use but cannot be distinguished from the effect of prolonged inflammation in relapsing cases.

The available data on bone health in LVV typically predate the introduction of the IL6-inhibitor Tocilizumab as a steroid-sparing agent. Adjunctive use of Tocilizumab alongside GC in trials facilitated faster reduction in GC treatment and lower cumulative GC doses in the treatment of GCA (38). Widespread use of Tocilizumab is expected to lead to fewer GC-related adverse events in GCA, including osteoporosis and fractures. However GC alone remains the primary treatment for GCA. BSR and EULAR guidelines recommend Tocilizumab for relapsing patients and those who have already developed, or are at high risk of developing, a complication related to GC (123, 124). The EULAR guideline emphasises that the addition of Tocilizumab must be balanced against the risk of treatment-related adverse effects in comorbid elderly patients. Recent ACR guidance however recommends Tocilizumab plus GC over GC alone for all new patients with GCA (125). As more patients at risk of osteoporosis and fracture are treated with Tocilizumab, the incidence of these outcomes is anticipated to reduce.

ANCA Associated Vasculitis

AAV is a necrotizing vasculitis that predominantly affects small vessels and is associated with ANCA specific for MPO and/or PR3. AAV mostly present as systemic disease affecting multiple organs. The main clinicopathologic subgroups of AAV are microscopic polyangitis (MPA), granulomatosis with polyangiitis (GPA) and eosinophilic granulomatosis with polyangiitis (EGPA). Although these AAV variants are distinct entities, the clinical manifestations can be overlapping and available data on bone health and fracture risk mostly refers to a pooled AAV group (2, 126). A cross-sectional study (127) showed that amongst 99 AAV patients with an average age of 55 years, 57% had osteopenia and 21% had osteoporosis. Over two thirds (69%) of patients were treated with prolonged high dose GC with an average cumulative dose of 10.7 g. The cumulative GC dose was inversely related to Z-score of lumbar spine and proximal femur confirming the link between high cumulative GC dose and systemic bone loss. In addition to the negative impact of GC on BMD other factors were identified as potential contributors such as low dietary calcium intake and previous cyclophosphamide treatment. This study however was performed almost 20 years ago when the availability, knowledge and use of GC sparing therapies and osteoporosis treatments such as bisphosphonates was scarce.

A population based cohort study from Southern Sweden (128) found that osteoporosis was 4 times more commonly diagnosed in patients with AAV when compared to an age and sex matched general population control cohort (rate ratio 4.6,

95% CI 3.0-7.0). Two long-term follow up studies in SVV including AAV demonstrated that osteoporosis was one of the most commonly reported comorbidities affecting 14-16% of patients when followed up over 7 to 8 years (129). A recent study compared the bone mineral density of 35 treatment naïve AAV patients with 35 healthy, age and sex matched controls. The diagnosis with AAV was associated with osteopenia however when adjusting for other variables such as BMI the association was lost (130).The bone health in newly diagnosed treatment naïve AAV patients is however an interesting question and larger scale studies could provide valuable information on baseline bone status and fracture risk.

Fractures are more common in AAV patients than the general population, with one case control study of 543 AAV patients having twice the risk of hip fracture compared to age and sex matched controls (131). In a retrospective cohort of 22,821 AAV patients, Miyano et al. reported 0.6% developed fractures following diagnosis, with a median time to fracture of 52 days (70). In two further retrospective cohorts of 246 AAV patients 11/246 (4.5%) and 24/278 (8.6%) developed fractures following diagnosis (132, 133), whilst in a cohort of 83 AAV patients aged 65 and over, 8 (9.6%) developed fractures (134).

Bone Health in Miscellaneous Vasculitic Disorders

Several other forms of small and medium vessel vasculitis can affect children and/or adults [e.g., Behcet's Disease (BD), Polyarteritis Nodosa (PAN), IgA-associated vasculitis (IgAV)]. These miscellaneous vasculitides are relatively rare, occurring in approximately 1:500,000 people across Europe (135). High doses of GC, often administered to induce remission in the early phases of these rare vasculitides, are highly probable to be detrimental to BMD and fracture risk in affected patients. This is particularly true in these rarer disorders as they often occur in childhood or early adulthood when peak bone mass attainment may not have been achieved.

BD, a multi-system disorder characterized by the presence of recurrent oro-genital inflammation, most commonly occurs between the ages of 20 and 40 years. Typically, it follows a relapsing-remitting course and can affect multiple organ systems. Inflammatory ocular, vascular, neurological or gastro-intestinal disease is associated with a poorer prognosis and usually requires high dose corticosteroid treatment to promptly prevent irreversible end-organ damage. The current literature examining bone health in BD and the impact of corticosteroids is limited. However, two studies have compared BMD between patients with BD and age- and gender-matched healthy controls. Tekin and colleagues investigated differences in BMD and bone turnover markers between 30 patients with BD (mean age 37 years) and 30 healthy controls (mean age 35 years) (136). Lumbar spine and total hip BMD was no different between the two groups and there were no significant differences in markers of bone turnover. Another case-control study by Bicer and colleagues in Turkey compared BMD between patients with BD (n=35) and healthy controls (n=33) (137). This study excluded patients receiving oral corticosteroid therapy and post-menopausal women. Mean age in the BD group was 38 years and in the control group was 40 years. Mean disease duration in the BD group was 6.7 years. Similar to the study by Tekin, BMD was not significantly different between patients with BD and healthy control subjects. The European League Against Rheumatism (EULAR) guidelines for the management of BD advises that if required, high-dose corticosteroids should always be used in combination with concurrent immunosuppressives such as azathioprine, interferonα, or anti-TNFα therapy (138). This ensures that the requirement for long-term high dose corticosteroids in BD is minimized and attenuates the impact of corticosteroids on BMD and fracture risk.

For the management of systemic PAN, the French vasculitis group advise corticosteroid therapy starting at a dose of 1 mg/kg/day of prednisone to a maximum of 60 mg daily (139). There is no agreed or widely accepted reduction strategy and several different regimens are currently being employed worldwide, often for up to 6 or 12 months (140). A prospective study of patients with SVV assessing the long-term outcomes in patients with PAN or MPA identified osteoporosis as one of the three most common sequelae (129). Over a mean follow-up of 98 months, 18% of patients with PAN developed an osteoporotic vertebral fracture compared with 15% of those with MPA highlighting the importance of consideration of bone health in systemic vasculitis.

BONE PRESERVING TREATMENTS IN VASCULITIS: THE ROLE OF STEROID-SPARING THERAPIES

Prevention and management of GIOP is addressed in several guidelines and has been extensively reviewed in other articles and is beyond the scope of this review (141–144). It is worth highlighting that a dual-energy X-ray absorptiometry (DXA) scan for BMD measurement is required in the majority of cases for a fracture risk assessment. As glucocorticoids are particularly associated with osteoporosis of trabecular bone, vertebral fracture assessment (VFA) should be included routinely when DXA scans are performed (145).

Across the spectrum of systemic vasculitis, new, more targeted immunosuppressive and immunomodulatory treatments have been developed to assist with the treatment of systemic vasculitis.

ANCA Associated Vasculitis Treatment

In AAV, steroid-light and steroid-free regimens are beginning to be used with some success (134, 146). Use of the targeted complement 5a inhibitor Avacopan offers promise as a GC substitute in AAV but more work is required (147). Likewise, Mepolizumab, a monoclonal antibody against IL5 has also demonstrated promise as a treatment adjunct to facilitate greater chances of remission and a faster reduction in GC in EGPA (148, 149).

Publication of the GiACTA study heralded a new era for the treatment of GCA (38). The use of an IL6 inhibitor

(Tocilizumab) in GCA has facilitated a significantly more rapid reduction in corticosteroid treatment compared with corticosteroid therapy alone. Significantly the GiACTA trial showed reduced cumulative GC doses by 43.5% and 51.2% in the two arms where Tocilizumab was used alongside GC taper as compared to placebo plus GC taper. Evidence for the glucocorticoid sparing effects of older, more conventional disease modifying immunosuppressants such as methotrexate, azathioprine or mycophenolate mofetil in systemic vasculitis is extremely limited and merits further attention.

CONCLUSION

Osteoporosis and fragility fractures are significant long-term complications in vasculitis and most data is available for GCA. High dose GC are undoubtedly one of the main contributing factors. Other factors may increase fracture risk however further research is required to define the role of inflammation, medications and organ involvement on fracture risk in vasculitides. Expansion of non-corticosteroid options for the treatment of systemic vasculitis offers a great hope that in the future, higher fracture rates and impaired bone health will not be a significant problem for our patients suffering from vasculitis.

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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Bone Mineral Density Assessment by Quantitative Computed Tomography in Glucocorticoid-Treated Boys With Duchenne Muscular Dystrophy: A Linear Mixed-Effects Modeling Approach

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Objective: Boys with Duchenne muscular dystrophy (DMD) are at risk of bone damage and low bone mineral density (BMD). The aim of the study is to examine lumbar BMD values measured by QCT and identify the factors associated with BMD loss using a multilevel mixed-effects model.

Methods: Lumbar BMD was evaluated by quantitative computed tomography (QCT) at diagnosis, 1 and 2 years follow up in patients with DMD who were treated with GC. Demographic data, functional activity scores (FMSs), laboratory parameters and steroid use were recorded. A multilevel mixed-effects model was used to analyze BMD loss.

Results: Nineteen patients with DMD who had a total of sixty complete records between January 2018 and October 2021 were retrospectively analyzed. At baseline, 15.8% of patients (3/19) had low lumbar BMD (Z score ≤ –2), and the mean BMD Z score on QCT was -0.85 (SD 1.32). The mean BMD Z score at 1 and 2 years postbaseline decreased to -1.56 (SD 1.62) and -2.02 (SD 1.36), respectively. In our model, BMD Z score loss was associated with age (β=-0.358, p=0.0003) and FMS (β=-0.454, p=0.031). Cumulative GC exposure and serum levels of calcium, phosphorus, 25(OH)-vitamin D and creatinine kinase did not independently predict BMD loss.

Conclusions: This study demonstrates that in DMD patients, lumbar BMD decreased gradually and progressively. Age and FMS are the main contributors to BMD loss in boys with DMD. Early recognition of risk factors associated with BMD loss may facilitate the development of strategies to optimize bone health.

Keywords: Duchenne muscular dystrophy, osteoporosis, bone mineral density, glucocorticoids, quantitative computed tomography

INTRODUCTION

Duchenne muscular dystrophy (DMD) is an X-linked disorder that is associated with progressive muscle wasting and weakness, occurring in 1 of 3000-5000 live male births (1, 2). Boys with DMD usually present symptoms before six years of age, lose independent ambulation during the teenage years and are life-limited by the third decade of life, usually due to cardiorespiratory compromise (3). Low bone mineral density (BMD) is a common feature in patients with DMD and is associated with poor clinical outcomes and quality of life. Osteoporotic fractures may occur during normal activities of daily living in these patients, with a reported incidence of 21-44% (4, 5).

Although recent DMD care guidelines recommend serial spine radiographs to assess changes in spine morphology to monitor and diagnosis osteoporosis (6), BMD values still play a critical role in determining the overall trajectory of bone health, regardless of whether a patient has bone fragility. Baseline and annual dual-energy X-ray absorptiometry (DXA) scans for DMD patients have also been suggested (6). However, there are limitations of DXA in evaluating BMD that should be noted. Several studies have shown that spine deformities or anatomical changes can cause inaccuracies in BMD measurements made with DXA (7–9). Therefore, BMD Z scores adjusted for age-matched, height, bone age or bone size have been used to more accurately estimate the actual BMD and evaluate bone health in boys with DMD (10–14). In contrast, quantitative computed tomography (QCT) is not subject to these limitations because it is able to correct the scoliosis curves and directly measure the true volumetric BMD at lumbar trabecular bone. In addition, trabecular bone tends to be more metabolically active than cortical bone and responds quickly to treatment (15). To our knowledge, only one study about QCT-based BMD data in boys with DMD was published in 2020, and it showed that QCT markedly increased the diagnostic rate of osteoporosis compared to DXA (16).

Factors negatively affecting bone health in DMD patients include progressive muscular weakness, loss of weight bearing activity and potent osteotoxicity of long-term glucocorticoid (GC) therapy (17). To date, GC therapy is the only disease-modifying therapy. However, prolonged use of GC predisposes patients to osteoporosis by increasing bone resorption, decreasing bone formation and growth and delaying puberty (18, 19). On the other hand, mechanical stimulation may play a vital role in stimulating bone growth (20, 21). Furthermore, low levels of vitamin D cause abnormalities in osteoblast function and imbalances in calcium metabolism and can worsen this process. However, to our knowledge, which of these factors has the greatest impact on BMD loss in boys with DMD has not been well described.

Therefore, this study aimed to examine lumbar BMD values measured by QCT and identify the factors associated with BMD loss using a multilevel mixed-effects model, which might be helpful in developing strategies to optimize bone health and decrease the risk of fragility fractures in DMD.

MATERIALS AND METHODS

Study Participants

We conducted a retrospective longitudinal study using data from the electronic medical records of patients with DMD at West China Second University Hospital from January 2018 to October 2021. A total of forty-two boys with DMD were confirmed by means of genetic testing and/or muscle biopsy during this period. Boys were excluded if they were not taking glucocorticoid therapy. Additionally, patients who were lost to follow-up or did not have BMD information were also excluded. Nineteen boys were selected who had undergone annual "bone health" assessments during at least 2 consecutive follow-ups irrespective of their age (i.e., they had at least 2 years follow up). This study was approved by the Institutional Review Board of West China Second University Hospital. (IRB# 20200021gc).

