Review



# Side Effects of Glucocorticoids: In Vivo Models and Underlying Mechanisms

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# Side Effects of Glucocorticoids: In Vivo Models and Underlying Mechanisms

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**Abstract:** Glucocorticoids are widely used in the therapy of inflammatory and autoimmune disease as well as cancer. They realize the therapeutic effects via cytotoxic action on the immune cells; however, glucocorticoids are characterized by multiple severe side effects including metabolic and atrophic complications as well as glucocorticoid resistance development. With the progression in the field of steroid research and their mechanism of action, several steroid and non-steroid GC analogues with decreased adverse effects were developed for potential treatment on cancer, inflammatory and autoimmune diseases. Therefore, the important criteria of the evaluation of the efficacy of such molecules is the proof-of-concept studies in vivo on the proper models of the main disease as well as potential GC-related side effects. Here we summarized the current experience of the research groups worldwide in modeling of GC undesirable effects. The presented review will be useful for the translational research of GC and their analogues in vivo.

**Keywords:** glucocorticoid; glucocorticoid receptor; selective glucocorticoid receptor agonists; glucocorticoid side effects; osteoporosis; diabetes; myopathy; skin atrophy; obesity; in vivo models

## Introduction

Synthetic glucocorticoids (GCs) are widely used in clinical practice for their powerful anti-inflammatory and immunosuppressive properties [1–4]. GCs represent the standard therapy for various diseases, as well as allergic, inflammatory, rheumatoid, oncological, vascular, dermatological, and other systemic diseases [5–8]. However, long-term administration of GCs at high doses is frequently associated with severe adverse effects, in particular, metabolic disorders and atrophic effects, including musculoskeletal side effects (glucocorticoid-induced osteoporosis (GIO), osteonecrosis, myopathy), disorders of carbohydrate and fat metabolism (diabetes mellitus, dyslipidemia, obesity), gastrointestinal, cardiovascular, neuropsychiatric side effects [9–11]. Despite multiple studies in the field, there is still no consensus on the most suitable animal models that could representatively demonstrate the pathogenesis of GC side effects. Relevant and reproducible animal models are critical for further studies of the side effects of GCs as well as novel ligands of glucocorticoid receptor (GR). In this review, we summarize current data on design of GC side effects in animals over the past years.

## Glucocorticoid-Induced Osteoporosis (GIO)

### *Pathogenesis*

Both the cumulative dose and duration of exposure to GCs are risk factors for bone fractures[9,12]. Aseptic osteonecrosis is a severe complication of GC therapy. 1,6-7,6% of pediatric

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patients with acute leukemia develop the severe complications after long-term GC therapy [13]. The incidence of GIO reaches 30-50% in patients with prolonged GC therapy [14]. GCs affect the development and viability of osteoclasts, osteoblasts and osteocytes, leading to increased bone resorption and impaired bone formation subsequently associated with an increased fragility due to bone tissue loss [15].

The pathophysiology of secondary GIO involves direct and indirect mechanisms [16]. GC treatment decrease the viability of bone-forming osteoblasts and increase the proliferation of osteoclasts associated with the resorption [17]. Osteoblasts are primary cells responsible for bone development via the regulation of bone matrix synthesis and direct interaction with osteocytes and osteoclasts [18]. It was demonstrated in in vivo and in vitro studies that supraphysiological levels of GCs inhibit proliferation and differentiation of osteoblasts via interaction with multiple signaling pathways [19]. GCs suppress the proliferation of osteoblast precursors until their complete differentiation. In immature osteoblasts in vitro, GCs exposure was associated with cell cycle arrest in G1 phase due to down-regulation of cell cycle activators such as cyclin A, cyclin D, cyclin-dependent kinase 2 (CDK2), CDK4 and CDK6 [20–22], as well as activation of cell cycle inhibitors such as p53, p21 and p27 [20,23]. Suppression of the murine osteoblasts MC3T3-E1 proliferation by dexamethasone (Dex) was associated with G1 phase delay and apoptosis induction accompanied by p53-dependent activation of p21 and the up-regulation of proapoptotic genes *NOXA* and *PUMA* [23]. In addition, GCs inhibit the proliferation of osteoblast precursors via suppression of intracellular mitogenic signaling pathways, specifically mitogen-activated protein kinase (MAPK) signaling [24,25]. In MBA-15.4 murine bone marrow stromal osteoblast cells GCs cause rapid activation of MAPK-1 and dephosphorylation of extracellular signal-regulated kinase (ERK) as well as impaired proliferation [25]. Another mechanism of GC-mediated inhibition of osteoblast differentiation is their direct interference with the Wnt/β-catenin signaling [26,27].

