Animal Models to Explore the Effects of Glucocorticoids on Skeletal Growth and Structure

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Abstract

Glucocorticoids (GCs) are effective for the treatment of many chronic conditions but their use is associated with frequent and wide-ranging adverse effects including osteoporosis and growth retardation. The mechanisms that underlie the undesirable effects of GCs on skeletal development are unclear and there is no proven effective treatment to combat them. An *in-vivo* model that investigates the development and progression of GC-induced changes in bone is, therefore, important and a well characterized pre-clinical model is vital for the evaluation of new interventions. Currently, there is no established animal model to investigate GC effects on skeletal development and there are pros and cons to consider with the different protocols used to induce osteoporosis and growth retardation. This review will summarize the literature and highlight the models and techniques employed in experimental studies to date.

Introduction and background

It is estimated that, at any one time, over 250,000 people are exposed to systemic glucocorticoids (GCs); approximately 10% of children will require GCs at some stage during their childhood (Mushtaq & Ahmed 2002) and 5% of the population aged 80 years or over have used GCs in the past (Kanis *et al.* 2004). Long-term GCs are effective in many conditions, such as inflammatory bowel disease (Pappa *et al.* 2011), chronic renal disorders (Olgaard *et al.* 1992), lung conditions, hematological malignancies(El-Hajj Fuleihan *et al.* 2012) and connective tissue disease, and in some, such as Duchenne muscular dystrophy (DMD) (Matthews *et al.* 2016), they are the mainstay of long-term treatment. Unfortunately, GCs are associated with frequent and wide-ranging side-effects, many of which are dose-related and associated with considerable morbidity. Of these, two of the potentially most serious and challenging to manage are glucocorticoid-induced osteoporosis (GIO) and growth retardation. Osteoporosis is characterized by a reduction in bone mass and loss of bone microarchitecture, leading to impaired bone strength and increased fracture risk (Reinwald & Burr 2008). GIO is the most prevalent type of secondary osteoporosis and accounts for about 25% of cases (Eastell *et al.* 1998). It is associated with considerable morbidity and mortality; a reduction in bone mineral density (BMD) of up to 40% can occur with GC therapy and it is estimated that up to half of those on long-term GC therapy will experience fractures (Reid 1997). In those with DMD, 75% are predicted to have a vertebral fracture after 8 years of GC therapy(Bothwell *et al.* 2003) and this event is often followed by loss of ambulation (McDonald *et al.* 2002). The General Practice Research Database has shown that daily prednisolone doses of as little as 2.5mg can cause an increased risk of fracture (Van Staa *et al.* 2000). A recent meta-analysis also showed that there is only weak evidence for the use of common osteoporosis

drugs in the prevention of fractures (Amiche *et al.* 2016), suggesting that there is great need for preclinical work to inform the development of new therapies.

As healthy children have high rates of bone growth, their skeleton is particularly vulnerable to the adverse effects of GCs on bone formation. GC-induced growth retardation was first described 60 years ago after an equivalent cortisone dose of only 1.5mg/kg/day (Blodgett *et al.* 1956) and can be considerable; by 15 years of age, boys with DMD who are treated with deflazacort are 21 cm shorter on average than untreated boys (Biggar *et al.* 2006). GC-induced growth retardation can also occur following GC exposure by several alternative routes including inhaled GC in asthma (Allen *et al.* 1994) and intra-articular GC injections in juvenile arthritis (Umlawska & Prusek-Dudkiewicz 2010). GC-induced growth retardation is dose-dependent and alternate-day or weekend dosing is associated with less growth retardation (Escolar *et al.* 2011; Ricotti *et al.* 2013). In children, although compensatory catch up growth may occur after cessation of GC therapy(Crofton *et al.* 1998), prolonged exposure may reduce the potential for catch up (Simon *et al.* 2002).

Skeletal Development

The fetal skeleton develops in two distinct ways; intramembranous ossification occurs within flat bones including the skull and facial bones, whereas endochondral ossification accounts for the linear development of the long bones such as the femur and tibia. Appositional growth also occurs, whereby bone lining the medullary cavity is reabsorbed and new bone tissue is laid down beneath the periosteum, thus increasing bone diameter. This can still occur even after longitudinal growth ceases. In this review we shall focus on endochondral ossification, which is driven by the actions of the chondrocytes within the epiphyseal growth plate and is

the process responsible for bone formation and longitudinal growth of the majority of the skeleton. During the initial, patterning phase of skeletal development, mesenchymal cells condense into tissue elements at specific sites that form the structure of future bones (Karsenty & Wagner 2002). By 5 weeks gestation in humans, these pre-cartilaginous anlagen reflect the shape, size, position and number of skeletal elements that will be present in the mature skeleton (Javaid & Cooper 2002). Following this, differentiation to either chondrocytes or osteoblasts occurs within the condensations. Chondrocytes within each element organize into growth plates and move through their associated orderly pattern of resting, proliferative and hypertrophic phases (Mackie *et al.* 2011). Once they reach the hypertrophic phase, chondrocytes promote invasion of blood vessels and the production of an extracellular matrix (ECM) that is rich in type II collagen, aggrecan, cytokines and vascular growth factors which facilitates vascular invasion and gradual mineralization of the ECM surrounding the hypertrophic chondrocyte. The cartilaginous ECM is gradually replaced by a bony ECM (rich in type I collagen), when apoptosis of the hypertrophic chondrocytes occurs and osteoblasts invade the cartilaginous scaffold. As osteoblasts lay down new bone, to form the periosteum, the primary ossification centre expands towards the ends of the cartilage model. In long bones, a secondary ossification centre subsequently forms at each end of the bone, leaving a cartilaginous growth plate in between the two ossification centres. Growth is orchestrated at the growth plates but at puberty, bony bridges form between the ossification centers, resulting in the cessation of growth due to the fusion of the growth plate and its replacement by bone. After birth, a continuing cycle of modelling (or remodelling in adults when it occurs without a change in bone shape) occurs and there is a fine balance between bone formation and bone resorption to ensure that bone can sense and adapt to alterations in functional, metabolic and mechanical demands.