Data Collection

All data were collected from the electronic medical records, including age, height, weight, functional activity scores (FMSs), laboratory parameters, status of steroid use, and BMD values at each clinic visit. The results of laboratory tests, including serum levels of calcium, phosphorus, 25(OH)-vitamin D, creatinine kinase (CK), creatine kinase isoenzyme (CK-MB) and intact parathyroid hormone (PTH), were recorded. The FMS reflecting their functional activity level was defined by Swinyard and Deaver's 8-grade scale (22).

In GC-treated boys with DMD, the initial dose was usually given in the form of either prednisolone or deflazacort at 0.75 mg/kg/d or 0.9 mg/kg/d, respectively, and then the dose was increased according to body mass. At the same time, once the hormone was taken, the children were given oral vitamin D3 (400 IU/d) and elemental calcium (400 mg/d) in the form of dietary supplements. The conditions of GC use, including age at initiation of GC use, daily dose and length of treatment, were also recorded. The cumulative GC dose was calculated using information recorded in the subjects' medical records.

QCT Scanning and BMD Measurement Procedures

We used a Neusoft 128-slice helical CT scanner (NeuViz128, China) to acquire CT images of the lumbar spine (L1-L3; 120 kV, 70 mAs, 3-mm slice thickness). Quality control was ensured throughout the study through daily calibration and cross calibration with the European spine phantom (ESP-145) on 10 repeated scans acquired following a prescribed QCT scanning protocol. The quality assurance (QA) results showed that the ESP volumetric BMD measured at our center differed by less than 5 mg/cm^3 on average.

Asynchronous BMD calibration in combination with QCT Pro analysis software (Mindways Software, Inc.) was used to obtain lumbar spine (L1–L3) trabecular volumetric BMD (mg/cm^3) (**Figure 1**), as previously reported (16). References for vertebral BMD Z scores based on age and sex were provided by the manufacturer of the QCT software (Mindways Software) (23). A low BMD was defined as a Z score of ≤ -2.0 according to the current

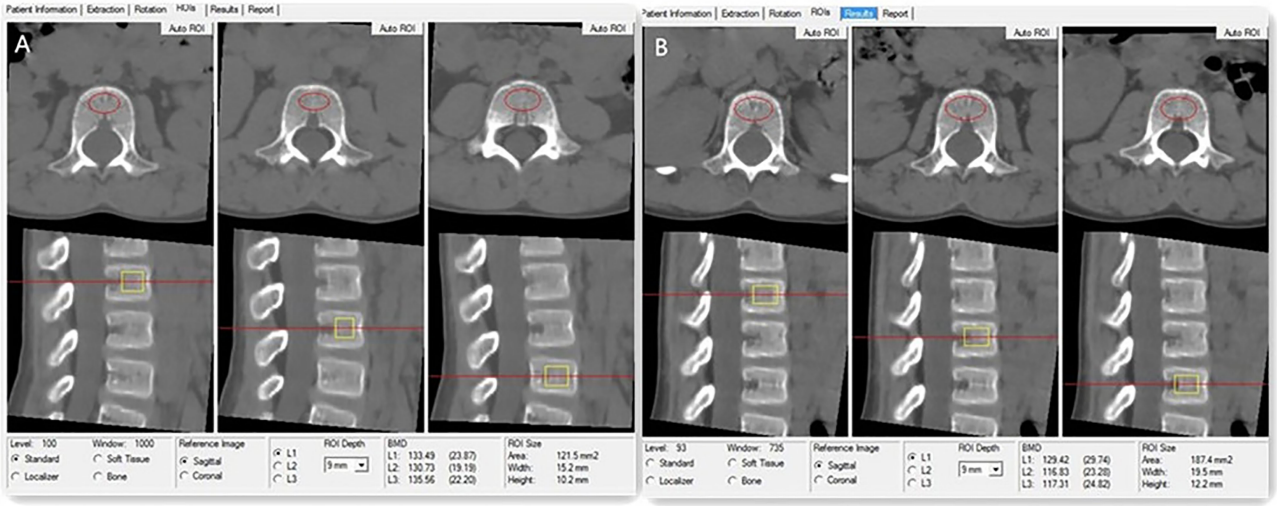

FIGURE 1 | **(A)** Images for a 7-year-old boy with DMD who has been treated with GC for 2 years. The measurements of L1, L2, and L3 vertebral trabecular volumetric bone mineral density (BMD) are shown. The BMD of L1, L2, and L3 is 133.49 mg/cm^3, 130.73 mg/cm^3, and 135.56 mg/cm^3, respectively; the average lumbar volumetric BMD is 133.26 mg/cm3, and the Z score is -1.55. **(B)** The same boy with DMD was followed up after 1 year of GC therapy. The measurements of L1, L2, and L3 vertebral trabecular volumetric bone mineral density (BMD) are shown; the BMD of L1, L2, and L3 is 128.42 mg/cm^3, 116.83 mg/cm^3, and 117.31 mg/cm^3, respectively. The average lumbar volumetric BMD is 121.19 mg/cm3, and the Z score is -1.97.

ISCD recommendations for children (15). All patients or their families provided written informed consent to undergo QCT scanning.

Statistical Analysis

Statistical analysis was performed using SPSS software (version 22.0). A multilevel mixed effect model was used to analyze BMD loss. Level one was the patient's relevant measurements at different time points, and level two was the patient. The outcome variable was BMD Z score. First, the intraclass correlation coefficient (ICC) was used to determine whether the dependent variable was significantly different among individual levels and consider the necessity of establishing a multilevel model. When high levels were significant, independent variables including age, serum calcium, phosphorus levels, 25 (OH)-vitamin D, creatinine kinase (CK), FMS, and cumulative GC dose were added to the fixed-effect portion of the model. Interaction effects between each independent variable and time were also considered. If the interaction term was not statistically significant, it was omitted from the model. A two-tailed p value <0.05 was considered as statistically significant.

RESULTS

Baseline and Follow-up Characteristics

Nineteen boys with GC-treated DMD who had a total of sixty complete records from January 2018 to October 2021 were included in this study. At baseline, the mean age was 8.58 ± 1.87 years, and the mean age at GC therapy initiation was 6.67 ± 2.19 years. Serum CK and CK-MB levels increased gradually with increasing follow-up time. The serum calcium levels were in the normal range in 19 patients, and only 1 patient had decreased levels of serum calcium at the second year of follow-up. The serum phosphorus levels decreased in 4 patients (21.05% decrease) at baseline, and serum phosphorus levels decreased in 7 and 8 patients after 1 and 2 years of follow-up, respectively. The levels of serum 25(OH)-vitamin D were in the range of deficiency, with a mean of 19.90 ± 4.28 ng/dL at baseline; 11 patients (57.89%) had vitamin D deficiency, and 8 patients (42.11%) had insufficiency. The mean levels of serum 25(OH)-vitamin D showed a decreasing tendency with increasing follow-up. The demographic and clinical characteristics at baseline and follow-up in patients with DMD are summarized in **Table 1**.

Assessment of Lumbar BMD

At baseline, QCT scans showed reduced lumbar BMD (Z score ≤ −2) in 3 (15.8%) patients and normal lumbar BMD (Z score >−2) in 16 (84.2%) patients; the mean volumetric BMD of the lumbar spine was 127.83 ± 38.49 mg/cm^3, and the corresponding BMD Z score was -0.85 ± 1.32, ranging from 1.68 to -3.34. During follow-up, the BMD Z score at year 1 and year 2 postbaseline decreased to a mean of -1.56 (SD 1.62) and -2.02 (SD 1.36), respectively. **Figure 2** illustrates the longitudinal changes in BMD value and Z score at baseline and follow-up in these patients.

Multilevel Mixed Model Analysis With BMD Z Score as the Outcome

It can be seen from the covariance parameter estimate of the null model and the results of the Z test that the dependent variable is not independent, and the measured values for a given individual are similar (Z=2.74, P=003). The ICC was 73%; that is, 73% of

TABLE 1 | Demographic and clinical characters at baseline and follow-up for patients with DMD.

Clinical variables	Baseline (n = 19)	Year 1 (n = 19)	Year 2 (n = 19)	Year 3 (n = 3)
Age (years)	8.58 ± 1.87	9.69 ± 2.06	11.15 ± 1.89	12.73 ± 2.08
BMI	18.25 ± 2.39	19.50 ± 3.94	19.88 ± 4.22	19.54 ± 3.53
Lumbar BMD (mg/cm^3)	127.83 ± 38.49	123.90 ± 39.54	116.53 ± 38.58	115.43 ± 10.92
T value	-1.75 ± 1.36	-1.87 ± 1.38	-2.15 ± 1.47	-2.13 ± 0.52
Z score	-0.85 ± 1.32	-1.56 ± 1.62	-2.02 ± 1.36	-2.41 ± 0.51
≤–1.5	4 (21.1%)	9 (47.4%)	13 (63.2%)	3 (100.0%)
≤–2	3 (15.8%)	6 (31.6%)	6 (31.6%)	2 (66.7%)
Age started GC (years)	6.75 ± 2.34			
Duration (M)	18.39 ± 10.78	33.89 ± 10.58	44.78 ± 14.45	61.33 ± 15.28
Cumulative exposure (g)	5.79 ± 3.77	11.9 ± 4.21	16.81 ± 5.92	23.72 ± 2.98
Decreased of calcium	0 (0%)	0 (0%)	1 (5.26%)	0 (0%)
Decreased of phosphorus	4 (21.05%)	7 (36.84%)	8 (42.11%)	1 (33.33%)
CK-MB (U/L)	158.32 ± 94.48	108.64 ± 77.19	99.65 ± 74.09	79.47 ± 57.97
CK (x10^3 UG/L)	15.81 ± 7.74	11.42 ± 5.82	12.87 ± 9.06	10.72 ± 2.72
25 (OH)-Vitamin D (ng/dL)	19.90 ± 4.28	19.14 ± 3.99	18.30 ± 4.74	15.17 ± 1.55
20-30 ng/dL	8 (42.11%)	6 (31.58%)	6 (31.58%)	0 (0%)
≤20 ng/dL	11 (57.89%)	13 (68.42%)	13 (68.42%)	3 (100.00%)
FMS	1.37 ± 0.68	1.37 ± 0.60	1.89 ± 0.94	1.67 ± 1.16
FMS>1	5 (26.32%)	6 (31.58%)	10 (52.63%)	1 (33.33%)

Data are presented as mean means ± standard deviation or n (%).
DMD, duchenne muscular dystrophy; BMI, body mass index; BMD, bone mineral density; GC, glucocorticoid; CK-MB, creatine kinase isoenzyme; CK, creatinine kinase; FMS, functional activity score.

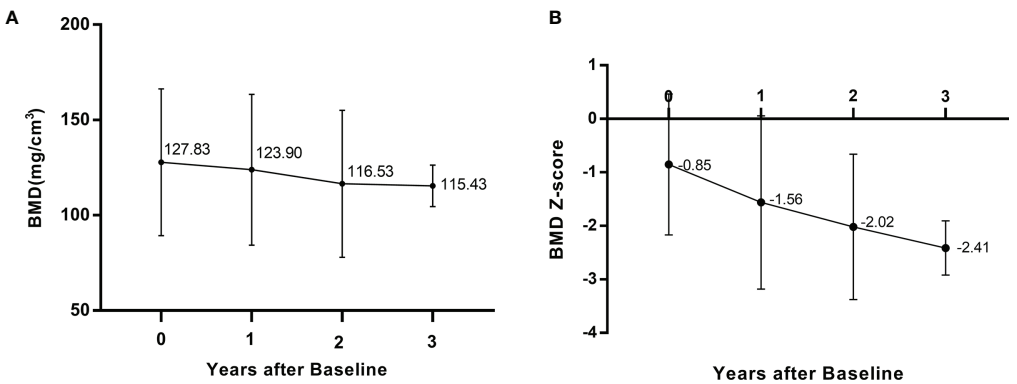

FIGURE 2 | Longitudinal changes lumbar intrabecular BMD **(A)** and lumbar BMD Z score **(B)** at baseline and follow-up.

the variation in BMD Z score was caused by variation at the individual level. Therefore, a multilevel mixed effect model was adopted for the analysis. In the multilevel mixed-effects model with BMD Z score as an outcome, after controlling for potential confounders, cumulative GC exposure, serum levels of calcium (decreased vs. normal), phosphorus (decreased vs. normal), 25 (OH)-vitamin D (deficiency vs. insufficiency) and CK were not statistically significant predictors of the BMD Z score; only age (β=-0.358, p=0.0003) and FMS (β=-0.454, p=0.031) were associated with a decrease in BMD Z score (**Table 2**).

DISCUSSION

Previous studies have confirmed the value of QCT in assessing BMD in pediatric patients (24–26). In DMD patients, obtaining accurate measurements of BMD is usually difficult. The most common causes for this difficulty are severe spinal rotation, scoliosis, and other musculoskeletal changes, which are present in 70%-90% of patients with advanced DMD (27). Nevertheless, QCT software can directly correct for scoliosis curves and accurately measure trabecular bones. Therefore, this study is significant because it is the first study to explore the factors associated with lumbar BMD loss in boys with DMD using QCT data.