An important mechanism in GIO pathogenesis is associated with increased osteclastogenesis resulting from activation of macrophage colony-stimulating factor (M-CSF) and receptor activator of nuclear factor-kappa B (NF-κB) ligand (RANKL) [28]. In addition, GCs promote osteoclast survival by modulation of receptor activator of NF-κB ligand (RANKL)-induced signaling and inhibition of the expression of osteoprotegerin (OPG) [29]. In addition, GCs have recently been shown to be associated with osteoclast autophagy via inhibition of the PI3K/AKT/mTOR signaling [30]. Association of supraphysiological doses of GCs and increased bone resorption is supported by increase in serum level of resorption markers including tartrate-resistant acid phosphatase (TRAP), carboxy-terminal telopeptide of type I collagen (CTX), urinary calcium/creatinine, cAMP, urinary crossover, and soluble osteoclastogenic cytokine receptor interleukin-6 (sIL-6R$\alpha$ ) [31].

*Animal Models*

Animals including mice, rats, rabbits, dogs, sheep and Zebrafish have been used for GIO modeling [32–36]. Osteoporosis research requires skeletally mature animals to avoid the many complicating factors that arise in young animals with developing bone tissue. Rodent models including Sprague Dawley, Wistar and rarely Albinos/LEW CrlCrlj rats, as well as C57BL/6 mice and New Zealand rabbits, remain preferential for in vivo research. Sex dimorphism has not been reported, however, it is important to note that many osteoporosis models have been developed to study postmenopausal osteoporosis in females with ovariectomy [37–39]. Appropriate age for rats varied from eight weeks to six months. In C57BL/6 mice, the age range was from eight weeks to four months. The advantages of rodent models are high reproducibility, rapid turnover of bone tissue, and relatively low cost. At the same time, short period of remodeling and incapability of achieving truly skeletal maturity should be noted as disadvantages [40]. Adult Zebrafish model for the measurement of the activity of cathepsin K, TRAP and ALP against the background of Dex-induced osteoporosis [41] was recently described with the changes in vertebral bone density, associated osteogenic markers and Zebrafish mobility [35].

*Experimental Protocols: Drugs, Doses, Administration Routes*

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The most widely described drugs for osteoporosis induction are dexamethasone (Dex), prednisolone (Pr), and methylprednisolone (MPr). GIO models described over the past five years are presented in Table 1. Route of administration vary depending on animal species. Large animals (sheep, rabbits) require i.m. or s.c. treatment, i.v. and i.p. with less frequency. At the same time, i.p., i.m. and s.c. (with the option for surgically placing pellets) treatments are more suitable for The optimal dose ranged from 0.1 mg/kg to 100 mg/kg over a time period of 4 to 8 weeks.