GCs and their mechanisms

Figure 1

a) GC-induced osteoporosis

The aetiology of GC-induced osteoporosis is complex and a detailed review of the underlying mechanisms as recently reported (Henneicke *et al.* 2014) is beyond the scope of the current review. Instead we will summarise the key mechanisms and the differing effects of GCs in osteoblasts, osteoclasts and osteocytes. There are two distinct phases of GC-induced bone loss, resulting from the suppressive effects of GCs on both osteoblastogenesis and osteoclastogenesis. The initial acute period of increased bone resorption is followed by a more indolent phase of bone loss caused by a reduction in bone formation (Canalis *et al.* 2004). Indirect effects of GCs on the skeleton such as decreased calcium absorption, increased renal calcium clearance, reduced growth hormone (GH) secretion and suppression of sex steroid metabolism were previously thought to play a fundamental role, but the main mechanisms underlying GIO are now known to result from the direct effect of GCs on the resident bone cells, see figure 1.

Glucocorticoids and mineralocorticoids act through corticosteroid receptors - the mineralocorticoid receptor (MR) and the glucocorticoid receptor (GR). These receptors have often been referred to as Type 1 and Type 2 corticosteroid receptors, respectively(Eberwine 1999; Stewart 2007). The GR is expressed in many bone cells, including osteoblasts, osteoclasts and osteocytes (Bouvard *et al.* 2009) and also in chondrocytes within the growth plate. Once GCs bind to the GR in the cytoplasm, the GR translocates to the nucleus, where it acts as a transcription factor and modifies gene expression, via the GC-response element, either by causing transactivation or transrepression. Transactivation accounts for most of the

GC-associated adverse effects and in-vitro and murine studies demonstrate that selective GR modulators can alter the extent of these adverse effects (Owen *et al.* 2007; Thiele *et al.* 2012). However, studies using transgenic mice with a GR gene mutation that prevents dimerization and therefore transactivation still have reduced bone formation. This suggests that transrepression is probably also at least partly responsible (Rauch *et al.* 2010). Polymorphism of the GR gene is associated with varying susceptibility to GCs (Huizenga *et al.* 1998) which may in part explain the heterogeneity in GC-associated fracture rates in humans.

Micro RNAs (MiRNAs) are endogenous RNAs made up of 18-25 nucleotides that interact with messenger RNA to change protein expression. Recent work has shown that several MiRNAs have differential expression in GC-treated bone. For example, a reduction in MiRNA-29a expression, which interacts with Wnt signalling components and Dkk-1 during osteoblast differentiation was associated with GC-associated bone loss. Gain of MiRNA-29a function by a MiRNA-29a precursor (Wang *et al.* 2013) attenuated the deleterious effects of GC treatment on bone mass, microarchitecture, and biomechanical strength.

Effects of GC on osteoblasts

The chronic bone loss in GIO predominantly results from the ability of GCs to decrease both the number and functionality of osteoblasts. Osteoblasts and adipocytes are both derived from mesenchymal stem cells. By changing the fate of osteoprogenitor cells, GCs effectively reduce the pool of cells that can become mature, differentiated osteoblasts and bone marrow stromal cells are instead directed along the adipogenesis pathway. This has been shown to occur via the transactivation of CCAAT/enhancer binding protein in murine stromal cells(Pereira *et al.* 2002), which increases expression of peroxisome proliferator-activated receptor gamma 2 (PPARγ2) and suppresses expression of Runx2 (Canalis *et al.* 2004, 2007). GCs may, therefore, increase bone marrow adipose tissue at

the expense of mature osteoblasts and cancellous bone (Weinstein & Manolagas 2000). Outside of bone, GCs also promote preadipocyte conversion to mature adipocytes and thus cause hyperplasia of adipose tissue. A 2-fold increase in cancellous adipocyte area in GC-treated mice compared to placebo has been reported, alongside a significant increase in adipocyte production in bone marrow cultures (Weinstein & Manolagas 2000). The exact mechanism(s) by which the reduction in osteoblastogenesis occurs is unclear, however, it is known that GCs cause suppression of bone anabolic factors such as bone morphogenetic proteins (Pereira *et al.* 2002), osteoblast-specific factor 2 (OSF-2) and insulin-like growth factor 1 (IGF-1) (Jones & Clemmons 1995) and TGF-β which activate osteoblastic transcription factors such as Runx2 and β-catenin. In cultured human osteoblasts, exogenous GC administration also results in suppression of the canonical Wnt-β-Catenin signaling pathway which prevents osteoblast apoptosis and encourages progression through the osteoblast cell cycle and thus proliferation (Ohnaka *et al.* 2005). Furthermore, murine GC exposure has been shown to upregulate sclerostin gene expression, which antagonises Wnt stimulation of osteoblast differentiation (Yao *et al.* 2016). Using a transgenic mouse line, GCs have also been shown to suppress interleukin 11 expression, which further inhibits osteoblast differentiation (Rauch *et al.* 2010). As well as inhibiting osteoblast differentiation, GCs also prevent bone matrix synthesis by inhibiting osteoblast-driven synthesis of type I collagen, which forms most of the ECM (Canalis 2005) and osteocalcin. GC administration to mice has also been shown to induce osteoblast apoptosis and suppress terminal differentiation (Weinstein *et al.* 1998).

Effects of GC on osteoclasts and osteocytes

Osteoclasts are derived from haematopoetic stem cells and resorb bone by creating an acidic environment and producing collagen-degrading enzymes. GCs exert an early direct effect on

osteoclasts by increasing both their number and activity, with a corresponding increase in bone resorption, seen after only 7 days of GC treatment in mice (Jia *et al.* 2006). This overall increase in osteoclast number occurs despite a reduction in osteoclast production in the bone marrow, suggesting that GC treatment increases the lifespan of pre-existing osteoclasts. However, the longer term role of the osteoclast in glucocorticoid-induced osteoporosis remains controversial; despite an initial increase in bone resorption, prolonged GC excess appears to suppress osteoclast number and function. For example, after 4 weeks of prednisolone treatment in mice, bone resorption fell to or below normal levels (Weinstein *et al.* 1998). GCs also directly block the induction of cytoskeletal changes in the osteoclast required for the resorptive capabilities of the cell (Kim *et al.* 2007). There is also evidence that GCs suppress the proliferation of osteoclast precursors (Kim *et al.* 2006) However, GC also cause an increase in Receptor Activator of Nuclear Factor Kappa Beta Ligand (RANKL) (Hofbauer *et al.* 2009), which is produced by both osteoblasts and osteocytes (Nakashima *et al.* 2011; Xiong *et al.* 2011) and down-regulation of osteoprotegrin (OPG), which is a decoy receptor for RANKL. This skews the ratio of RANKL: OPG towards osteoclastogenesis. Overall, the long-term effect of exogenous GCs on osteoclastogenesis still requires clarification but it appears that the osteoblast is the main target of exogenous GCs.