We observed that lumbar BMD decreased longitudinally in GC-treated boys with DMD. Up to 31.6% (6/19) of patients had lumbar BMD Z scores ≤ −2 during follow-up. Suthar et al. (28) reported a reduction in BMD measured with DXA (height adjusted Z score ≤ −2) in 57% of boys with DMD in North India, which was greater than that observed in our study. Aparicio et al. (29) found osteopenia in the lumbar region in

TABLE 2 | Multilevel mixed model of lumbar BMD Z score in patients with DMD.

Predictors	β (95%CI)	P value
Constant	2.118 (0.395,3.841)	0.018
Cumulative GC exposure (g)	0.0006 (-0,038,0.039)	0.974
FMS (FMS=1 vs. FMS>1)	-0.454 (-0.866,-0.043)	0.031
Calcium (normal vs. decreased, mmol/L)	0.443 (-0.697,1.584)	0.434
Phosphorus (normal vs. decreased, mmol/L)	-0.213 (-0.588,0.161)	0.255
Creatinine kinase (x10^3 U/L)	0.013 (-0.019,0.046)	0.408
Age (year)	-0.358 (-0.537,-0.178)	<0.001
25(OH)-Vitamin D (deficiency vs. insufficiency, ng/dL)	-0.077 (-0.404,0.248)	0.630

CI, confidence interval; FMS, functional activity score.

30% of DMD patients who were not using any steroids. Therefore, the discrepancies among the results of these studies may be incomparable due to differences in population characteristics or study designs.

Our findings suggest that age is an important risk factor for lumbar BMD loss (β=-0.358, p=0.0003). Older patients with DMD may have more significant bone health impairment than younger patients who have better mobility and walking ability. Age might be useful for estimating the risk of lumbar BMD loss in boys with DMD. A previous study noted that age ≥10.5 years was associated with a reduction in BMD in boys with DMD (30). Summer et al. (31) observed that the age of appendicular lean mass loss in DMD children was approximately 12 years. However, their results may not be applicable to all DMD patients because their studies were not investigating the effects of GC exposure. Our study considered the effects of hormones and obtained similar results to other studies. Therefore, early interventions in boys with DMD could prove valuable.

It is well known that GC therapy is used to maintain muscle strength and mobilization and protect cardiac and respiratory functions in DMD (32), but potential side effects and consequent toxicity related to bone health need to be taken into account. Long-term GC treatment induces severe osteoporosis, resulting in a deregulation in bone turnover (10). The effect of GC on bone is highly dose- and time-dependent. Pharmacological doses of GC induce bone loss, which becomes evident after 6–12 months of chronic use (33). Dilber et al. found a cutoff value of 2100 mg/kg for the cumulative dose, above which adverse effects on bone were expected (34). However, Van Staa et al. found that the adverse effects of oral GC on bone were related to the daily dose rather than the cumulative dose of GC (35). In our study, we did not observe any contribution of cumulative GC dose to BMD Z score decrease using multilevel mixed-effects model analysis, which could be due to the small number of patients in the study or to the younger age and higher functional level of the patients still taking GCs. On the other hand, the use of different GC regimens may impact bone health outcomes in patients with DMD (36). Crabtree et al. (37) observed that lumbar BMD in boys with DMD was not significantly different between daily and intermittent GC regimens either at baseline or over the duration of follow-up, but a higher frequency of vertebral fracture and greater linear growth impairment were found in those receiving daily GC treatment.

In our study, an association between FMS and BMD Z score was observed in multilevel mixed-effects model analysis (β=-0.454, p=0.031). A previous study also observed that a Vignos scale ≥6 for lower extremity function can predict BMD loss (30). Progressive decline in muscle function with age is frequently accompanied by a decline in BMD and bone quality, leading to increased bone fragility fractures (13). It is still unclear whether bone defects are due to a direct mechanical effect or nonmechanical factors that also contribute to poor bone status (38). A "muscle-bone interactions" model has been introduced to explain the relationship between bone and muscle in children with neuromuscular diseases (39). Physical therapy interventions may have a positive effect in preserving and improving motor function and muscle strength in boys with DMD (21, 40), whereas the effect on improving BMD needs to be further explored.

Among the clinical characteristics, serum CK, as a diagnostic marker of DMD, was significantly higher than the normal value. Furthermore, we did not observe any contribution of CK to BMD Z score loss (β=0.013, p=0.408). The serum CK levels in children with DMD usually reached a peak at the age of three and then gradually stabilized and decreased, with an average annual decrease of 8.7%-20% (41). The changes in serum CK reflect the degree of muscle damage rather than bone damage.

Vitamin D is essential for skeletal health. It mediates the mineralization of newly synthesized osteoid tissue within bone. In the present study, we observed that the levels of serum vitamin D were low in our subjects, and vitamin D deficiency was found in 68.42% of the group at the 2-year follow-up, which can lead to decreased intestinal calcium absorption and even an imbalance in calcium metabolism (42). On the other hand, vitamin D deficiency stimulated PTH secretion. PTH promoted calcium release from bones to maintain calcium homeostasis, as observed in DMD patients in previous studies (10, 42). In our study, the mean levels of serum calcium and phosphorus were almost within normal ranges. Therefore, in multilevel mixed-effects model analysis, prediction of reduced BMD Z scores was not possible with the serum calcium (β=0.443, p=0.434) or phosphorus (β=-0.213, p=0.255) level.

Vitamin D deficiency also results in muscle weakness, a problem from which boys with DMD already suffer. Therefore, it is essential to ensure adequate daily intake of calcium and vitamin D in DMD patients even if the patients do not show increased levels of PTH or bone markers. In our analysis, we did not observe any contribution of vitamin D deficiency (vs. insufficiency) to BMD Z score loss (β=-0.077, p=0.630).

This was probably due to the routine use of dietary calcium and vitamin D supplements in patients with DMD. Recent studies have shown beneficial effects of vitamin D therapy on bone health in DMD patients (42, 43). Cholecalciferol plus adequate dietary calcium intake seems to be an effective first-line approach that controls bone turnover, corrects vitamin D deficiency, and increases BMC and BMD in most patients with DMD.

The strength of this study is that mixed-effects model analysis is a valid statistical method for assessing changes in BMD over time, both within a patient and between patients. Since the data in this study are hierarchical and the measurement results for dependent variables within individuals are correlated, the multilevel model can obtain more accurate parameter estimates than the traditional linear regression model (44). Another advantage of this study is that lumbar BMD was obtained by QCT, which is more accurate than the DXA method. However, there is a lack of comparable data, as QCT equipment is not widely available. Our study has several limitations. First, a main limitation of this study is the small number of participants. Thus, some of our analyses may have been underpowered. Second, QCT has a higher radiation dose than DXA, which might limit its applicability to clinical use. Third, this retrospective study used demographic and clinical data extracted from medical records in a single institution. We are unable to assess the condition of fractures because the data on long bone and vertebral fractures are incomplete. Similarly, some data that may affect BMD were not included in the analysis, such as nutrition status, vitamin D treatment and testosterone therapy.

In conclusion, our study suggests that in DMD patients, BMD showed a gradual and progressive decreasing trend. Age and muscle function are the main contributors to lumbar BMD loss in boys with DMD. Early recognition of BMD changes may help in developing strategies for optimizing bone health, especially in patients with the risk factors identified in this study.

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 human participants were reviewed and approved by the Institutional Review Board of West China Second University Hospital. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

CL and H-BQ designed the study. CL, D-DY, X-GL, and WL collected the data. CL and LZ analyzed the data. YL, F-LJ, X-JC, and GN gave critical comments on the writing. CL wrote and prepared the original draft. H-BQ supervised the study. All authors contributed to the article and approved the submitted version.

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REVIEW
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Bad to the Bone: The Effects of Therapeutic Glucocorticoids on Osteoblasts and Osteocytes

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Despite the continued development of specialized immunosuppressive therapies in the form of monoclonal antibodies, glucocorticoids remain a mainstay in the treatment of rheumatological and auto-inflammatory disorders. Therapeutic glucocorticoids are unmatched in the breadth of their immunosuppressive properties and deliver their anti-inflammatory effects at unparalleled speed. However, long-term exposure to therapeutic doses of glucocorticoids decreases bone mass and increases the risk of fractures – particularly in the spine – thus limiting their clinical use. Due to the abundant expression of glucocorticoid receptors across all skeletal cell populations and their respective progenitors, therapeutic glucocorticoids affect skeletal quality through a plethora of cellular targets and molecular mechanisms. However, recent evidence from rodent studies, supported by clinical data, highlights the considerable role of cells of the osteoblast lineage in the pathogenesis of glucocorticoid-induced osteoporosis: it is now appreciated that cells of the osteoblast lineage are key targets of therapeutic glucocorticoids and have an outsized role in mediating their undesirable skeletal effects. As part of this article, we review the molecular mechanisms underpinning the detrimental effects of supraphysiological levels of glucocorticoids on cells of the osteoblast lineage including osteocytes and highlight the clinical implications of recent discoveries in the field.

Keywords: glucocorticoids, osteoblasts, osteocytes, glucocorticoid-induced osteoporosis (GIO), anti-resorptive treatment, osteo-anabolic treatment

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INTRODUCTION

Harvey Cushing first described the development of 'osteoporosis of the skeleton' in the spine of patients suffering from endogenous hypercortisolism 90 years ago (1). Two decades later, clinicians observed the same phenomenon in patients receiving synthetic glucocorticoids (GCs) (2). GC-induced osteoporosis (GIO) is considered the third most common condition of pathological bone loss following post-menopause and aging, and is the most frequent cause of secondary osteoporosis. For instance, in the Global Longitudinal Study of Osteoporosis in Women (GLOW), about 2.7-4.6% of women from 10 different countries received treatment with GCs (3). Although a considerable proportion of GC-induced fractures remain asymptomatic and thus difficult to detect, exposure to exogenous GCs has been linked to a high incidence of fractures, particularly in the spine. A rapid

reduction in bone mineral density (BMD) is generally observed as early as 3-6 months after initiation of GC treatment and persists during continued GC exposure (4–9). Aside from the spine, typically locations of GC-induced fractures include the ribs and pelvis (8, 10–12), indicating that sites rich in trabecular bone are more affected than the cortical structures (10). Interestingly, some studies observed a rapid development of fractures in patients receiving GCs, even before any detectable decreases in the bone mineral density (9, 13, 14), suggesting that not just bone mass but also bone quality is compromised in the presence of supra-physiological levels of GCs (**Box 1**).

Several molecular mechanisms underlying GIO have been identified through *in vivo* and *in vitro* studies. Overall, the effects of excess GCs in the skeleton are complex owing to the multifaceted nature of interactions between local and systemic factors. Generally, GCs act *via* the glucocorticoid receptor (GR), which is ubiquitously expressed in all skeletal cell types. The molecular nature of GC-GR interactions and their interplay with target cells are manifold and complex. Briefly, upon ligand binding the GR translocates to the nucleus where it either acts as a dimer by binding directly to the DNA in the promotor region of target genes or it may act as a monomer by interfering with other transcription factors such as activator protein 1 (AP-1) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB). A detailed review of the molecular action of the GC-GR complex is provided by Hartmann et al. (18) or Vandewalle et al. (19).

The skeletal effects of therapeutic GC use have to be separated from the role of physiological GCs in the skeleton. Physiological concentrations of GCs are critically required for differentiation of stromal progenitors towards the osteoblast lineage – and away from adipocytes – (20, 21) and thus support bone formation (22) and the accrual of bone mass (23–25). Overall, physiological concentrations of GCs exert anabolic effects throughout the skeleton particularly during growth, whereas supraphysiological (or therapeutic) levels of GCs result in loss of bone mass and quality (26, 27). Early studies on GIO have described several extra-skeletal effects, which may mechanistically underpin GC-induced bone loss, such as i) a dysregulation of calcium homeostasis through decreased intestinal calcium absorption and increased renal calcium clearance; ii) a reduction in the growth hormone/insulin-like growth factor axis; iii) alteration in gonadal steroid hormones; or iv) the potential development of secondary hyperparathyroidism. Also, the catabolic effects of GCs on skeletal muscle have been marked as a contributor to increased fracture risk *via* increased incidence of falls secondary to muscle weakness (28–30). Interestingly, over the last two decades, advances in mouse genetics have enabled the detailed characterization of the mechanisms of GC-induced bone loss. This led to the discovery that the direct effects of supra-physiological levels of GCs on bone cells represent a significant part of the pathogenesis of GIO. Generally, the pathogenesis of GIO is characterized by two phases: an initial phase of accelerated bone loss owing mainly to increased osteoclast-mediated bone resorption; followed by a slow but continuous phase of qualitative and quantitative bone loss as a result of the compromised function of both osteoblasts and osteocytes. While all skeletal cell types – namely osteoblasts, osteocytes and osteoclasts – are targeted by GCs, it is now understood that cells of the osteoblast lineage are the main effectors of GC-induced bone loss and the GC-induced rise in fracture risk.

Here we review the molecular and cellular targets of therapeutic doses of GCs with a particular focus on osteoblasts and osteocytes as well as the implications for clinical therapy of GIO.