**Table 1.** Main characteristics of the proposed experimental protocols for GIO induction.

| GC | Dose | Route of administration | Duration of administration | Animal species | Age, sex | Reference |
|---|---|---|---|---|---|---|
| MPr | 10 mg/kg | i.p. | 1 per day/4 weeks | C57BL/6 mice | 8 weeks, males | [32] |
| Dex | 1 mg/kg | | 1 per day/8 weeks | | | |
| Pr | 5 mg/kg | s.c. | 1 per day/60 days | C57BL/6 mice | 8 weeks, males | [98] |
| Dex | 50 mg/kg | i.p. | 1 per day/5 weeks | C57BL/6 mice | 6 weeks, males | [99] |
| Dex | 2 mg/kg | i.m. | 3 per day/8 weeks | C57BL/6 mice | 3 months, males | [100] |
| Pr | 5 mg/kg | s.c. (pellet implanted) | 60-day slow-release | C57BL/6 mice | 15 weeks, females | [101] |
| Dex | 100 mg/kg | i.m. | 1 per day/4 weeks | C57BL/6J mice | 8 weeks, males | [102] |
| Dex | 10 mg/kg | i.p. | 3 weeks | C57BL/6J mice | 8 weeks, males | [103] |
| Dex | 25 mg/kg | s.c. | 1 per day/4 weeks | Balb/c mice | 9–10 weeks, females | [104] |
| Dex | 1 mg/kg | i.m. | 1 per day/8 weeks | Sprague Dawley rats | 12 weeks, males | [33] |
| Dex | 5 mg/kg | i.m. | twice a week/6 weeks | Sprague Dawley rats | 8 weeks, males | [105] |
| MPr | 10 mg/kg | per os | 1 per day/3 weeks | Wistar rats | 3 months, males | [106] |
| Pr | 0.42 mg/day | s.c. (pellets containing 25 mg) | 60-day slow-release | LEW CrlCrlj rats | 6 weeks, females | [107] |
| Dex | 1 mg/kg | i.m. | 1 per day/60 days | Sprague Dawley rats | 8 weeks, males | [108] |
| MPr | 0.5 mg/kg | i.m. | 1 per day/4 weeks | New Zealand White rabbits | 12 weeks, females | [109] |

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| | | | | | | |
|---|---|---|---|---|---|---|
| MPr | 1 mg/kg | s.c. | 1 per day/6 weeks | New Zealand White rabbits | 5–7 months, females | [34] |
| Pr | 2 mg/kg | per os | 1 per day/2 weeks | Beagle dogs | 2-3 years, males | [36] |
| | 1 mg/kg | | 1 per day/4 weeks | | | |
| | 0.5 mg/kg | | 1 per day/3 weeks | | | |

*GIO Evaluation Criteria*

To evaluate GIO development level, mechanical, histomorphometric and biochemical tests are used including dual-energy X-ray absorptiometry (DXA), micro-computed tomography (μCT), histomorphometry and biochemistry analysis in serum [42–46]. μCT is currently a gold standard for assessing morphology and microarchitecture of bone in mice and other small animals ex vivo. Description of trabecular areas using μCT is carried out with the minimum set of parameters including bone volume fraction, trabecular thickness, trabecular separation, and trabecular number. Secondary end-points including structure model index, connectivity density, degree of anisotropy could be added in the study. The that should be specified For cortical areas minimum set of variables are specified as total cross-sectional area, cortical bone area, cortical thickness, and cortical bone fraction [47].

DXA is a simpler and faster method for quantitative analysis of changes in trabecular bone in small animals. DXA allows measurement of bone mineral content (BMC) and bone mineral density (BMD) [48]. DXA application require careful selection of positions with confirmation of control points to minimize errors [49]. Table 2 summarizes the examples of mouse positions in DXA.

The comparison of different approaches for GIO evaluation are summarized in the Table 3

**Table 2.** Variants of mouse position during DXA.

| | |
|---|---|
| Head | Measurement by site: no reposition |
| Spine | Keep your tail and head as close as possible to a straight line. |
| Fore legs | Head direction/not overlap or rotate |
| Back legs | Head direction/not overlap or rotate |
| Tail | Included in the scan range/not overlap |

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**Table 3.** Comparison of methods of GIO evaluation in cat model.

| DXA | μCT | Histomorphometry | Biochemistry analysis in serum |
|---|---|---|---|
| Bone mineral content (BMC) | Bone volume/tissue volume) (BV/TV) | Percent labeled perimeter (%L.Pm) | Alkaline phosphatase (ALP) |
| Bone mineral density (BMD) | Bone surface/bone volume (BS/BV) | Mineralization apposition rate (MAR) | Tartrate-resistant acid phosphatase (TRAP) |
| | Trabecular number (Tb.N) | Bone formation rate/bone surface referent (BFR/BS) | Osteocalcin (OCN) |
| | Trabecular thickness (Tb.Th) | Bone formation rate/bone volume referent (BFR/BV) | C-terminal telopeptide of type 1 collagen (CTX-I) |
| | Trabecular separation (Tb.Sp) | Bone formation rate/tissue volume referent (BFR/TV) | Bone-specific alkaline phosphatase |
| | Structure-model index (SMI) | Total tissue area (T.Ar) | |
| | Degree of anisotropy (DA) | Cortical area (Ct.Ar), Marrow area (Ma.Ar) | |