Osteocytes are terminally differentiated osteoblasts that play an important role in the repair of bone micro-damage. GCs alter the osteocyte-canalicular network by changing the elastic modulus surrounding the lacunae of osteocytes and cause reduced mineralisation (Lane *et al.* 2006). Autophagy may be responsible for these observed localised osteocyte perilacunar changes, occurring as a self-protection mechanism during GC treatment (Xia *et al.* 2010). High dose GC therapy in several animal and human models has also been shown to induce osteocyte apoptosis (Zalavras *et al.* 2003).

222 b) **GC-induced growth retardation**

223 The growth-suppressing effects of GCs are multifactorial and result from both systemic and
224 local actions on all types of bone cell. The GH/IGF-1 axis is the main determinant of
225 postnatal longitudinal growth and GH and IGF-1 have interdependent roles in growth
226 regulation. The rate of longitudinal bone growth is principally controlled through the
227 regulation of chondrocyte proliferation, differentiation and hypertrophy at the growth plate
228 (Wong *et al.* 2016). GH promotes chondrocyte differentiation, the secretion of IGF-1 by liver
229 cells and the amplification of local IGF-1 synthesis by chondrocytes, which induces clonal
230 expansion of chondrocyte columns within the growth plate (Zezulak & Green 1986).
231
232 GCs also affect the expression of various components of the GH/IGF-1 axis (Price *et al.*
233 1992; Jux *et al.* 1998; Klaus *et al.* 2000; Smink *et al.* 2002). Seven days of dexamethasone
234 treatment in pre-pubertal mice reduced gene expression of IGF-1 throughout chondrocytes in
235 all phases within the growth plate (Smink *et al.* 2003a) as well as causing a significant
236 increase in the number of apoptotic cells within the hypertrophic zone. Different mechanisms
237 of GC-induced apoptosis have been proposed such as activation of caspase 3 and suppression
238 of Bcl-2 (Chrysis *et al.* 2003; Espina *et al.* 2008). GCs block the activation of GH and IGF-1
239 receptors in chondrocytes as well as reducing IGF-1 and GH receptor expression by
240 chondrocytes (Wong *et al.* 2016). Glucocorticoids also impair IGF-1 signaling, mainly via
241 the phosphoinositide 3-kinase pathway within the growth plate. Furthermore, GCs suppress
242 prostaglandin E2 synthesis (Harada *et al.* 1995) as well as vascular endothelial growth factor
243 expression in chondrocytes, thus preventing blood vessel invasion of the ossification center,
244 which is crucial for degradation of the ECM and subsequent ossification and growth (Smink
245 *et al.* 2003a). The intrinsic effect of GC on the mouse growth plate was evident when a local
246 dexamethasone infusion significantly reduced tibial growth compared to the contralateral

247 limb (Baron *et al.* 1992). GCs also act systemically to inhibit the pulsatile secretion of GH
248 from the anterior pituitary gland by increasing somatostatin tone (Mazziotti & Giustina
249 2013).
250
251 **Animal models of GIO and GC induced growth retardation**
252 It is essential to utilise animal models that show similar pathology to the human disease
253 process that is under scrutiny, in order to effectively carry out pre-clinical studies and test
254 novel compounds. GCs may lead to some localized changes in bone strength that are similar
255 to other causes of osteoporosis, but they also display some unique effects which explains why
256 GC exposure is associated with a higher risk of fracture at equivalent BMD and hence
257 reinforcing the need for an appropriate animal model to specifically investigate GIO (Lane
258 2005; Xia *et al.* 2010). In addition, the search continues to find selective GR agonists that
259 possess the anti-inflammatory benefits of traditional GCs without the associated adverse
260 effects (Sundahl *et al.* 2015). Suitable pre-clinical models are also vital to this process.

261 It remains a challenge, however, to find an appropriate animal model for preclinical studies
262 of skeletal development as there is no single animal model that exactly mimics the human
263 pathology. Whilst larger animals such as primates and dogs may have the most similar
264 reproductive, anatomical and physiological characteristics, there are ethical issues to consider
265 as well as difficulties with their maintenance and costs (Reinwald & Burr 2008). Sheep,
266 rabbits, and pigs have also been developed as large animal models of GIO in previous studies
267 (Scholz-Ahrens *et al.* 2007; Baofeng *et al.* 2010; Ding *et al.* 2010) but these too have
268 limitations. The following section will discuss the various animal models used to investigate
269 both GIO and GC-induced growth retardation.

270 **Animal species used for GC-induced osteoporosis models**

Different animal species have been used to explore the effect of GCs on the development of osteoporosis and to search for substances that prevent the observed deleterious effects. The inquiry performed on PubMed, with “osteoporosis”, “glucocorticoids” and “animal name” used as MeSH terms, retrieved 70 papers for rats, 34 for mice, 16 for rabbits, 11 for sheep, 5 for pigs and 3 papers for zebrafish. Although the popularity of rats is related to their established position in postmenopausal osteoporosis research, as evidenced by FDA guidelines (Thompson *et al.* 1995), murine models are increasingly used nowadays. Mice are considered to be an appropriate pre-clinical model of GIO. They share more than 95% of the human genome and can be readily genetically manipulated to simulate specific human diseases. It is also possible to control for the variability found in humans and undertake experiments that would otherwise be impossible in humans. They also have the added advantage of being relatively easy and cost-effective to maintain. The adult mammalian skeleton undergoes a continuous remodeling cycle and some of the early pre-clinical studies using different species failed to appreciate this. More recent work has shown that the mouse shows a similar pattern to human GIO, with an early phase of osteoclast mediated bone resorption, followed by a more indolent phase of decreased osteoblastogenesis and bone formation (Yao *et al.* 2008). Unlike in humans, however, mice lack osteons (or the Haversian system) in cortical bone and therefore remodelling within this structure does not occur as it does in humans (Jilka 2013). Marked effects on bone structural parameters caused by GCs are more frequently observed in younger animals, but in order to avoid complications in bone measurements due to loss of weight caused by GC, it has been suggested that skeletally mature animals should be used to investigate GIO. Gene knockout and transgenic approaches have also established the usefulness of the mouse in determining which genes are critical for bone turnover (Rauch *et al.* 2010). The mouse has also been used effectively in other models of bone loss, such as androgen or estrogen loss and ageing (Pogoda *et al.* 2005). However,

with regard to bone density and quality, dogs appear to be most similar to humans and rats the least (Aerssens *et al.* 1998). Interestingly, *in vivo* and *in vitro* bone mineral imaging as well as scale mineralization studies in zebrafish were described as a very simple alternative to explore alterations in mineralization pathways to GC challenge (Barrett *et al.* 2006).