THE OSTEOBLAST LINEAGE AS A KEY TARGET FOR EXCESS GCs

Skeletal cells continually interact with one another through the process of bone remodeling. Bone remodeling includes the coordinated processes of bone formation and bone resorption. Formation of new bone is performed by osteoblasts, whereas bone resorption is carried out by osteoclasts. Osteocytes act as mechanosensors and orchestrate the skeletal remodeling process by initiating and governing the remodeling cycle (31, 32). While exogenous GCs affect all cells of the remodeling process – either directly or indirectly (**Figure 1**) –, cells of the osteoblast lineage, and therefore bone formation, are key targets of GCs in the skeleton.

Generally, exposure to supra-physiological levels of GCs results in a strong suppression of bone formation and the anabolic function of osteoblasts in both humans and rodents. Treatment of patients with therapeutic doses of GCs rapidly suppresses serum markers of bone formation such as osteocalcin, bone-specific alkaline phosphatase (ALP) and procollagen type I N-terminal propeptide (P1NP) (33–40). Similarly, prolonged exposure of rodents to excess GCs decreases the systemic markers of bone formation and the osteoblasts' anabolic function, such as osteocalcin and P1NP (17, 41–46). Histomorphological analysis of bones from GC-treated rodents confirms these findings and reveals compromised bone

Abbreviations: 11β-HSD2, 11β-hydroxysteroid dehydrogenase type 2; AP-1, activator protein 1; ALP, alkaline phosphatase; BMP, bone morphogenic protein; BMD, bone mineral density; CTX, carboxy-terminal collagen crosslinks; C/EBPα, CCAAT-enhancer-binding protein alpha; CDK, Cyclin-dependent Kinase; DUSP1, Dual-specificity phosphatase 1; DKK1, dickkopf1; ER, endoplasmic reticulum; ERK, extracellular-signal-regulated kinases; Eif2a, Eukaryotic Translation Initiation Factor 2A; FAK, focal adhesion kinase; GR, glucocorticoid receptor; GCs, glucocorticoids; -GRE, negative GC-response element; GIO, GC-induced osteoporosis; GLOW, Global Longitudinal Study of Osteoporosis in Women; IL-11, interleukin-11; IGF-1, insulin-like growth factor I; JNK, c-Jun N-terminal kinase; JAK2, Janus kinase 2; LncRNAs, long non-coding RNAs; LIF, leukemia inhibitory factor; MKP1, MAPK phosphatase 1; MAPK, mitogen-activated protein kinase; MMP, matrix metalloproteinase; M-CSF, Macrophage colony-stimulating factor; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; OPG, osteoprotegerin; OCN, osteocalcin; P1NP, procollagen type I N-terminal propeptide; PPARγ, Peroxisome proliferator-activated receptor gamma; PTH, parathyroid hormone; PYK2, Protein-tyrosine kinase 2 beta; RUNX2, runt-related transcription factor 2; RANKL, receptor activator of nuclear factor kappa-B ligand; ROS, reactive oxygen species; SOST, sclerostin; sRFP1, Secreted frizzled-related protein; STAT3, Signal Transducer And Activator Of Transcription 3; TRAP-5b, tartrate-resistant acid phosphatase-5b; TBS, trabecular bone score.

BOX 1 | Bone mineral density as a surrogate parameter in GIO.

GCs have been shown to substantially increase fracture risk in humans. Interestingly, the increase in fracture risk manifest itself immediately after the commencement of GC therapy (8), leading to the hypothesis that GCs may damage bone beyond the loss of bone mass. And indeed, studies were able to establish that in patients suffering from GIO fractures occurred more frequently compared to patients with postmenopausal osteoporosis even when BMD scores were taken into account (13). Similarly, it has been established that the commonly used FRAX algorithm underestimates the occurrence of fractures in subjects treated with GCs (15). More recently the use of trabecular bone score (TBS) has been shown to potentially remedy some of these concerns (16); however, its use has not been widely adopted and/or established as a diagnostic tool in GIO. Overall, the predictive value of BMD is reduced in GIO compared to postmenopausal osteoporosis. This is of particular concern as virtually all studies assessing the use of anti-osteoporotic medication in GIO utilize BMD as a surrogate parameter for fractures. Studies were not adequately powered to allow for an analysis of fracture risk. This should be taken into account when evaluating the results of clinical trials comparing therapeutic agents in the context of GIO.

Preclinical studies have attempted to assess the underlying reason for the particularly high fracture risk in GIO compared to postmenopausal osteoporosis. Studies in rodents were able to link the high fracture risk in GIO as well as the rapid onset of fractures following commencement of GC-therapy to their detrimental effects on osteocytes. Lane et al. highlighted the role of the lacunar-canalicular network in this context, which is largely maintained by osteocytes (17). Others have built on this idea and highlighted the role of the skeletal vasculature in GIO, see section '*The Effects of Excess GCs on the Function of Osteocytes'* for further details. However, the rapid increase in fracture risk with commencement of GC-therapy may also be the result of systemic effects of supraphysiological levels of GCs; i.e. GCs may decrease muscle strength and adversely affect coordination and/or lead to an increase in falls (and thus fractures) due to their effects in the central nervous system. Hence, whether the rapid and strong increase in fractures following commencement of therapeutic GCs is a result of bone-intrinsic effects of GCs or GC-action elsewhere in the body remains to be determined.

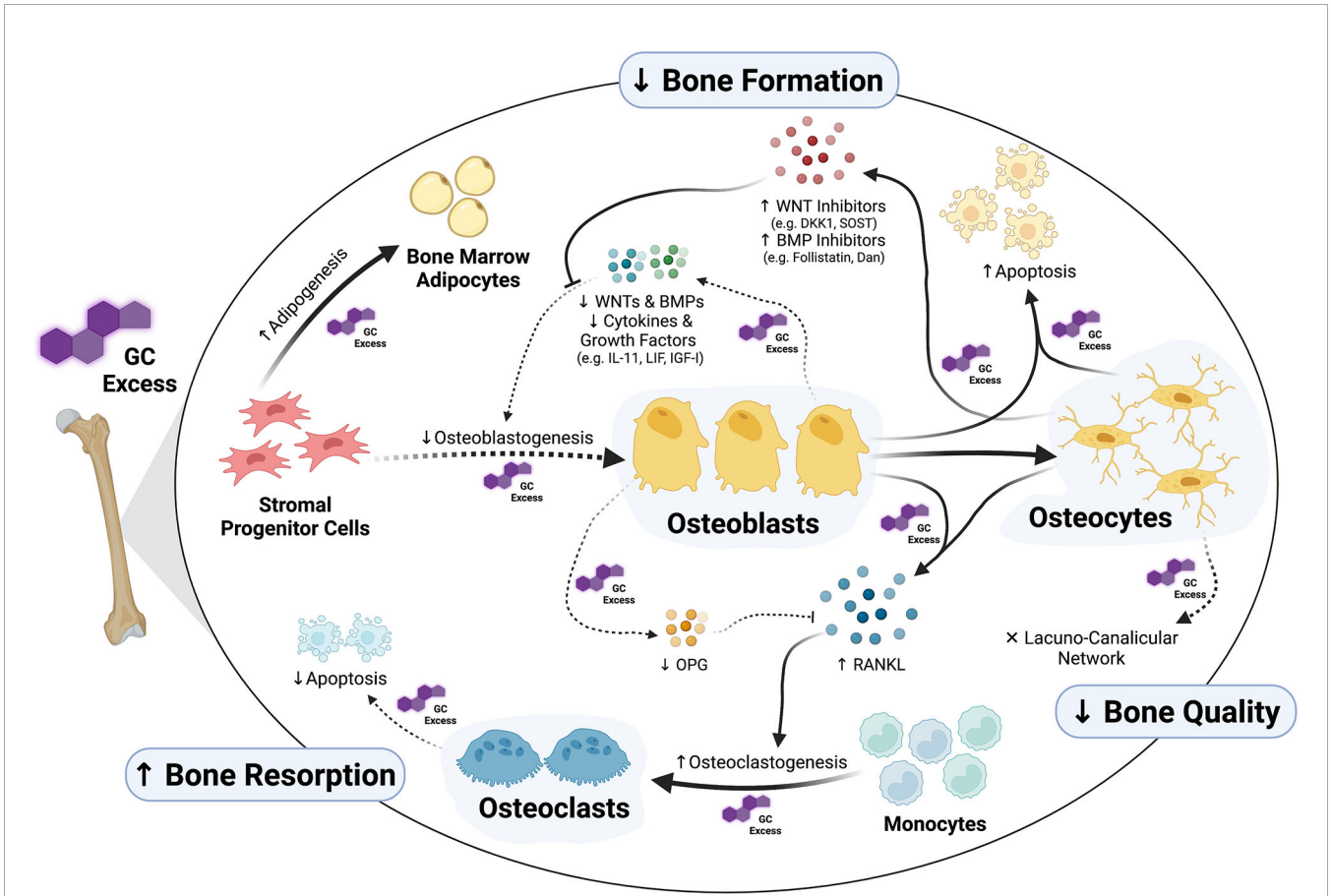

FIGURE 1 | Osteoblasts and osteocytes as main targets of glucocorticoid (GC) excess in the skeleton. Exposure to supra-physiological levels of GCs affects many aspects of osteoblast formation and function. Whereas GCs inhibit osteogenic commitment of stromal progenitor cells by diversion into adipogenesis, they inhibit proliferation and differentiation of pre-osteoblasts through direct as well as autocrine/paracrine effects. Together with suppression of osteoblast function, all these GC-induced alterations in osteoblasts suppress bone formation. Additionally, GCs induce apoptosis of both osteoblasts and osteocytes and cause disruptions in osteocytic lacuna-canalicular network affecting bone quality. Osteoclast-mediated bone resorption is affected by GCs as well, especially through the regulation of the RANKL/OPG system *via* osteoblasts and osteocytes. The figure was created with BioRender.com.

formation and mineralization as well as a reduction in the number and surface of osteoblasts (17, 23, 43, 45, 47–49). Similar effects were observed in bone biopsies from GC-treated patients (50–53). Overall, GIO occurs in both rodents and humans with similar cellular and molecular features. Thus, rodents may act as a suitable model organism to investigate the molecular and cellular mechanism underlying GIO (54).

The significance of osteoblasts in the pathogenesis of GIO has been made clear through the utilization of genetically modified mouse models, in which GC-GR signaling has been disrupted in a cell-specific fashion. Protection of osteoblasts from excessive GC signaling by osteoblast-specific overexpression of the GC-inactivating enzyme, 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2), not only prevented GC-induced osteoblast apoptosis but also preserved osteoblast function and bone formation (43, 55). Similarly, specific deletion of GR in osteoblasts prevented both GC-driven bone loss as well as compromised bone formation (23). Some – though not all – studies investigating the disruption of GC signaling in osteoblasts/osteocytes during GC excess showed that not only osteoblast function and bone formation were preserved in this setting but also the GC-induced increase in osteoclast number and activity was prevented (43). Collectively, these results suggest that the adverse skeletal effects of exogenous GCs result to a large degree from their detrimental action on cells of the osteoblast lineage. Quantifying the overall contribution of osteoclasts to the development of GC-induced osteoporosis remains challenging. The selective abrogation of GC-GR signaling in osteoclasts (by GR knock-out) resulted in preserved bone resorption and preserved bone formation, indicating a prominent role for osteoclasts in GC-induced bone loss (56). However, – in the hands of different researchers – the osteoclast-specific disruption of GCs (either by 11β-HSD2 overexpression or conditional GR knockout) had no discernible protective effects against GC-induced bone loss since osteoblasts were readily affected by excess GCs (23, 57). Collectively, the weight of the evidence strongly points to the osteoblast lineage as a more impactful target of GCs in the skeleton compared to the cells of the osteoclast lineage.