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| | | | |
|---|---|---|---|
| | Connectivity density (Conn.D) | Cortical width (Ct.Wi) | |
| | Total cross-sectional area (Tt.Ar) | Percent periosteal-labeled perimeter (%P-L.Pm) | |
| | Cortical bone area (Ct.Ar) | Periosteal-MAR (P-MAR) | |
| | Cortical thickness (Ct.Th) | Osteoclast number/bone surface (Oc.N/BS) | |
| | Cortical bone fraction (Ct.Ar/Tt.Ar) | Percent endocortical-labeled perimeter (%E-L.Pm) | |

**Glucocorticoid-Induced Myopathy and Skin Atrophy**

*Pathogenesis*

GCs induce catabolic/atrophic changes in multiple tissues including muscle, subcutaneous fat, and bone [50,51]. Osteosarcopenia in rheumatoid arthritis treated with GCs occurs in 37.1% of patients with rheumatoid arthritis, and the incidence of sarcopenia increased with age: from 14% in patients aged 40–49 years to 78,6% in patients aged 80–89 years [52]. GC-induced myopathy is the most common type of non-inflammatory toxic drug myopathy characterized by muscle weakness and atrophy, fatigue and fatigability [9,53]. In muscles, GCs decrease the protein synthesis and increase the rate of protein catabolism via various molecular pathways [9]. Detailed molecular mechanisms underlying GC-induced muscle atrophy still remain unclear. Impaired protein synthesis is associated with GC-dependent inhibition of ribosomal protein S6 p70 kinase (p70S6K) [54]. Moreover, insulin-like growth factor 1 (IGF-1) deficiency is assumed to contribute to GC-induced muscle atrophy [55]. IGF-1 activates the proliferative PI3K/Akt signaling blocking GC effects and preventing muscle atrophy [56]. In addition, overexpression of myostatin (MSTN), an inhibitor of muscle growth, leads to muscle cell atrophy by suppression of protein synthesis [57]. Additionally, activation of the ubiquitin-proteasome system and the lysosomal system leads to increased proteolysis and, accordingly, muscle destruction via up-regulation of *atrogin-1, MuRF-1, cathepsin-L, PDK4, p21, Gadd45* and *4E-BP1* [58,59].

Skin atrophy is a side effect of the use of topical and systemic GCs with the changes in all skin compartments: severe hypoplasia, loss of elasticity, increased fragility, telangiectasia, bruising, and barrier dysfunction [60,61]. Several studies have shown that one of main GC-dependent atrophogene in skin and muscle is regulated in development and DNA damage responses 1 (REDD1) gene, negative regulator of mTOR [62–65].

*Animal Models and Protocols*

Glucocorticoid-induced myopathy models described in the literature involves both males and females aged 4 months. Dex was administered i.p. at a dose of 10 mg/kg/day for 15 days. The tibialis

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anterior, gastrocnemius complex, quadriceps, biceps, triceps, and soleus muscles were harvested from the animals and weighed to assess absolute muscle mass. Dynamic treadmill exhaustion test and energy expenditure analysis were used to assess the severity of myopathy [66].

In another model, 6-week-old female Swiss mice (25 g) received Dex i.p. at a dose of 15 mg/kg every 24 hours for 10 days. 10 days after Dex treatment skeletal muscle samples (tibialis anterior, extensor digitorum longus, gastrocnemius, soleus, and diaphragm muscles) were analyzed by hematoxylin and eosin staining to quantify myofibril area [67].

Muscle atrophy of three-week-old male C57BL/6 mice was induced by i.p. administration of Dex at a dose of 15 mg/kg/day for 38 days. DXA was used to determine muscle mass. Muscle performance measurements were performed using a grip strength meter, muscle grip strength was measured by having mice grasp a net with their forelimbs, calculating the average of five consecutive measurements for each animal. Total running distance and time were also assessed using a rodent treadmill set at a 10° incline, with the endpoint set when mice were in contact with the impact grid for 10 s. [68].

Models of GC-induced myopathy in large animals are also described. In particular, beagle dogs, with a median weight of 13.7 kg and a median age of 5 years, were treated with prednisolone p.o. at a dosage of 1 mg/kg once daily for 4 weeks. After skeletal muscle scanning using CT, a skeletal muscle sample was obtained from the biceps femoris muscle and stained with antibodies against myosin heavy chain specifically expressed in fast-twitch and slow-twitch muscle fibers [69].