Techniques to measure GIO

Osteoporosis is defined as an alteration of bone structure leading to increased fragility and fracture rate. In humans, clinically significant fractures and inappropriately low BMD serve as diagnostic criteria for osteoporosis. There is no such consensus on criteria defining osteoporosis in animal models. As spontaneous fractures do not occur in most animal models, unlike in humans, suitable proxy outcome measures need to be utilized. The following methods have been used to describe changes in bone health after GC exposure:

Bone histomorphometry

Traditional methods to assess changes in bone structure include the evaluation of histological sections of mineralized bone. In basic osteoporosis research, lumbar vertebral bodies and long-bone (typically, femoral and tibial) metaphyses are examined to investigate trabecular (cancellous) bone changes, whereas cortical bone alterations are assessed within the diaphysis of long-bones. In addition to the primary static measures, so-called dynamic parameters can also be calculated using the primary measures assessed on bone histological sections after appropriate fluorochrome labeling.

Dual-energy x-ray absorptiometry

Dual-energy x-ray absorptiometry (DXA) is widely used for BMD evaluation in the clinical as well as research setting. DXA assesses areal BMD (aBMD = bone mineral content/bone area). The precision of *in vivo* DXA scans has been shown to be very good in mice

(coefficients of variation < 2 %) at total body (excluding head), lumbar spine (L4-L5), whole femur and whole tibia sites (Iida-Klein *et al.* 2003). This enables longitudinal BMD observations to be used in murine osteoporosis studies. However, in studies, DXA scans have often been performed on different skeletal sites *ex vivo* as an outcome measure (see Table 1). The main drawback of DXA is that there is no information on bone structure or quality. Bone mass increases with body mass, therefore, smaller and younger animals will have lower BMD compared to larger and older ones, but not necessarily more fragile bones. Since experimental drugs, such as GCs, may affect body weight or growth (as discussed later), size should be taken into account to prevent the introduction of bias regarding the effect on BMD. However, bone size adjustments are rarely undertaken in murine osteoporosis studies (none of the studies listed in Table 1).

Peripheral quantitative computerized tomography and Micro-computerized tomography

By using peripheral quantitative computerized tomography (pQCT), true volumetric BMD can be assessed, that, together with bone architecture and geometry, allows for calculation of bone strength and structural indices. These indices correlate very well with whole bone strength when tested *ex vivo* (Siu *et al.* 2003; Kokoroghiannis *et al.* 2009). Micro-computerized tomography (μCT) is normally used at a resolution of 1-10μm in rodents (Bouxsein *et al.* 2010). Major advantages compared to 2D histological sections are the 3D nature of the data, so that real mineralized bone matrix volumes in whole bone tissue volumes (BV/TV) can be assessed, faster data acquisition and larger bone region under investigation.

Biomechanical testing and biochemical markers of bone metabolism

Although the primary aim may be focused at the molecular, cellular, tissue or whole bone organ level, the crucial clinically relevant outcome of the numerous papers focusing on osteoporosis research is to increase bone strength and reduce fracture risk. Bone tissue is a complex and metabolically active structure and, at the organ level, bone continuously adapts to mechanical loading and other environmental factors to mitigate the stress and sustain its function. Therefore, none of the above mentioned parameters alone can sufficiently mirror actual bone health. Biomechanical testing is the only method capable of verifying whether a treatment may cause or prevent bone fragility. In laboratory animals, bone competence is usually tested through axial compression of the vertebral bodies or three-point bending of long bones (Jepsen *et al.* 2015).

Distinct biochemical markers in serum/plasma are also used to follow disease or drug-mediated changes in bone formation (Glendenning 2011).

GC type and dose to induce osteoporosis

Prednisolone (or prednisone), methylprednisolone and dexamethasone are the most frequent synthetic GC used in osteoporosis animal models (see Table 1). However, they have distinct differences in potency. Although the following order from the most to least potent is in agreement with several studies (*i.e.*, dexamethasone > methylprednisolone > prednisolone/prednisone > hydrocortisone/corticosterone), the relative efficacy may vary based on the assay or method of evaluation (Meikle & Tyler 1977; Tanaka *et al.* 1994; Buttgereit *et al.* 2002). The relative efficacy and potency of GC may also depend on the system studied, for example the potency for effects on bone metabolism may be quite different to those on glucose and fat metabolism (Ahmed *et al.* 2002; Wallace *et al.* 2003). In addition, it is not yet clear whether genomic or non-genomic pathways play the major role in GIO (Hartmann *et al.* 2016). Altered bone structure was observed in two-month-old male

mice treated with 15 mg/kg/day of corticosterone (Herrmann *et al.* 2009), but only 2.8 mg/kg/day of methylprednisolone was needed to induce similar changes in mice of same age and sex (Yao *et al.* 2016). Therefore, methylprednisolone appears to be more potent than corticosterone in osteoporosis induction. Another study showed decreases in bone density, bone formation rate and bone strength in 6-month-old C57BL/6 male mice treated with prednisolone 2.1 mg/kg/day over 28 days, but the same dose was not sufficient to induce significant changes in female mice (Weinstein *et al.* 2011). By contrast, the same prednisolone dose was used in female mice of similar age, but different strain (i.e., Swiss Webster), and significant decreases were observed in bone density, bone formation and bone strength after only 10 days (Plotkin *et al.* 2011). This highlights that sex- as well as strain-specific efficacy may be present with different GCs. Controlling for sex (male), strain (Swiss Webster) and route of administration (slow release subcutaneous pellets), 3-month-old mice required 5.6 mg/kg/day of prednisolone, the highest dose tested, to induce a significant decrease in mineralizing surface/ bone surface (MS/BS) and bone strength (Jia *et al.* 2011) whereas a decrease in MS/BS and BMD was observed in 7-month-old mice challenged with 2.1 mg/kg/day of prednisolone (Weinstein *et al.* 1998). Therefore, mouse age and pubertal status may be an additional factor influencing the potency of the tested GCs. In humans a dose of dexamethasone of 1mg is equivalent to 6mg of prednisolone, therefore consideration of the dose used relative to clinical application is important.

It is also important when investigating GIO to describe the impact on both trabecular and cortical bone as there are discrepancies between data obtained at different sites, see table 1.