THE EFFECTS OF GC EXCESS ON THE FORMATION AND FUNCTION OF OSTEOBLASTS

GCs cause alterations in the formation and apoptosis of osteoblasts as well as their function, all of which contribute to the pathogenesis of GIO. *In vivo* and *in vitro* studies have determined that supra-physiological levels of GCs exert their deleterious effects on cells of the osteoblast lineage at all stages of differentiation, leading to reduced osteoblast formation. Moreover, GCs limit both function and lifespan of osteoblasts, ultimately resulting in compromised bone formation. Furthermore, through the intrinsic link between bone formation and bone resorption, GCs may alter osteoblast activity and function through their action in osteoblasts and osteocytes. The effects of exogenous GCs on molecular pathways within osteoblasts are manifold and the relative contribution of each identified pathway is not always quantifiable. Nevertheless, the main effects of GCs on osteoblasts can be outlined as follows:

a) Decreased Osteogenic Cell Fate of Stromal Progenitor Cells

Given the multipotent nature of stromal progenitor cells in the bone marrow, supra-physiological levels of GCs induce diversion of these stem cells away from the osteoblast lineage towards the adipocyte lineage. Ultimately, this diversion of stem cell commitment leads to a decrease in the pool of osteoblast progenitors and limits bone formation. Accordingly, it has been shown that exposure to exogenous GCs in humans and rodents is associated with increased bone marrow adiposity (58–60). In line with these results, gene expression profiling of bone tissue from GC-treated mice displayed an induction of adipogenesis-related genes whereas osteogenic genes were downregulated (49). Moreover, bone marrow stromal progenitor cells from GC-treated rodents displayed reduced osteoblastogenesis *ex vivo* (23, 45, 48), with enhanced direction towards adipogenesis even in osteogenic media (59, 60). Similarly, exposure of bone marrow stromal progenitor cells to pharmacological levels of GCs results in decreased expression of essential osteogenic transcription factors such as runt-related transcription factor 2 (RUNX2), accompanied by concurrent increased expression of adipogenic transcription factors such as peroxisome proliferator- activated receptor gamma (PPARγ) and CCAAT-enhancer-binding protein alpha (C/EBPα) (61–66).

b) Suppressed Proliferation of Osteoprogenitors

Acting also on committed osteoblast precursors, GCs have been shown to inhibit and suppress their proliferation prior to full differentiation. In pre-osteoblast cultures, exposure to pharmacological 'micromolar' concentrations of GCs was associated with cell cycle arrest at the G1 phase due to downregulation of cell cycle activators such as Cyclin A, Cyclin D, Cyclin-dependent kinase 2 (CDK2), CDK4 and CDK6 (67–70) as well as upregulation of cell cycle inhibitors such as p53, p21 and p27 (67, 69, 71). In addition, GCs were shown to suppress the proliferation of osteoblast precursors through suppression of intracellular mitogenic signaling pathways, such as mitogen-activated protein kinase (MAPK) signaling *via* a rapid increase in the expression of a tyrosine phosphatase, MAPK phosphatase 1/dual specificity protein phosphatase 1 (MKP1/DUSP1), leading to dephosphorylation of extracellular-signal-regulated kinases (ERK), p38 and c-Jun N-terminal kinase (JNK) (72–74). Interestingly, while non-specific tyrosine phosphatase inhibition reversed GC-induced suppression of pre-osteoblasts *in vitro* and partly prevented deleterious bone effects (of GCs) in a rat model of GIO, *Mkp1* knockout mice were not protected against the adverse effects of methylprednisolone treatment (72–76). In a different study *Mkp1* deletion was shown to exacerbate inflammatory bone loss (77). These results suggest that targeting MKP1 may not represent a viable strategy for the prevention of GC-driven bone loss.

c) Inhibited Differentiation of Osteoblast Precursors Into Mature Osteoblasts

GC-induced inhibition of osteoblastogenesis is mediated mainly *via* suppression of signaling pathways involved in promoting osteoblast differentiation, importantly WNT and bone morphogenetic protein (BMP) pathways. First, GCs have been shown to inhibit the production of autocrine/paracrine WNT proteins, such as WNT7b, WNT10 and WNT16 (22, 78), as well as BMP proteins, such as BMP2, from mature osteoblasts (79–82). Conversely, the GC-driven suppression of osteoblast differentiation *in vitro* was corrected by supplementation of culture media with WNT and BMP proteins. Second, GCs increase the expression of inhibitory factors of the WNT and BMP signaling pathways from osteoblasts as well as osteocytes including WNT antagonists such as dickkopf1 (DKK1), sclerostin (SOST), secreted frizzled-related protein 1 (sRFP1) and axin-2 (22, 41, 49, 79, 83–89), as well as BMP antagonists, such as Follistatin and Dan (63, 79, 90). Third, exposure of pre-osteoblasts to supra-physiological levels of GCs suppresses the canonical WNT pathway through inducing degradation and inactivation of β-catenin, therefore inhibiting osteoblastogenesis (68, 91, 92). Moreover, suppression of growth factor pathways, such as insulin-like growth factor I (IGF-I), may contribute to the suppressive effects of GCs on osteoblastogenesis (93–96). GCs also suppress anabolic cytokines such as interleukin-11 (IL-11) and leukemia inhibitory factor (LIF) thereby reducing Janus kinase 2 (JAK2) – signal transducer and activator of transcription 3 (STAT3) signaling *via* inducing interaction of the monomeric glucocorticoid receptor with the transcription factor AP-1 (23, 97). Not only did supplementation of GC-treated osteoblasts with IL-11 (23, 97) and LIF (98) reverse the suppression in STAT3 signaling and osteoblast differentiation *in vitro*, treatment with LIF protected mice against GC-driven bone loss (98). Interestingly, reduced IL-11 expression was observed in other models of bone loss such as age-related suppression of bone formation, suggesting that IL-11 may be generally implicated in bone diseases (99, 100). Nevertheless, IL-11 is known to affect osteoclasts as well (101). Beside the direct targeting of key bone-anabolic pathways such as WNT and BMP signaling, GCs modulate the expression of miRNAs, including miR-29a, miR-34a-5p and miR-199a-5p, which regulate proliferation and differentiation of osteoblasts (102). A study by Wang and colleagues showed an association of GC-induced osteoporosis with miR-29a in rats, as GCs reduced the levels of miR-29a leading to a subsequent increase in deacetylation and ubiquitinylation of β-catenin, thus attenuating the pro-osteogenic impact of WNT signaling on differentiation of osteoblasts (103, 104). However, osteoblast-selective deletion of *Dicer*, an important enzyme in miRNA biogenesis, did not affect GC-induced suppression of osteogenesis both *in vitro* and *in vivo* (105).

d) Decreased Function of Osteoblasts

In addition to suppressed osteoblast formation, GCs decrease the anabolic function of osteoblasts, i.e., secretion of osteoid matrix proteins (e.g., collagen and osteocalcin) and subsequent mineralization of the matrix itself. For instance, GCs downregulate *OCN* (the gene encoding osteocalcin) gene expression in human and rat osteoblasts through direct binding of the GC-GR complex to a negative GC-response element (-GRE) in the enhancer region of the osteocalcin gene leading to trans-repression (106–108). Also, the expression of collagen from osteoblasts was shown to be suppressed by excess GCs *via* transcriptional and post-transcriptional mechanisms (109, 110). Apart from the synthesis of bone matrix proteins, supra-physiological levels of GCs were shown to provoke matrix degradation through upregulating expression of metalloproteinases such as matrix metalloproteinase 13 (MMP13) from osteoblasts (49, 111).

THE EFFECTS OF EXCESS GCs ON THE LIFESPAN OF OSTEOBLASTS AND OSTEOCYTES

Aside from suppression of osteoblast differentiation and activity, exposure to pharmacological levels of GCs triggers apoptosis in osteoblasts as well as their descendants, osteocytes, limiting their lifespan. Apoptotic osteoblasts and osteocytes were clearly detectable in the bones not only from GC-treated rodents (17, 45, 48, 55, 112) but also from patients undergoing therapy with GCs (45, 52, 113). It may be inferred that the GC-induced osteoblast apoptosis, similarly to suppressed osteoblast differentiation, likely contributes to the compromised bone formation, ultimately leading to GC-induced loss of bone mass and increase in fracture risk. More importantly, prevention of GC-driven apoptosis in osteoblasts and osteocytes has been associated with preservation of bone mass as well as strength in mouse models of GIO. For instance, co-treatment of mice with bisphosphonates (48, 114), intermittent parathyroid hormone (PTH) (115) or osteoprotegerin (OPG) (116) alleviated the adverse effects of pharmacological GCs on osteoblast and osteocyte apoptosis as well as bone formation and mineralization resulting in protection from bone loss.

Despite the evidence outlined above, some studies failed to detect a GC-induced increase in apoptosis of osteoblasts and osteocytes despite the detrimental effects of GCs on bone formation (23). This might be related to differences in the mouse strain and/or the dose of GCs utilized in the study. Importantly, the induction of apoptosis in osteocytes and osteoblasts has been shown to be dose- and time-dependent. In response to low 'nanomolar' concentrations of GCs, osteocytes and osteoblasts rely on autophagy to repair cellular damage and maintain viability (112, 117–120). In mice treated with low dose GCs, an upregulation of the expression of anti-oxidant and autophagy genes as well as an appearance of autophagic osteocytes and osteoblasts was observed in the skeleton (112, 119). However, prolonged exposure and/or high 'micromolar' doses of GCs result in suppression of autophagy as well as excessive intracellular damage due to accumulation of autophagosomes inside osteocytes and osteoblasts, which ultimately lead to the activation of pro-apoptotic pathways and

programmed cell death (112, 119, 121). Induction of autophagy in osteocytes and osteoblasts has been hypothesized to underpin a protective mechanism to preserve cellular viability (120, 122, 123); however, prolonged exposure to GCs is associated with suppressed autophagy leading to apoptosis (117, 123, 124). Indeed, enhancing autophagy *in vivo* by administration of the phytoecdysteroid, β-ecdysone, to GC-treated mice prevents GC-induced bone loss by reversing the suppression of bone formation and the induction of apoptosis in osteoblasts and osteocytes (121, 124). Likewise, pharmacological inhibition of autophagy was associated with an increase in GC-induced osteoblast apoptosis *in vitro* (117, 120). Nevertheless, the significance of autophagy in the detrimental effect of GCs on cells of the osteoblast lineage remains overwhelming (122, 125). Targeting apoptosis and autophagy of osteoblasts and osteocytes has been highlighted as a therapy for not only GC-driven bone loss (125), but also in age-related osteoporosis (126, 127).

Several studies using *in vitro* osteoblast and osteocyte cultures revealed some of the molecular mechanisms underpinning GC-induced apoptosis. Not only mechanisms related to regulation of transcription, but also rapid non-genomic mechanisms have been attributed to the apoptotic impact of GCs on the osteoblast lineage. The most evident subcellular apoptotic pathways in osteoblasts and/or osteocytes influenced by genomic GR actions have been upregulation of pro-apoptotic proteins such as BIM, BAK, p53 and p21 (67, 71, 128–130), as well as the suppression of survival, anti-apoptotic factors such as BCL-2, BCL-Xl and MCL-1 (67, 112, 131, 132). In addition, suppression of MAPK – ERK pathway through upregulation of MKP1/DUSP1 may act as another mechanism for GC-driven apoptosis in osteocytes and osteoblasts, as a non-selective protein tyrosine inhibitor was able to prevent GC-driven osteoblast apoptosis *in vitro* and *in vivo* (133). An increase in oxidative stress in the endoplasmic reticulum (ER) is one of the non-genomic pathways implicated in accumulation of reactive oxygen species (ROS), which may activate JNK signaling and programmed cell death in osteoblasts (84, 131, 134–136). Generally, prevention of oxidative stress exerts protective effects on osteoblasts and osteocytes thus preserving bone formation in addition to mediating anti-resorptive effects on osteoclasts (137). Prevention of ER stress and ROS accumulation *via* knocking down *Eif2a* (Eukaryotic Translation Initiation Factor 2A) not only prevented GC-induced apoptosis *in vitro* and *in vivo*, but also was associated with protection against bone loss (138). Inducing the protein tyrosine kinase 2 beta (PYK2) pathway and blocking focal adhesion kinase (FAK) signaling may contribute to GC-induced apoptosis in cells of the osteoblast lineage (136). In a recent report, genetic and pharmacological inactivation of Pyk2 signaling was proven effective in preventing not only apoptosis in osteoblasts and osteocytes, but also GC-induced bone loss, although reversing compromised osteoclast function was shown to likely contribute to such protective effects (139). Moreover, induction of Fas receptor/CD95 may advance apoptotic pathways in osteoblasts and osteocytes (140). Two recent studies hypothesized that long-non coding (lnc) RNAs are involved in GC-induced osteoblast apoptosis. Long-non coding RNAs are a large family of RNA molecules that are able to regulate protein expression and/or function. Lnc-MALAT1 and lnc-EPIC1 expression were shown to be altered in human osteoblasts treated with dexamethasone and to interact with AMP-activated protein kinase signaling and MYC [a regulator of osteoblast survival] (141, 142). However, the role of lncRNA in GIO remains to be validated *in vivo*.

THE EFFECTS OF EXCESS GCs ON THE FUNCTION OF OSTEOCYTES

Osteocytes play a crucial role in bone homeostasis through modulating the formation and activity of osteoblasts and bone formation *via* the release of WNT signaling inhibitors, sclerostin and dickkopf1 (DKK1) (143). In a number of studies, an upregulation of sclerostin gene and protein expression has been observed in the cortical-rich bones from GC-treated mice, where osteocytes are generally more abundant than osteoblasts (39, 49, 87, 144). Strong evidence for the significant contribution of the GC-driven upregulation of sclerostin in osteocytes to GIO has come from studies of abrogated sclerostin action in rodent models of excess GCs. Administration of anti-sclerostin antibodies to rats and mice prevented the development of GC-induced bone loss largely *via* preserving the function and number of osteoblasts and maintaining bone formation and mineralization (46, 145). In addition, knocking out *Sost* (the gene encoding sclerostin) in mice provided protection from GC-driven bone loss (144). In humans, the contribution of sclerostin to GC-induced bone loss is less clear. One study described a trend increase in serum levels of sclerostin in patients receiving pharmacological GCs (36). However, the serum levels of sclerostin were decreased in the patients treated with GCs in comparison to matched controls (39), and similar results were observed after acute treatment with therapeutic GCs in another study (146). DKK1, another WNT inhibitor expressed in osteocytes, is upregulated in GC-treated animals, and anti-sense silencing of *Dkk1* in mice was effective in preserving bone mass as well as bone formation during GC excess (49, 89). In a recent study, conditional knockout of *Dkk1* in osteoblasts and/or osteocytes prevented the development of GC-induced bone loss *via* reversing the adverse effects of GCs on osteoblasts and bone formation (41). Notably, both sclerostin and DKK1 have emerged as promising therapeutic targets in a number of bone diseases (147), and may be utilized clinically for the management of GIO in the future.