Rodent models are the most frequently used GC-induced skin atrophy models. Specifically, B6D2 (F1 C57Bl×DBA) mice were treated with topical GCs, for example, fluocinolone acetonide 1μg every 72h for 2 weeks [70] or 24 hours for four consecutive days [71]. Histological analysis was used to evaluate changes in dermal collagen fibers, immunostaining with anti-BrdU antibodies assessing the proliferation and morphometric analysis measuring epidermal width were used for the evaluation of skin thinning skin thinning) [71]. Proliferative index was calculated as the ratio of the number of BrdU+ basal keratinocytes to the total number of basal keratinocytes [70].

## Steroid-Induced Diabetes

### *Pathogenesis*

GCs are diabetogenic hormones inducing peripheral insulin resistance, hyperglycemia, and dyslipidemia. The incidence of steroid-induced diabetes in patients with rheumatologic disorders receiving GCs is 12,7% [72]. For lymphoma malignancy survivors the steroid-induced diabetes rate is 1,5-9% [73].

GC-induced hyperglycemia has a multifactorial origin and can be explained by increased gluconeogenesis in the liver, inhibition of glucose uptake by adipose tissue, and changes in receptor and post-receptor functions [74]. Long-term use of systemic GCs leads to the development of insulin resistance in skeletal muscles [75]. Although GCs are important in maintaining lipid homeostasis, an excess of GCs can lead to an increase in circulating free fatty acids and cause lipid accumulation in skeletal muscle and liver also associated with insulin resistance [76]. Moreover, GCs induce a post-receptor defect by decreasing key mediators of insulin action in peripheral tissues (insulin receptor substrate-1, PI3K, and protein kinase B) [77]. The mechanisms of GC-dependent inhibition of insulin release in β-cells are probably include changes in the expression of TA-related subsets of genes important for glucose sensitivity and insulin secretion [78].

### *Animal Models and Corresponding Protocols*

In vivo models demonstrate wide heterogeneity in pancreatic responses to GC exposure, associated with a complex metabolic effects in many organs and tissues.

The model of transgenic 8-week-old *Klf9fl/fl and Klf9alb*$^{-/-}$ male mice received Dex 1 mg/kg every other day for 2 months was recently described. The steroid-induced diabetes was analyzed by glucose tolerance test as well as evaluation of the metabolites such as triglycerides, cholesterol, serum ketone

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body, and FFAs in the blood serum. Liver tissue was studied by histology and proteome analysis [79].

In another study, *Ehmt2* mutant mice were used for the glucocorticoid-induced insulin resistance model, treated with Dex at a dose of 2 mg/kg body weight for 2 weeks. Glucose, insulin, and pyruvate tolerance tests were performed after 1 week of Dex treatment [80].

8-10 weeks old male Swiss mice were treated with Dex (2 mg/kg, i.m.) for 30 days. Glucose, insulin, and pyruvate tests were performed for evaluating the development of the diabetes [81]. In the similar model of insulin resistance, Wistar-Albino male rats were used. Dex was applied in the dose of 1 mg/kg/day i.p. for 7 days. Biochemical analysis included the determination of glucose, ALT (alanine aminotransferase), AST (aspartate aminotransferase), ALP, total cholesterol, total protein, urea and creatinine in the serum [82].

Male Wistar 3 month old rats received i.p. Dex injection 1mg/kg/day for ten days. Food and water consumption, plasma insulin and glucose concentrations were primary end-points of the study. HOMA- (Homeostasis Model Assessment) β and HOMA-IR (Insulin Resistance) were calculated on the day 10 of the study. HOMA-β evaluates the ability that pancreatic β cells have to secrete insulin (smaller values indicate low ability); HOMA-IR indicates sensitivity to insulin (smaller values indicate bigger insulin resistance) [83].

## Glucocorticoid-Induced Fat Metabolism Disorder

### *Pathogenesis*

Obesity is a key feature of GC-induced metabolic syndrome [84] and also is a common feature of Cushing's syndrome in patients receiving long-term GCs therapy [85,86]. The incidence of obesity is 40% in pediatric acute leukemia survivors treated with GCs [87].