Route of administration in GIO models

Osteoporosis is induced by systemic administration of GC. Many studies implemented regular intramuscular, intraperitoneal or subcutaneous injections, but single implantation of

slow release subcutaneous pellet or oral gavage have also been used (see table 1). In rats of the same strain and age, daily oral gavage of GCs over a 90 day period (Lin *et al.* 2014) led to similar adverse effects on bone (as assessed by histomorphometry and aBMD) as thrice weekly subcutaneous injections of GC over 56 days (Iwamoto *et al.* 2008). By contrast, a much shorter period of intervention is necessary to induce osteoporosis with daily injections (Ogoshi *et al.* 2008) or continuous infusion through subcutaneously implanted osmotic pumps (King *et al.* 1996). Daily injections are stressful for the animals, which may negatively influence the outcome and ethical regulations in some countries may not allow multiple repeated injections over a long time period. For example, the injection of carrier alone (PEG 400) caused a 3-fold increase in serum corticosterone levels in mice, compared to a 5-10 fold increase induced by an intraperitoneal injection of 10 mg corticosterone/kg body weight, 1 hour after injection (Herrmann *et al.* 2009). This technique of administration would also not be acceptable to most patients in the clinical trial setting. Micro-osmotic pumps were found to have a large variation in residual volumes 21 days after implantation. With a filling volume of 250 μL, residual volumes containing active drug ranged from 50 to 180 uL, which indicated major differences in the flow-rate of individual pumps (Herrmann *et al.* 2009). Subcutaneous insertion of slow release pellets containing corticosterone leads to more consistent drug levels as compared to subcutaneous injections of corticosterone. Oral gavage seems to be less effective compared to daily injections or slow release subcutaneous pellets, but has the most translational relevance, as this would be the most accepted method of GC administration in the clinical setting. Whilst slow release pellet insertion may reduce unnecessary repetition of periodical injections over the study period their safety and efficacy needs further validation.

Animal models of GC-induced growth retardation

It is likely that different animal models are required to investigate GIO and growth retardation. Poor choice of model may result in misinterpretation of results and limited translational promise. For example, the young growing rat does not show any bone loss or changes in microarchitecture of trabecular bone and modelling is the prevailing activity, therefore it is a poor model for human GIO (until at least 9 months of age when the transition to remodelling occurs). It does appear, however, to be a good model to mimic the growth retardation seen in children exposed to GC (Lelovas *et al.* 2008). For growth studies, the age and status of sexual maturity at the time of growth plate closure must also be considered. Unlike humans, bone acquisition and longitudinal bone growth continue in mice and rats after sexual maturity. Linear bone growth in rodents increases during the largest proportion of life expectancy in comparison with other species (Kilborn *et al.* 2002). Humans and primates (showing the second highest ratios of age at growth plate closure to life expectancy), cows and sheep are also considered adults at the age when growth plate closure occurs. By contrast, rabbits, dogs, and cats would be described as very young adults at the time of physis closure. In mice, whilst the highest growth phase is from weaning until sexual maturation, body weight continues to increase in the mouse up to the end of the 52nd week and long bone growth continues slowly after puberty (Jilka 2013). By contrast, New Zealand white rabbits begin sexual maturation at approximately 2 months of age and undergo epiphyseal fusion by approximately 6 months of age. Therefore in order to induce growth retardation and allow for subsequent catch-up growth in one study, GC challenge was commenced when the rabbits were 5 weeks of age (Weise *et al.* 2001). Nevertheless, using rabbits at a young age proved problematic for Kugelberg and colleagues who were unable to sex them at 3 weeks of age and therefore had to use both males and females in their study (Kugelberg *et al.* 2005). This is important as imprinting (Jansson *et al.* 1985) by androgen secretion of the neonatal rodent

444 brain has been shown to result in sex differentiation of body growth and, therefore, it is also
445 important to consider which sex of animal is most relevant to the research question.
446
447 **Techniques to assess bone growth rate**
448 When studying mammalian growth, simple gross parameters such as weight, body or tail
449 length have historically been used as proxies for growth rates (Hughes & Tanner 1970), and are
450 still routinely recorded when assessing growth in pre-clinical studies. These measurements
451 can be very inaccurate however, and dependent on other confounding factors (Melin *et al.*). X-
452 ray determination of the length of different long bones with the aid of anatomical landmarks
453 (Weber *et al.* 1968) is a simple but more accurate proxy. Recent advances in imaging also
454 mean that tibial/femoral length can be accurately measured using micro (μ)CT. This is often
455 performed in conjunction with other measures of trabecular and cortical bone structure
456 (Waarsing *et al.* 2004; Bouxsein *et al.* 2010). In addition, in *vivo* μCT is a non-invasive
457 imaging technique that allows longitudinal bone growth to be evaluated over a period of
458 weeks or months in the same animals and would therefore be well suited for monitoring GC-
459 induced growth retardation. This can be a cost effective and ethical method as it reduces the
460 number of animals required for a study and also minimizes intra-subject variability. Potential
461 drawbacks include the dose of ionizing radiation delivered through multiple scans and the
462 potential for radiation associated tissue effects on the growing skeleton (Klinck *et al.* 2008;
463 Laperre *et al.* 2011). Inclusion of a non-irradiated contralateral limb would clarify the
464 magnitude of this potential issue. Also, by administering fluorescent labels (Owen *et al.*
465 2009) at known time intervals, the bone formation rate (BFR) at the chondro-osseous
466 junction can be assessed visually under UV light, without the need for further staining or
467 decalcification (Dobie *et al.* 2015). In addition to the methods used to assess the growth rate

of the entire bone, measures of the tibial epiphyseal growth plate width have been used for over 50 years as a reliable proxy indicator of growth rate (Interlichia *et al.* 2010).

More recently, a number of investigators have used *ex vivo* models such as rodent metatarsals in culture (Mårtensson *et al.* 2004; Mushtaq *et al.* 2004). For example, when fetal mouse metatarsals were cultured for up to 10 days with either daily or alternate day dexamethasone at 10^{-6}M, dexamethasone treated bones paralleled control bone growth rate until day 8 when their rate of growth decreased resulting in a total length that was significantly reduced from controls at days 8 and 10 (Mushtaq *et al.* 2004).

It is well established that the rate of linear bone growth is dependent on growth plate chondrocyte proliferation, matrix turnover and changes in chondrocyte shape and size (Hunziker & Schenk 1989; Farquharson & Jefferies 2000). Advances in quantitative histology now enable the growth plate to be scrutinized in greater detail to assess the contribution of the different chondrocyte activities to overall growth rate. Whilst quantitative histology techniques were developed in the 1970's to assess the relationship between cell division in growth cartilage and overall bone growth, chondrocyte proliferation is now routinely quantified by the immunohistochemical detection of BrdU incorporation into proliferating cells in tissue sections of the growth plate (Farquharson & Loveridge 1990). Cell death of hypertrophic chondrocytes within the growth plate is also required for physiological bone growth and the TUNEL assay allows the detection and quantification of apoptotic cells within a population of chondrocytes (Kyrylkova *et al.* 2012).