Aside from affecting the regulatory role of osteocytes through sclerostin and DKK1, several alterations in the bone environment around the osteocyte-lacunar environment have been reported in response to pharmacological levels of GCs. In bones from GC-treated mice, changes in the bone matrix surrounding osteocyte lacunae were observed, specifically an increased lacunae size as well as perilacunar hypomineralization (17). Additionally, these effects were associated with compromised bone strength (17). Moreover, osteocyte perilacunar remodeling was shown to be adversely affected by exogenous GCs: a GC-induced suppression of the expression of matrix metalloproteinases (MMPs) leads to collagen disorganization and degeneration of the lacuno-

canalicular network (148). In the *in vitro* setting, Gao et al. were able to show that the gap-junction connectivity of osteocytes was adversely affected by dexamethasone treatment of an osteocyte cell line (MLYO-cells). These dexamethasone-induced changes resulted in a suppressed amount of Connexin 43 due to degradation by autophagy, thus leading to shortening of osteocyte dendrites, which likely contributes to the compromised connectivity between osteocytes (149). Furthermore, GCs were shown to impair the skeletal vasculature leading to a reduction in solute transport from the circulation to the osteocyte-lacunar-canalicular network and a decrease in the interstitial fluid, thereby compromising bone strength (150). Interestingly, PTH treatment was able to rescue skeletal vascularity during GC exposure (151). More recently, two studies highlighted the role of the skeletal vasculature in the context of GCs during growth. GC-exposure in young mice (typically around 3 weeks of age) impaired angiogenesis and osteogenesis simultaneously (152, 153). Liu et al. were able to show that osteoclast-derived angiogenin was decreased in response to elevated levels of GCs, leading to an increase in blood vessel senescence (153).

In summary, GCs exert a detrimental impact on the function and lifespan of osteocytes leading not only to compromised bone formation but also to disruptions in the lacunar-canalicular network (**Figure 1**). The GC-induced dysfunction of the osteocyte-canalicular network may represent a potential mechanism underlying the predisposition to developing fractures shortly after initiation of GC treatment prior to any significant decreases in BMD – a frequent clinical observation (8). The role of the skeletal vasculature in GIO has been highlighted through recent studies and its role needs further exploration – particularly its connection to bone cells (i.e. osteoblast, osteocytes and osteoclasts) as well as its link to fracture risk.

THE EFFECTS OF GC EXCESS ON OSTEOCLASTS

While the adverse effects of GCs on osteoblasts and osteocytes contribute to the long-term phase of bone loss and compromised bone strength in GIO, the initial rapid phase of bone loss typically observed in humans and rodents originates from a rapid induction of osteoclast-mediated bone resorption. In a number of *in vivo* studies, treatment of rodents with GCs results in a rapid elevation of systemic parameters of bone resorption including serum and/or urinary bone resorption markers, such as carboxy-terminal collagen crosslinks (CTX) and tartrate-resistant acid phosphatase-5b (TRAP-5b), upon exposure to supra-physiological levels of GCs (17, 41, 43, 46, 49). In addition, in the bones from GC-treated rodents, an increase in the number of osteoclasts, as well as an increase in gene expression of osteoclast-mediated bone resorption have been reported shortly after exposure to exogenous GCs (17, 45, 46, 48, 49). While some studies also showed upregulation of osteoclast activity and bone resorption markers at later time-points (41, 47, 154, 155), other studies failed to detect increases in bone resorption especially after prolonged GC exposure (45, 156). In addition, one study by Henneicke et al. showed that treatment with corticosterone affected osteoclasts in a site-specific manner in rodents: an increase in osteoclasts was detected in the endocortex, while they were reduced in the pericortex of tibia from GC-treated mice (43).

Several *in vivo* and *in vitro* studies have determined that the mechanisms of elevated osteoclast-mediated bone resorption in GIO originate not only from direct effects of GCs in osteoclasts, but also from indirect effects *via* the osteoblast lineage. It has been shown that the early increase in osteoclastic bone resorption may be accounted for by an increase in the survival of mature osteoclasts and reduced predisposition to apoptosis (48, 56, 57, 157). However, the direct impact of excess GCs on osteoclastogenesis and osteoclast activity has been controversially discussed due to conflicting results from *in vitro* studies. While some authors observed that pharmacological GCs augmented osteoclast formation and resorptive activity (158–160), others reported a reduction in proliferation of osteoclast precursors (56, 157). Additionally, bone marrow macrophages (osteoclast precursors) from GC-treated animals gave rise to a lower number of osteoclast precursors *ex vivo* than their placebo controls (45, 48). Furthermore, exposure of *in vitro*-formed osteoclasts to GCs increased their longevity, yet, in the same study, it decreased their resorptive function due to defects in cytoskeleton reorganization (56, 157). Interestingly, a recent study found that dexamethasone delayed the formation of multinucleated osteoclasts on plastic surfaces yet increased the formation of resorption pits on dentin slides (161). Ultimately, the contribution of direct effects of GCs on osteoclasts to the overall phenotype of GIO remains unclear due to the large amount of conflicting data.

In contrast, the indirect effects of GC excess on osteoclastogenesis and bone resorption have been well characterized across both *in vivo* and *vitro* studies. The receptor activator of NF-κB ligand (RANKL) – osteoprotegerin (OPG) system, which plays a crucial role in the differentiation of osteoclasts, is affected to a large degree by pharmacological levels of GCs. Several studies demonstrated that supraphysiological levels of GCs induce the expression and production of RANKL from osteoblasts in culture (162–165), a finding also confirmed *in vivo* (144, 166). Administration of a human anti-RANKL antibody to mice expressing human RANKL conferred protection from GC-induced bone loss (166). Some studies suggest that osteocytes – rather than osteoblasts – are the principle source of RANKL *in vivo* (167, 168); however, a more recent study failed to show an increase in RANKL in the osteocyte-enriched bones from GC-treated rodents (47). Interestingly, in the same study a genetic knockdown of *Rankl* specifically in osteocytes provided partial protection from GC-induced bone loss *via* reversal of the osteoclast induction (47).

Aside from RANKL, GCs have been shown to reduce the production of OPG, the decoy receptor of RANKL, from osteoblasts and/or osteocytes, which may aide GC-driven osteoclastogenesis (47, 144, 162–165, 169, 170). Additionally, administration of OPG was able to reduce GC-induced bone resorption in calvarial organ culture (165) as well as prevent GC-induced bone loss in rodents (116). Indeed, some studies suggest

that the increase in the ratio between RANKL and OPG in bone may be largely due to suppressed OPG rather than due to increased RANKL (47, 144, 169). Other indirect contributors to GC-induced bone resorption include macrophage colony-stimulating factor (M-CSF): exposure of osteoblasts to pharmacological levels of GCs was shown to induce the expression of M-CSF, which acts as an essential factor for osteoclast differentiation (171).

In summary, GCs certainly exert direct effects on osteoclasts; however, whether these direct effects contribute to the phenotype of GC-induced bone loss remains controversial. In contrast, *in vivo* and *in vitro* studies clearly demonstrate that GCs readily induce osteoclast formation indirectly through upregulation of pro-osteoclastogenic factors derived from cells of the osteoblast lineage (**Figure 1**).

TARGETING OSTEOBLASTS AS A THERAPEUTIC APPROACH FOR THE MANAGEMENT OF GIO

As the mainstay of osteoporosis therapy anti-resorptive bisphosphonates have been widely used in the therapy of GIO. Generally, the use of bisphosphonate in GIO leads to an increase in bone mineral density compared to placebo or calcium and vitamin D supplements (15). Thus, three different bisphosphonates are currently approved for the treatment of GIO, namely risedronate (172, 173), alendronate (174) and zoledronic acid (175). Zoledronic acid has been shown to be superior to risedronate in GIO and postmenopausal osteoporosis (175) and is generally considered the most potent bisphosphonate. Although not an osteoanabolic therapy, denosumab, a RANKL inhibitor, counteracts a key mechanism of GCs in bone – the induction of RANKL release from osteoblasts and osteocytes. Clinical studies showed a larger increase in bone mineral density (BMD) during denosumab therapy compared to risedronate confirming its superiority to one of the bisphosphonates in GIO (176, 177). Unfortunately, denosumab has not yet been evaluated against the most potent bisphosphonate zoledronic acid in the context of GC use, but its value in the treatment of GIO is undeniable.

While bisphosphonates and denosumab have been successfully utilized to combat GIO, they only offset the GC-induced activation of osteoclasts – which is of particular importance during the initial stage of GC-therapy. However, as outlined above, bisphosphonates fail to address the suppression of osteoblast and osteocyte function, which are a crucial part of the pathogenesis of GIO. The development of targeted osteoporosis therapies opens up the possibility of targeting the mechanism underlying GIO more specifically.

Currently only one osteoanabolic agent, targeting bone formation directly, is approved for the treatment of GIO. As a parathyroid hormone (PTH) analog (1-34 PTH), teriparatide primarily stimulates bone formation – even though bone resorption is activated in response to teriparatide as well. However, bone resorption is initiated much later than bone formation resulting in an 'anabolic window', during which new bone is formed (178). Mechanistically, as an anabolic therapy it mitigates the GC-induced suppression of osteoblast (and osteocyte) activity, which forms a key part of the mechanism underpinning GIO. In the clinical setting, teriparatide has been shown to increase BMD to a larger extent than risedronate (179) and alendronate (180, 181) during GC exposure, thus highlighting the key role of osteoanabolic therapy for GIO. At this stage, no adequate comparison between teriparatide and denosumab exists during GIO (182), hence, no conclusions may be drawn regarding their relative potency in the context of GC therapy.

Novel osteoanabolic therapies such as the PTH-related protein analogue abaloparatide (183) and the anti-sclerostin antibody romosozumab (184, 185), which have been approved for the use in postmenopausal osteoporosis, have not yet been evaluated in GIO. Given their osteoanabolic properties, they may prove similarly effective as teriparatide.

In summary, all available pharmacological therapies are effective in GIO, this includes bisphosphonates, denosumab as well as teriparatide (**Table 1**). Therapies, which target the molecular and cellular mechanisms of GCs in the skeleton such as denosumab and teriparatide, have been shown to be superior to bisphosphonates in GIO. Some (186) but not all (187) guidelines reflect this by recommending the use of teriparatide in severe cases of GIO or following the occurrence of fractures under treatment with bisphosphonates.

SUMMARY

Glucocorticoids affect the three main cell types within the skeleton – osteoblasts, osteocytes and osteoclasts – ultimately leading to a loss of bone mass and bone quality as well as causing

TABLE 1 | Current and future pharmacological GIO therapy.

Drug	Administration	Mechanism of action	Renal function	Approval for GIO
Risedronate	oral, 5 mg daily or 35 mg weekly	anti-resorptive (bisphosphonate)	avoid if GFR < 50 (35) mL/min/1.73	yes
Alendronate	oral, 70 mg weekly	anti-resorptive (bisphosphonate)	avoid if GFR < 50 (35) mL/min/1.73	yes
Zoledronic acid	i.v., 5 mg every 12 months	anti-resorptive (bisphosphonate)	avoid if GFR < 50 (35) mL/min/1.73	yes
Denosumab	s.c., 60 mg every 6 months	anti-resorptive (RANKL antibody)	no adjustment	yes
Teriparatide	s.c., 20 µg daily	osteo-anabolic [recombinant PTH (1-34)]	no adjustment	yes
Abaloparatide	s.c., 80 µg daily	osteo-anabolic [recombinant PTH (1-34)]	no adjustment	no
Romosozumab	s.c., 210 mg every month	osteo-anabolic (synthetic PTHrp analog)	no adjustment	no

(Of note, in addition to calcium and vitamin D supplementation).

a substantial increase in fracture risk. Preclinical studies have highlighted the key role of osteoblasts and osteocytes in the pathogenesis of glucocorticoid-induced osteoporosis and emerging clinical evidence supports the superiority of osteoblast-targeted therapies. Future studies should develop and evaluate therapeutic strategies that not only alleviate GC-induced bone resorption but also prevent the GC-induced damage to osteoblasts and osteocytes and activate bone formation. Furthermore, novel aspects of GIO such as the role of the skeletal vasculature ought to be explored in greater detail.

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Therapy With Intravenous Methylprednisolone Pulses Is Associated With Loss of Bone Microarchitecture in Trabecular Bone Score -Assessment Among Patients With Moderate-to-Severe Graves' Orbitopathy: A Pilot Study

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Background: Therapy with intravenous glucocorticoids (GCs) is associated with various side effects, however, the impact on bone remains elusive. Trabecular bone score (TBS) is a diagnostic tool providing information on bone microarchitecture based on images obtained from dual-energy X-ray absorptiometry. We investigated the influence of the intravenous methylprednisolone (IVMP) pulse administration on TBS in patients with moderate-to-severe Graves' orbitopathy (GO).

Methods: Fifteen patients with GO were treated with 12 IVMP pulses (6x0.5g, 6x0.25 g on a weekly schedule). They received supplementation with 2000 IU of vitamin D and 1.0 g of calcium throughout the study period. TBS was assessed at baseline and after last IVMP pulse. To determine the difference between values at baseline and after treatment the least significant change (LSC) methodology was used. We compared pre- and posttreatment mean TBS values.

Results: We found a significant decrease of TBS in 5 out of 15 (33%) patients. Mean TBS value decreased becoming 2.4% lower than at baseline ($p<0.05$).

Conclusions: IVMP pulse therapy exerts negative effect on bone microarchitecture in TBS assessment. The analysis of the clinical risk factors for osteoporosis and the evaluation of bone mineral density and TBS should be considered before initiating IVMP therapy.