Pathogenesis of fat redeposition by GC excess, includes the following mechanisms: 1) increased appetite and high calorie consumption [88][89]; 2) increased blood glucose levels due to stimulation of gluconeogenesis caused by GCs [90–92]; 3) stimulation of *de novo* lipogenesis enhanced by high levels of glucose and insulin [93], and 4) increased release of free fatty acids from fat stores and stimulation of their liver uptake [94,95].

### *Animal Models*

In animal models of Cushing's syndrome and associated obesity, the simplest model is the excessive administration of exogenous GCs to rodents by s.c., i.p., i.m. and p.o. route as well as slow-release pellet implantation and osmotic mini-pumps [96,97]. Several animal models are described in Table 4.

**Table 4.** Animal models of GC-induced fat metabolism disorder.

| Animal species, sex | Model | Design | References |
|---|---|---|---|
| Male Wistar rats | Dex 0.25 mg/kg/day during 4 weeks | Subcutaneous, retroperitoneal and mesenteric) fat pads were excised, weighed and processed for adipocyte isolation, | [110] |

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|---|---|---|---|
| | | morphometric cell analysis and incorporation of glucose into lipids | |
| Male Wistar rats | 6 weeks of continuous infusion of 0.6mg/kg/day of hydrocortisone | Subcutaneous and visceral (retroperitoneal and mesenteric) fat pads were analyzed for: lipogenic enzymes activity; molecular changes of 11-hydroxysteroid dehydrogenase type 1 (11βHSD1) enzyme; enzymes involved in lipid uptake, incorporation, and metabolism and in fatty acids esterification. | [111] |
| Male CD1 Swiss white mice | 4 weeks either via the drinking water (25-100μg/mL) or through weekly surgical implantation of slow release pellets containing 1.5mg corticosterone | Insulin tolerance tests, Measurements of bone mineral content, bone area, lean mass and fat mass | [112] |
| Male Wistar rats | Dex 120 μg/kg s.c. for 7 days | The level of 11β-HSD1 dehydrogenase activity in adipose tissue homogenates was determined by measurement of the rate of corticosterone to 11-dehydrocorticosterone conversion | [113] |

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| | | | |
|---|---|---|---|
| Male broiler chickens (Gallus Gallus Domesticus) | Dex 2 mg/kg/day for 3 days | The concentrations of glucose, urate, non-esterified fatty acids, triglyceride, and LPL were measured. The activities of fatty acid synthesis and malic enzyme in liver and adipose tissues were measured. | [114] |

## Conclusions

Experimental models of GC side effects are numerous and may vary depending on specific complication. We analyzed the animal species, the methods and criteria for the evaluation of the pathology induction. These models and their combination allow studying the main side effects of novel steroid and non-steroid GC analogues, in particular, potential selective glucocorticoid receptor agonists (SEGRA) with beneficial therapeutic profile. In turn, it makes possible proper design of clinical trial synopsizes and protocols.

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## List of Abbreviations

| | |
|---|---|
| μCT | micro-computed tomography |
| ALT | alanine transaminase |
| ALP | alkaline phosphatase |
| AST | aspartate transaminase |
| BMC | bone mineral content |
| BMD | bone mineral density |
| cAMP | cyclic adenosine monophosphate |
| CDK | cyclin-dependent kinase |
| CTX | carboxy-terminal telopeptide |
| Dex | dexamethasone |
| DXA | dual-energy X-ray absorptiometry |
| ERK | extracellular signal-regulated kinase |
| FFA | free fatty acids |
| GCs | glucocorticoids |
| GIO | glucocorticoid-induced osteoporosis |
| GR | glucocorticoid receptor |
| IR | insulin resistance |
| MAPK | mitogen-activated protein kinase |
| M-CSF | macrophage colony-stimulating factor |
| MPr | methylprednisolone |
| MSTN | myostatin |
| NF-κB | nuclear factor kappa B |

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| | |
|---|---|
| OPG | osteoprotegerin |
| Pr | prednisolone |
| RANKL | receptor activator of nuclear factor-kappa B (NF-κB) ligand |
| REDD1 | regulated in development and DNA damage response 1 |
| TA | transactivation |
| TRAP | tartrate-resistant acid phosphatase |

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