GC type and dose to induce growth retardation

The inquiry was performed on PubMed, with "growth retardation" or "growth", "glucocorticoids" and "animal name" used as MeSH terms. When summarising the data, we

have not included studies where only gross body measurement parameters were taken as a subset of a larger study. Studies where only an abstract was available were also excluded. Where the same groups have published multiple work using the same species and methodology, only the initial data has been represented in table 2.

As shown in Table 2, dexamethasone was the most frequently used GC in the growth retardation models that we reviewed. Method of administration and dosage varied greatly, consistent with the GIO models. Rodents were used in the majority of studies. Four of the studies administered subcutaneous injections of dexamethasone to mice of between 3 and 5 weeks of age. All used daily injections, except for one, where a 5-times weekly regimen was followed (Rooman *et al.* 1999). The length of course varied from 7 to 28 days and the dose used varied from approximately 0.02mg/kg/day to 5mg/kg/day. In one of the studies, where three varying doses were used, the lowest dose of 0.2μg (approximately 0.02mg/kg/day) did not cause significant growth reduction, but both the 2μg and 20μg doses caused similar growth retardation (Rooman *et al.* 1999). No differing side-effects were reported in the two groups. When a dose of 2mg/kg/day was used, body weight was reduced only in males and femur length only in females, whilst a significant reduction in body weight was demonstrated by day 3 using 5mg/kg/day in females in a different study (Owen *et al.* 2009). It would, therefore, appear that there is a sex difference in response to GCs and that an optimal dose would be greater than 2mg/kg/day to ensure significant growth retardation in both sexes. However, the rapid catabolic response with a reduction in body weight by day 3 seen with a dose of 5mg/kg/day would suggest the need for close monitoring (Owen *et al.* 2009).

We reviewed 8 studies using rats, usually either Wistar or Sprague-Dawley and up to 4 months of age at study induction. All except two studies used only male rats. Length of course varied greatly from 4 to 90 days. In one of the studies using prednisolone,

10mg/kg/day was originally chosen (after a previous study by the same authors demonstrated no effect on cortical bone using 5mg/kg/day (Ortoft *et al.* 1992)) but after observing unexpectedly high weight loss, the dose was decreased to 5mg/kg/day. Using 5mg/kg/day they were able to demonstrate reduced longitudinal bone growth of the lumbar vertebrae. This highlights one of the problems of using body weight as a reflection of growth. GC can show a dual metabolic effect on body weight, depending on the dosage, method of administration and length of treatment. High dosages can cause a catabolic effect and loss in body weight whereas lower dosages can cause an increase in appetite and associated weight gain (as frequently seen in humans). For example, 1mg/kg single dose of dexamethasone given to piglets caused accelerated growth at 18 days of age (Carroll 2001). Piglets are also noted to have a metabolic response to GCs that closely mimics the response observed in infants and children receiving long-term GC therapy (Ward *et al.* 1998). One of the studies using Wistar rats demonstrated inhibition of growth after only 10 days of either inhaled budesonide or fluticasone (Kemer *et al.* 2015), even at a dose of only 50mcg. This is particularly relevant when considering that inhaled GCs are the treatment of choice for persistent asthma symptoms in both children and adults.

Decreased bone growth has been demonstrated even at concentrations as low as 1mg/kg/day in a study of rats, where doses of up to 9mg/kg/day of methylprednisolone were used (Ortoft *et al.* 1998a). In this study there was no discernible dose-specific side-effects although serum insulin levels were reduced in all groups. These authors also noted that the catabolic effect of 9mg/kg/day of methylprednisolone (Ortoft *et al.* 1998b) by daily subcutaneous injection was less than that noted when a 5mg of depot prednisolone was used in rats of a similar age (Ortoft *et al.* 1998a). This suggests that routes of administration must also be considered.

Three studies were reviewed which used rabbits; each of these used dexamethasone, but via a different method of administration (eye drops, local infusion and daily subcutaneous injection) therefore they cannot be directly compared. However, all studies reported significant reductions in growth within the dexamethasone-treated groups. All rabbits were aged 5 weeks or less at study induction and all were aged 11 weeks or less at time of cull. In the only pre-clinical model to use a topical method of GC administration, significant effects on growth were demonstrated (Kugelberg *et al.* 2005).

Three studies used piglets, all of whom were less than 7 weeks of age at the end of the study. Again a variety of routes of GC administration were used. It would appear that a dose of 0.25 mg/kg/day of dexamethasone is insufficient to induce bone growth retardation in young piglets (Śliwa *et al.* 2005). In a similar study, a reduction in growth velocity persisted only when piglets were dosed with 0.3mg/kg/day and above (Ward *et al.* 1998) and when prednisolone, at an equivalent dexamethasone dose of 0.75mg/kg/day was used, a significant change in growth plate histology was seen (Smink *et al.* 2003b).

It appears that higher equivalent doses of GCs are used in rodents compared to larger mammals such as rabbits and piglets. In young mice, an optimal dose of dexamethasone when administered by daily subcutaneous injection seems to be between 2 and 5 mg/kg/day. This review demonstrates that there are a varied number of different methods that can be employed effectively to cause GC-induced growth retardation. However, unlike the review of GIO, we found no studies using implantable pellets or osmotic mini-pumps that measured growth parameters and therefore further studies are required to clarify their effectiveness of these delivery routes in causing growth retardation. Having highlighted the pitfalls of using the gross parameter body weight as a marker of growth; we propose that any future studies

568 should also use other confirmatory parameters of growth such as bone length measurements,
569 BFR or growth plate histology.