Keywords: graves' orbitopathy, graves' ophthalmopathy, trabecular bone score, EUGOGO, methylprednisolone

INTRODUCTION

Glucocorticoids (GCs) are highly effective and widely used for treatment of various autoimmune diseases. Therapy with GCs is associated with multiple side effects (1–5), including the negative impact on bone, leading to secondary osteoporosis and an increased risk of fractures (6, 7).

GCs affect bone in several ways: they decrease calcium absorption in the gut (8), reduce calcium reabsorption in the kidney (9), decrease production of sex hormones (10), cause proximal muscle weakness (11). However, the main negative mechanism involves the increase of the osteoclast lifespan, stimulation of the osteocyte and the osteoblast apoptosis, and the decrease of the osteoblastogenesis. These processes lead to enhanced bone resorption, decreased bone formation, and finally reduction of bone quality and structure (12, 13).

GCs have probably a greater impact on bone microarchitecture rather than on bone mass (14–16). Among patients treated with oral GCs individual fracture risk is increased independently of bone mineral density (BMD) as the fractures often occur with non-osteoporotic T-score values (10, 17). The effects of GCs on bone quality are not fully expressed by BMD measurement (14, 16, 18). Therefore, to identify GCs-treated patients with high risk of fracture, the analysis of other factors contributing to bone strength and resistance to fracture is needed.

The trabecular bone score (TBS) is a non-invasive technique that performs novel gray-level texture measurements on lumbar spine DXA images, and thereby enables estimating trabecular microstructure and assessment of bone quality (19). Low TBS values indicate weak bone, prone to fractures with fewer poorly connected trabeculae, whereas elevated TBS values reflect denser bone with stronger bone microarchitecture. As shown in previous studies, TBS might be a good indicator of bone health in patients treated with GCs, as it seems to be more sensitive than BMD in detecting the GCs-induced fractures (14, 20–22).

Although the negative impact of oral GCs on bone microarchitecture was demonstrated in several studies (18, 20–22), the influence of intravenous GCs remains elusive. A few studies suggest no negative impact of intravenous methylprednisolone (IVMP) on BMD (23–26). Others demonstrate loss of BMD (27, 28). There is only one pilot study reporting no change of TBS after IVMP pulse therapy (29).

GCs administered intravenously are commonly used in a variety of autoimmune diseases. IVMP therapy in weekly infusions is still the first-line treatment according to the latest European Group on Graves' Orbitopathy (EUGOGO) recommendations in patients with moderate-to-severe and active Graves' orbitopathy (GO) (30–32). Throughout the therapy bone protection is recommended (32). The aim of our study was to investigate early changes of TBS after IVMP therapy with cumulative dose of 4.5 g in euthyroid patients with active, moderate-to-severe GO.

MATERIALS AND METHODS

Patients

Consecutive patients with active, moderate-to-severe GO were enrolled to participate in the study between 2018 to 2021. GO was diagnosed and treated according to the EUGOGO recommendations (33). The study was conducted at the Department of Internal Medicine and Endocrinology, Medical University of Warsaw. Exclusion criteria were: age < 20 years; BMI < 17 kg/m2 or > 37 kg/m2; treatment with oral or intravenous GCs within the last 6 months; any other treatment known to significantly alter bone metabolism (i.e. bisphosphonates or other drugs with anti-fracture effects, heparin, vitamin-K antagonists, proton pump inhibitors, selective serotonin reuptake inhibitors, benzodiazepines, antiepileptic, antipsychotic drugs); clinical diagnosis of osteoporosis based on the presence of low-energy fractures, or BMD measurements (DXA T score below -2.5 SD), as defined by the World Health Organization (WHO) (34). We included to the study 15 patients: 14 patients diagnosed with Graves' disease and 1 patient with Hashimoto thyroiditis. Among patients with Graves' disease, 11 patients were treated with antithyroid drugs (alone or according to a "block and replace" schedule) and 3 individuals who were at least 6 months after the last radical treatment (thyroidectomy and/or radioiodine therapy) received levothyroxine. One patient had Hashimoto thyroiditis treated with levothyroxine. All patients remained clinically euthyroid, with free triiodothyronine (fT3) and free thyroxine (fT4) levels within the reference range at baseline, in the last month prior to the study as well as throughout the time of the IVMP therapy. All patients were treated with IVMP pulses in a 12-week protocol (six infusions of 0.5 g, followed by six infusions of 0.25 g; cumulative dose 4.5 g) according to the current EUGOGO recommendations (33). Supplementation with 2000 IU of vitamin D and 1.0 g of calcium daily was routinely initiated in all patients at the beginning of IVMP therapy and continued throughout the study. The 25-hydroxyvitamin D [25(OH) D] concentrations below 20 ng/mL were described as deficient, concentrations of 20-30 ng/mL as suboptimal, and concentrations higher than 30 ng/mL as optimal vitamin D status, based on the guidelines for vitamin D supplementation and treatment of deficits approved in Central Europe (35). The baseline characteristics of the study group are presented in **Table 1**. All procedures were performed in accordance with the 1964 Helsinki declaration. Informed and written consent was obtained from all individual participants included in the study. The study was approved by the Local Bioethics Committee (KB/197/2018).

BMD and TBS Evaluation

Areal BMD (grams per cm2) of the lumbar spine (L1–L4) and the femoral neck were assessed using dual-energy X-ray absorptiometry (DXA) at baseline (within 2 weeks before IVMP therapy) and within one month after the 12th IVMP pulse. DXA scans were performed by one technician using the

TABLE 1 | Baseline characteristics of patients (n = 15).

Characteristic	Number of patients (%) or mean ± SD (range)
Age, years	53.6 ± 10.6 (40 ÷74)
Male/female	2/13 (13%/87%)
Menopause (in women)	6 (46%)
Years after menopause (in women)	15.3 ± 15.3 (5.0 ÷ 30.0)
Body mass index (kg/m2)	28.0 ± 6.0 (20.3 ÷ 37.0)
Current smokers	6 (40%)
Thyroid disease	
Duration of thyroid disease (years)	3.6 ± 6.9 (0.3 ÷ 24.0)
Graves' disease treated for hyperthyroidism	11 (73%)
Graves' disease after radical treatment on levothyroxine	3 (20%)
Hashimoto thyroiditis on levothyroxine	1 (7%)
Duration of therapy with antithyroid drugs (months)[a]	8.6 ± 4.4 (4.0 ÷ 18.0)
TSH (reference range: 0.27–4.2 μIU/mL)	1.8 ± 1.7 (0.005 ÷ 5.1)
fT4 (reference range:12.0–22.0 pmol/L)	15.3 ± 2.7 (12.0 ÷ 21.1)
fT3 (reference range: 3.1–6.8 pmol/L)	4.3 ± 0.9 (3.1 ÷ 6.2)
TRAb (reference range: <1.8 IU/l)	13.5 ± 12.5 (1.7÷ 40.0)
25(OH)D (ng/mL)	32.3 ± 12.3 (13.8 ÷ 57.0)
DXA lumbar spine: BMD (g/cm2)	1.05 ± 0.1 (0.89 ÷ 1.25)
DXA lumbar spine: T-score (SD)	-0.01 ± 1.1 (-1.6 ÷ 1.8)
DXA lumbar spine: Z-score (SD)	0.99 ± 1.6 (-1.2 ÷ 4.1)
DXA femoral neck: BMD (g/cm2)	0.83 ± 0.1 (0.61 ÷ 0.93)
DXA femoral neck: T-score (SD)	-0.23 ± 0.7 (-2.1 ÷ 0.6)
DXA femoral neck: Z-score (SD)	0.61 ± 0.8 (-0.7 ÷ 2.5)
Densitometric osteopenia	4 (27%)
Lumbar spine: osteopenia	3 (20%)
Femoral neck: osteopenia	2 (13%)
TBS for vertebrae L1-L4	1.31 ± 0.2 (0.92 ÷ 1.50)
Partially disturbed microarchitecture	2 (13%)
Degraded microarchitecture	3 (20%)

Hologic Discovery A Densitometer. BMD measurements were calculated, and Z-scores and T-scores were subsequently analyzed by the same physician. Osteoporosis and osteopenia were diagnosed in individuals with a T-score of the lumbar spine and/or the femoral neck ≤−2.5 standard deviation (SD) and between <−1.0 and >−2.5 SD, respectively (34).

TBS was calculated using iNsight® Software (version 3.0, Med-Imaps, Pessac, France) on the DXA lumbar spine (L1-L4) images. The TBS absolute values for the sum of vertebrae L1-L4 were reported. The absolute TBS values <1.230 were considered as the degraded microarchitecture, TBS values between ≥ 1.23 and <1.31 indicated partially disturbed bone microarchitecture, whereas TBS values ≥ 1.31 were assessed as normal (36).

The least significant change (LSC) methodology was used to evaluate the differences in BMD expressed in g/cm^2 and TBS values before and after the IVMP treatment. LSC values for BMD and TBS were calculated for the DXA device in the Medical University of Warsaw's densitometry unit and were estimated to be 3% for the lumbar spine BMD, 5.4% for the femoral neck BMD and 4.6% for TBS. An increase or decrease of BMD or TBS equal to or exceeding the LSC was considered significant.

Laboratory Evaluation

Thyroid-stimulating hormone (TSH), fT3, fT4, thyrotropin receptor antibodies (TRAb) and 25(OH)D levels were assessed at baseline and after the last IVMP pulse. TSH, fT3, fT4, TRAb and 25 (OH)D were examined using an electrochemiluminescence immunoassay on Cobas 8000 Analyzer (Roche Diagnostics, Mannheim, Germany). The reference ranges for TSH, fT3, fT4 and TRAb were: 0.27–4.2 μIU/mL, 3.1–6.8 pmol/L, 12.0–22.0 pmol/L and <1.8 IU/mL, respectively.

Statistical Analysis

All analyses were performed using SPSS statistical software version 22.0 (IBM SPPS Statistics, New York, US). Continuous variables are expressed as means ± standard deviation (SD), while categorical variables are expressed as numbers (n) and percentages (%). The Shapiro-Wilk test was used to confirm or reject the normal distribution of each continuous variable. Comparisons between continuous data were performed using paired t-test (for parameters with normal distribution) or Wilcoxon rank sum test (for parameters with distribution deviations). Categorical data were analyzed using Fisher exact test. Comparisons between continuous data were performed with the Mann – Whitney U test. Pearson correlation test was performed to investigate correlations. Statistical significance was established for results with $p<0.05$.

RESULTS

Baseline Data

At baseline, 3 out of 15 patients (20%) had degraded, and 2 out of 15 patients (13%) had partially disturbed microarchitecture. We found osteopenia in 4 out of 15 patients (27%): in 2 patients only in the lumbar spine BMD, in 1 patient only in the femoral neck

BMD and in another patient in both measurement sites. The baseline TBS values correlated negatively with BMI (r=-0.72, p=0.003). We observed vitamin D deficiency in 2 patients.

Effect of IVMP Therapy on TBS and BMD

According to the LSC criteria, we found the following changes in TBS and BMD after 12 weeks of IVMP therapy (**Figure 1**):

- decrease in TBS in 5 out of 15 patients (33%)
- decrease of BMD in 2 out of 15 patients (13%; 1 in the lumbar spine BMD, 1 in the femoral neck BMD)
- increase of BMD in 7 out of 15 patients (47%; all in the lumbar spine BMD)
- no increase of the femoral neck BMD
- no increase of TBS.

Mean TBS value decreased becoming 2.4% lower than at baseline (p=0.04). Mean lumbar spine BMD increased becoming 1.6% higher after IVMP therapy than at baseline (p=0.047). There was no significant change in mean post-treatment femoral neck BMD as compared to the baseline. (**Figure 2**, **Table 2**).

The correlations between the baseline TBS values and the changes in TBS with selected parameters are presented in **Table 3**.

There were no significant differences between the groups with decreased TBS (drop in TBS equal to or exceeding the LSC) vs. no change in TBS after IVMP treatment as far as the selected characteristics were considered (**Table 4**).

In **Table 1** (of the **Supplementary Material**) we have included the results of BMD and TBS of our study group before and after treatment with IVMP pulses.

A decrease of TBS equal to or exceeding LSC value occurred in 2 out of 2 women and in none out of 2 men with osteopenia before IVMP therapy. In one woman with degraded TBS a decrease exceeding LSC value occurred. There was no correlation between the changes in TBS and the baseline TBS values (r =-0.03, p=0.91). However, no correlation was found between the changes of TBS and the baseline lumbar spine BMD, or the femoral neck BMD. Details are presented in **Table 3**.

There was no significant difference in serum 25(OH)D concentration. No correlation was found between changes in TBS and baseline vitamin D status.

DISCUSSION

The presented study revealed a decrease of the mean TBS value in patients with GO treated with IVMP. The reduction of TBS value equal to or exceeding the LSC occurred in 33% of the patients. In contrast, we observed the overall increase of the mean BMD in the lumbar spine as well as the increase in the lumbar spine BMD exceeding the LSC value in almost half of the study group.