570 **Genetically engineered animal models**

571 Global deletion of GR is lethal and mice die of respiratory failure due to lung atelectasis on
572 the first day of life (Cole *et al.* 1995) therefore it is not possible to create a complete GR-
573 knockout model. However, tissue-specific genetically modified mouse models can be useful
574 to tease out the effect of GCs on interlinked reactions between the different types of bone
575 cells. For example, deleting osteoblast-specific GR conferred protection from GIO, while
576 deleting osteoclast-derived GR had no effect (Rauch *et al.* 2010). Development of col 2.3 and
577 col 3.6 hydroxysteroid dehydrogenase (HSD)2 transgenic mouse models that activate 11β-
578 HSD2 in osteoblasts showed decreased vertebral trabecular and femoral cortical bone mass,
579 without any change in serum GC levels (Liu *et al.* 2004), thus implicating a role for
580 endogenous GC signaling within the osteoblast for optimal bone mass acquisition.
581

582 **Conclusion**

583 In this review we have demonstrated that there are specific outcome measures that should be
584 assessed when investigating either GIO or GC-induced growth retardation. We carried out a
585 literature review with the aim of determining the most appropriate animal model to use when
586 demonstrating the effects of GC on growth and bone structure, but results are too
587 heterogeneous to enable one specific model to be advocated over another in all situations.
588 However, there is sufficient evidence to recommend that investigation of GC-induced growth
589 retardation in mice should be performed using dexamethasone 2-5 mg/kg/day by daily
590 subcutaneous injection and the outcome measures should include serial lengths (using
591 consistent measuring technique) and/or growth plate width and BFR; the measurement of

592 body weight for assessing linear growth is too inaccurate. When investigating GIO, there is
593 insufficient evidence to recommend one specific mode of delivery over another but in most
594 studies a dose of prednisolone 2-5mg/kg/day in mice has been sufficient. Recommended
595 outcome measures include volumetric BMD (by pQCT or μCT rather than by DXA for
596 greater accuracy) and bone biomechanical testing to mimic fracture rate in clinical studies.
597

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Figure 1: systemic consequences of exogenous glucocorticoids and effects on different bone cells and adipocytes.

Legend- RANKL; receptor activator of nuclear factor kappa-B ligand, OPG; osteoprotegrin, BMP2; bone morphogenetic protein 2, OSF-2; osteoblast-specific factor- 2, IGF-1; Insulin-like growth factor-1, TGF□, transforming growth factor beta.

Table 1. Animal models of glucocorticoid-induced osteoporosis

Species	Sex + Age	GC type, duration, administration, dose	Body Weight (compared to baseline)	Bone site	Bone Imaging Technique	Histomorphometry (GC vs Controls)	µCT (GC vs control)	DXA (GC vs control)	Bone Strength Testing (GC vs control)	Ref
Mice, FVB	F, 3 w	Dex, 28 days working day SC 14.3 ug/mouse/d	NA	Fem	uCT	NA	BV/TV: no diff	NA	NA	(Postnov *et al.* 2009)
Mice, ICR	M, 6-8 w	Dex, 28 days, daily IP inj., 2.5 mg/kg/d	No change in GC group, + 15 % in controls	Tib	Histomorph, (tib), pQCT (tib diaphysis)	BV/TV - 45 %	pQCT: no difference in vBMD, Cortical Thickness - 57 %	NA	NA	(Du *et al.* 2011)
Mice, mod Swiss Webster backgrnd	M, 2 m	Pred, 21 days, sc pellet, 0.8, 2.8 and 4.0 mg/kg/d	-20 % in GC groups, +24 % in controls	Fem LS	Histomorph,(L4, fem shaft); uCT (L5, distal fem)	MS/BS - 50 %, BFR/BS - 65 % in highest GC group only	BV/TV - 22 % in highest GC group only	NA	Axial compression (L6), 4-point-bend test (fem) L6: Max Load -48 % and -61 % in 2 higher doses GC gps, resp; no diff at fem	(Yao *et al.* 2016)
Mice, Swiss Webster	M, 2 m	Pred, 21 days, sc pellet, 3.3 mg/kg/d	- 20 % in GC group; + 25 % in controls	Fem LS	Histomorph (L4, L5,fem diaphysis); uCT (fem diaphysis, L5)	L5: MS/BS -46 %, BFR/BS -60 %; Fem: diaphyseal endocortex: BFR/BS -91 %, diaphyseal periosteum -92 %	L5: BV/TV -32 %; distal fem: BV/TV: no diff	NA	Axial compression (L6), 3-point-bend test (femur) Max Load: L6: -24 %, fem: no diff	(Dai *et al.* 2015)
Mice, WT littermates of transgenic offspring	M, 8 w	Cort, 28 days, sc pellet, NA	NA	Tib LS	Histomorph(prox tibia); uCT (L3, tibia)	Zero endocortical BFR/BS at tib	L3: BV/TV: no diff; tibial metaphysis: BV/TV: no diff; tib diaphysis: Cortical thickness: no diff	NA	3-point-bend test (tib) Max Load: no diff	(Henneicke *et al.* 2011)
Mice, CD1 Swiss White	M, 7-9 w	Cort, 28 days, sc pellet, 15 mg/kg/d	NA	Tib LS	uCT (L3, tibia)	NA	L3: BV/TV - 33 %; tibia: BV/TV - 56 %	NA	NA	(Herrmann *et al.* 2009)
Mice, C57BL/6J + 129/SvJ	F+M, 9 w	Cort, 28 days sc pellet every week NA	+ 27 % in GC groups; + 3 % in controls	Tib	QCT	NA	Trab BMD:-12% F,-21% M BV/TV -20% F,-27%M cort vBMD decreased, but not cort thickness/ bone area	NA	NA	(Tamura *et al.* 2015)
Mice, C57BL/6	F, 8-10 w	Dex, 17 days working day IP 88 ug/mouse/d	NA	Fem	pQCT	NA	Trab vBMD :+30%, Cort thickness: -9%	NA	NA	(Grahnemo *et al.* 2015)
Mice, C57BL/6J	F, 3 m	Dex, 84 days thrice wk IM 2.1 mg/kg/d	NA (at end GC group +22 % v controls)	Tib	Histomorph,;CT	NA	BV/TV - 47 %	NA	NA	(Cheng *et al.* 2015)
Mice, Swiss Webster	M, 3 m	Pred, 28 days, sc pellet, 1.4, 2.8 and 5.6 mg/kg/d	NA	F	Histomorph	MS/BS -40-60 % (in two highest GC doses)	NA	NA	Axial compression (L6)	(Jia *et al.* 2011)