This is the first study showing the negative effect of the intravenous GCs on bone microarchitecture. The current results differ from research performed by Censi et al. involving GO patients treated with IVMP pulse therapy, in which no change in neither of TBS nor BMD was found (29). The possible explanations for the divergent results include different cumulative doses of IVMP (4.5 g of IVMP in the current research vs. 1.5-5.25 g of IVMP in Censi et al. study), different timing of follow-up and no analysis according to the LSC criteria

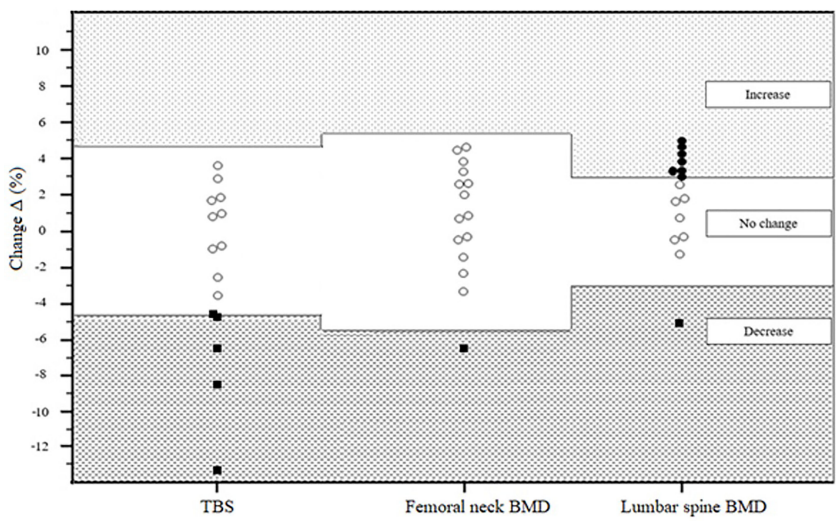

FIGURE 1 | Percentage of TBS and BMD changes according to the LSC criteria in 15 patients after IVMP therapy. TBS trabecular bone score BMD bone mineral density LSC least significant change IVMP intravenous methylprednisolone. Bullets and squares represent individual percentage of BMD and TBS changes (black squares represent a decrease in TBS or BMD – equal to or exceeding LSC calculated to be 4.6% change for TBS, 5.4% change for femoral neck and 3% change for lumbar spine; black bullets represent an increase in BMD equal to or exceeding LSC; white bullets represent no change in TBS or BMD). BMD, bone mineral density; IVMP, intravenous methylprednisolone therapy; LSC, least significant change; TBS, trabecular bone score.

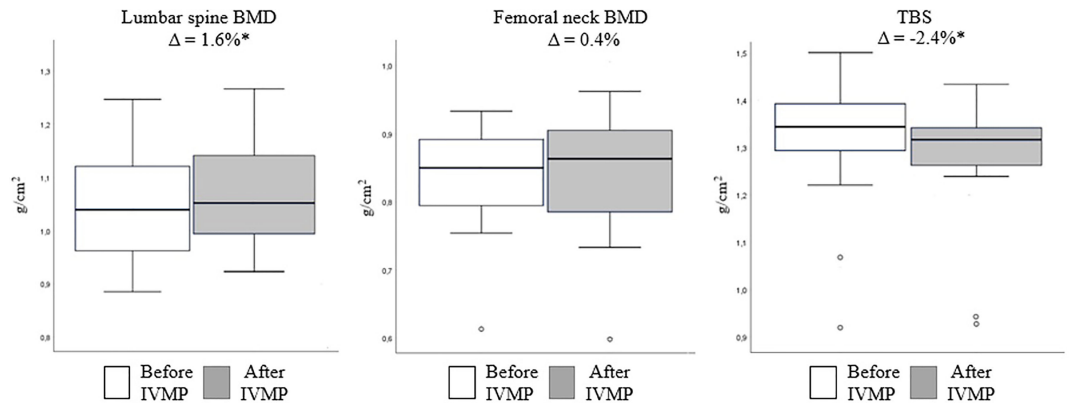

FIGURE 2 | Mean bone mineral density and trabecular bone score values before (white) and after (gray) intravenous methylprednisolone (IVMP) therapy. Values shown above are Δ, calculated as 100 x (after IVMP value – before IVMP value)/before IVMP value. Data are expressed as mean ± SD. *p<0.05 vs. baseline. BMD, bone mineral density; TBS, trabecular bone score; IVMP, intravenous methylprednisolone therapy; SD, standard deviation.

in Censi et al. research. The results of our research stay in agreement with recent studies involving patients treated with oral GCs that demonstrate the decline of TBS while BMD remains unchanged (20–22). The trabecular bone microarchitecture seems to be more affected than BMD not only in patients treated with oral but with intravenous GCs as well. The findings of our study bring evidence that the adverse effects of IVMP on bone are not fully captured with BMD evaluation and that TBS adds value to the BMD assessment.

Based on previous studies, the decrease in bone quality plays a significant role in the rapid increase in fracture risk occurring in GCs-treated patients. Fractures may be better predicted when TBS is used in addition to BMD (37–40). It highlights the clinical need for methods that can identify GCs-treated individuals particularly vulnerable to steroid-induced deterioration of bone quality, as those are at increased risk of fractures. The results of current study indicate that the decrease in TBS despite the increase of BMD might be an indicator that the intravenous GCs are not so safe in terms of bone safety. Further studies with a larger study group should include vertebral and non-vertebral fracture assessment during therapy as well as in the follow-up period.

Some of the clinical risk factors need to be taken into consideration when assessing the influence of GCs on bone microarchitecture. We found a negative correlation of baseline TBS with BMI. This stays in agreement with other reports suggesting that lower TBS values are present more frequently in patients with higher BMI (41). However, we noticed no correlation between change in TBS and BMI. We found no correlations between baseline TBS, or change in TBS and baseline levels of TSH, fT4, fT3, TRAb or duration of treatment with antithyroid drugs. There were no differences in baseline TSH levels between subjects with a decrease versus those lacking any change in TBS.

Another issue that should be considered is that Graves' disease itself has been demonstrated to have a strong correlation with decreased TBS due to the hyperthyroidism that increases bone resorption (42). Bone loss is enhanced because of the thyrotoxicosis as well as the excessive release of inflammatory cytokines (43). In the present study, all the patients had documented fT4 and fT3 within the reference range for at least one month before the first IVMP infusion as well as during the whole therapy. During the observation time the majority of patients were treated with antithyroid therapy, which had been initiated without delay in the past. Previous studies concerning the reversibility of bone loss after the initiation of antithyroid therapy have shown that bone quantity measured by BMD as well as bone quality measured using TBS improve simultaneously after thyroid function normalization (44, 45). The increase in BMD after IVMP therapy observed in current as well as in our previous study (23) were greater for sites rich in trabecular bone (lumbar spine) than with cortical bone (femoral neck), as trabecular bone is more metabolically active, and changes occur earlier than in cortical bone (46). Taking

TABLE 2 | Comparison of bone mineral density and trabecular bone score between baseline and after intravenous methylprednisolone therapy (n = 15).

Variable	Before IVMP	After IVMP	P
Lumbar spine BMD (g/cm^2)	1.05 ± 0.11	1.07 ± 0.11	0.047
Femoral neck BMD (g/cm^2)	0.83 ± 0.08	0.84 ± 0.10	0.43
TBS	1.31 ± 0.15	1.28 ± 0.16	0.04

Continuous variables are presented as means ± SD.
BMD, bone mineral density; IVMP, intravenous methylprednisolone; SD, standard deviation; TBS, trabecular bone score.

TABLE 3 | Correlations between the baseline trabecular bone score (TBS) and change in TBS with selected parameters.

Parameter	Baseline TBS	Change in TBS
Age	r=0.06, p= 0.84	r=-0.18, p=0.52
Baseline BMI	r=-0.72, p=0.003	r=-0.10, p=0.71
Baseline TRAb	r=0.18, p= 0.52	r=-0.32, p=0.25
Baseline TSH	r=-0.01, p= 0.97	r=0.01, p=0.99
Baseline 25(OH)D	r=0.35, p=0.20	r=-0.03, p=0.93
Baseline lumbar spine BMD	r=0.42, p=0.12	r=0.12, p=0.67
Change in lumbar spine BMD	r=0.23, p=0.41	r=0.26, p=0.35
Baseline femoral neck BMD	r=-0.27, p=0.33	r=0.04, p=0.90
Change in femoral neck BMD	r=-0.26, p=0.34	r=0.02, p=0.95
Duration of therapy with antithyroid drugs[a]	r=-0.15, p=0.65	r=0.31, p=0.31

[a]*Analysis performed in 11 patients treated with antithyroid drugs throughout the study.*

BMD, bone mineral density; BMI, body mass index; IVMP, intravenous methylprednisolone; TBS, trabecular bone score; TRAb, TSH receptor antibodies; TSH, thyroid-stimulating hormone.

everything into consideration, we cannot exclude that the BMD changes during the therapy with intravenous GCs were secondary to the ongoing restoration of BMD after the stabilization of thyroid function as well as the reduction of the inflammatory state. However, the decrease of TBS occurring simultaneously with the increase of lumbar spine BMD indicates that in the course of the IVMP therapy the restored bone density might have been unevenly distributed within the trabecular bone. Further study and analysis are needed in order to confirm our observations as well as to identify patients particularly vulnerable to bone quality deterioration during IVMP therapy.

There are studies indicating that the supplementation with vitamin D might be effective in preventing of bone loss in GCs-treated patients (47). In our study the mean vitamin D status at baseline was optimal, the supplementation with 1.0 g of calcium and 2000 IU of vitamin D was continued through the whole study period. We did not observe any correlations between changes in TBS and baseline vitamin D status. There was also no difference between groups with and without decrease of TBS equal to or exceeding LSC as far as baseline 25(OH)D concentration was taken into account. It is noteworthy that despite the optimal vitamin D status at baseline and the sufficient vitamin D prophylaxis, the decrease in TBS could not be prevented.

The biggest limitation of our study is a small sample size including men, pre- and postmenopausal women, different age groups and the reassessment of TBS after a relatively short follow-up period. However, the strength of our study is the therapy of all subjects according to the same protocol. We consider this research as a pilot study that allows us to design larger prospective research with longer follow-up period. More studies with additional measurements of TBS and fracture assessment are needed to determine whether TBS improves with time and if the fractures occur in patients after IVMP therapy. Patients with GO sometimes require the second-line treatment including, among others, the second course of IVMP with a higher cumulative dose (7.5 g) or oral GCs, which may potentially further exacerbate bone microarchitecture. Finally, TBS is an indirect index of bone microstructure state, and the accuracy of TBS measurements might be affected by body composition (48). However, each patient served as his or her own control.

It is noteworthy that according to the latest EUGOGO recommendations (32), in the most severe forms of moderate-to-severe and active GO, a higher cumulative dose of IVMP (7.5 g) is recommended as an alternative first-line treatment. Further studies aimed to assess bone microarchitecture in patients treated with different cumulative doses of IVMP (4.5 g

TABLE 4 | Comparison of selected baseline characteristics of patients with and without decrease of trabecular bone score (TBS) (decrease in TBS equal to or exceeding the least significant change) after intravenous methylprednisolone therapy.

Characteristic	Decrease of TBS 5/15 (33%)	No change of TBS 10/15 (66%)	P
Age (years), mean ± SD	59.0 ± 13.8	50.9 ± 8.1	0.33[b]
Women, n (%)	5 (100%)	8 (80%)	0.52[a]
Women after menopause, n (%)	3 (60%)	3 (30%)	0.59[a]
BMI (kg/m^2),	27.8 ± 6.3	27.4 ± 4.8	0.90[b]
Smokers, n (%)	2 (40%)	4 (40%)	1.00[a]
TSH (μIU/mL), mean ± SD	1.5 ± 2.0	1.9 ± 1.6	0.76[b]
TRAb (IU/L), mean ± SD	20.0 ± 17.4	10.3 ± 8.6	0.27[b]
25(OH)D (ng/mL), mean ± SD	27.5 ± 7.0	34.7 ± 14.0	0.46[b]
Osteopenia (T score -1.0 to >-2.5), n (%)	2 (40%)	2 (10%)	0.56 [a]
Degraded or partially disturbed microarchitecture (TBS <1.31), n (%)	1 (20%)	4 (40%)	0.60[a]
Lumbar spine BMD (g/cm^2), mean ± SD	1.040 ± 0.1	1.061 ± 0.1	0.81[b]

[a]*Chi-squared Test;*

[b]*Mann – Whitney U test.*

BMD, bone mineral density; BMI, body mass index; TBS, trabecular bone score; TSH, thyroid-stimulating hormone; TRAb, TSH receptor antibodies; SD, standard deviation; 25(OH)D, 25-hydroxyvitamin D.

vs. 7.5 g) are needed. The assessment of the impact of IVMP on bone markers would have also a great value as the mechanisms through which IVMP exerts its effects on bone remain not fully recognized.

In conclusion, our study revealed that IVMP pulse therapy with cumulative dose of 4.5 g is associated with loss of bone microarchitecture in TBS assessment with no negative effect on BMD among patients with GO.

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 human participants were reviewed and approved by Local Bioethical Committee of the Medical University of Warsaw. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

JR and PM were responsible for the concept and design of the study. JR and KP collected patient data and prepared the data for analysis. JR, KP, JP, and PM analyzed clinical data. JR was responsible for statistical analysis, tables, and figures. All authors drafted the manuscript. PM supervised all aspects of the study, critically revised the manuscript and approved the final manuscript as submitted. All authors contributed to the article and approved the submitted version.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2022.893600/full#supplementary-material

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