Mice, C57BL/6	F, 4 m	Pred, 28 days sc pellet 1.4, 2.1 mg/kg/d	No change	Fem LS	Histomorph (LS) DXA (LS; fem) uCT (LS)	MS/BS: no diff, BFR/BS: -36%	BV/TV: no diff Cort thickness: -22%	LS: aBMD: -5% Fem: no diff	Axial compression (LS) No diff	(Sato *et al.* 2016)
Mice, Swiss Webster	M, 4 m	Pred, 28 days, sc pellet, 2.1 mg/kg/d	NA	LS	Histomorph(L5); DXA (L? in vivo)	BV/TV - 66 %	NA	aBMD change from baseline - 9 % in GC gp and - 4 % in controls, (sig diff between grps)	NA	(Li *et al.* 2016)
Mice, Swiss Webster	F, 5 m	Pred, 10 days sc pellet 2.1 mg/kg/d	NA	LS	Histomorph, DXA	BV/TV: -23%, MS/BS: -86%, BFR/BS: -90%	NA	aBMD: - 18%	Axial compression (LS) Max Load: -34%	(Plotkin *et al.* 2011)
Mice, Swiss Webster	M, 5 m	Pred, 28 days, sc pellet, 5.0 mg/kg/d	No change by end (-15 % after 2 wks in GC gp)	Tib	Histomorph, ; uCT	BV/TV -22 %, MS/BS -61 %, BFR/BS -75 %	BV/TV no difference	NA	NA	(Bouvard *et al.* 2013)
Mice, C57BL/6	M, 6 m	Pred, 56 days, sc pellet, 2.8 mg/kg/d	NA	Fem LS	uCT (L3, femoral diaphysis)	NA	L3: BV/TV - 25 %; femoral diaphysis: Cortical thickness -20 %	NA	NA	(Fumoto *et al.* 2014)
Mice, C57BL/6	M, 6 m	Pred, 28 days, sc pellet, 2.1 mg/kg/d	NA	LS	Histomorph,(L1-L4); uCT (L5); DXA (L1-L4 in vivo)	BFR/BS - 49 %	BV/TV not diff	aBMD - 11 %	Axial compression (L6)	(Weinstein *et al.* 2011)
Mice, Swiss Webster	M, 6 m	Pred, 56 days, sc pellet, 5.0 mg/kg/d	NA	Fem	uCT	NA	BV/TV no difference (-30 % at day 28)	NA	NA	(Yao *et al.* 2008)
Mice, BALB/c	F, 7 m	Dex, 14 & 21 days daily IP 1.0, 5.0, 10 mg/kg/d	No change	Fem Tib LS	Histomorph (Fem, Tib, L5) uCT (Fem, Tib, LS)	Fem: BV/TV: no diff, Fem: MS/BS: -62%, Fem: BFR/BS: -74% (at mid GC dose)	Fem BV/TV: + 11%; LS: no diff	NA	NA	(McLaughlin *et al.* 2002)
Mice, Swiss Webster	M, 6 m	Pred, 21 days, sc pellet, 1.4 mg/kg/d	-10 % after 1 wk, regained initial weight by study end (no diff GC v control by end)	LS	Histomorph(L5); uCT (L5)	BV/TV -19 %, MS/BS - 31 %, BFR/BS - 80 %	BV/TV - 22 %	NA	Axial compression (L3) Max Load: no dif	(Lane *et al.* 2005)
Mice, Swiss Webster	M, 7 m	Pred, 27 days, sc pellet, 0.7 and 2.1 mg/kg/d	NA (tendency to lower weights in GC grps by end)	Fem LS	Histomorph(L?, femur), DXA (L? in vivo)	BV/TV - 39 %, MS/BS - 26 %, BFR/BS - 53 % (in higher GC group only)	NA	aBMD change from base - 3,- 7,- 9 % in controls, lower, higher GC dose groups, respectively (sig diff between higher GC v control)	NA	(Weinstein *et al.* 1998)
Mice, Black Swiss + 129SvJ	M, 7 m	Pred, 28 days sc pellet, 2.1 mg/kg/d	NA	Fem LS	Histomorph,(LS, Fem); pQCT (LS, Fem)	LS: BV/TV: -31% LS: BFR/BS: 84% Fem: no difference	pQCT: vBMD no diff	NA	Axial compression (LS); 3-point bend test (femur)	(Hofbauer *et al.* 2009)

									Max Load: LS: - 29 %, Fem: no diff	
Rabbits, Japanese white	F, 6 m	MP, 28 days, daily IM inj., 2.0 mg/kg/d	- 9 % in both control and GC groups	Fem LS	DXA (fem head and shaft), uCT (fem, L4)	NA	osteonecrosis after 8 wks (4-wk treatment + 4-wk wash out) in fem head	aBMD: femoral head - 33 %; fem shaft - 22 %	NA	(Lin *et al.* 2016)
Rabbits, New Zealand white	F, 8 m	MP, 56 days, daily IM inj., 1.0 mg/kg/d	No change	LS	DXA (L3-L4 in vivo), uCT (L3-4)	NA	BV/TV - 17 %	aBMD - 25 %	Axial compression (L3-4) Max Load - 19 %, no diff in Stiffness	(Baofeng *et al.* 2010)
Rabbits, New Zealand white	F, 8 m	MP, 28 days, daily IM inj., 1.5 mg/kg/d	No change (no details were shown)	LS Knee	DXA (L3-L4, knee)	NA	NA	aBMD: spine - 9 %; knee - 19 %	NA	(Castañeda *et al.* 2008)
Rabbits, New Zealand white	M, 8 m	Dex, 84 days, twice a week IM inj., 0.9 mg/kg/d	Slight increase in all groups (no numbers shown)	LS	Histomorph (L3), DXA (L3-L4)	BV/TV - 39 %	NA	aBMD - 27 %	Axial compression (L4) Max Load - 38 %, Stiffness - 34 %	(Yongtao *et al.* 2014)
Rats, Wistar	M, 2 m	Pred, 42 days, oral gavage every second day, 15 mg/kg/d	NA	Tib	pQCT (tibial diaphysis)	NA	Cortical vBMD -2 %, Cortical thickness: no diff, SSI - 25 %	NA	NA	(Yokote *et al.* 2008)
Rats, Sprague-Dawley	F, 3 m	Dex, 84 days, twice a week IM inj., 0.7 mg/kg/d	No change	Multi sites	DXA (head, upper limb, fem, trunk, rib, pelvis, spine, whole body)	NA	NA	aBMD: spine - 18 %	NA	(Jiang *et al.* 2016)
Rats, Sprague-Dawley	F, 3 m	MP, 56 days, thrice a week SC inj., 2.1 mg/kg/d	No change	Tib Fem	Histomorph,(tib diaphysis); DXA (fem)	MS/BS - 60 %, BFR/BS - 76 %	NA	aBMD - 5 %	NA	(Iwamoto *et al.* 2008)
Rats, Sprague-Dawley	M, 3 m	Pred, 90 days, daily oral gavage, 1.5, 3.0 and 6.0 mg/kg/d	+33 % in GC groups; +62 % in controls	Tib Fem LS	Histomorph (fem, tibia); DXA (fem, L5); uCT (L6)	tib: BV/TV: no diff, MS/BS - 27 % (high GC-gp only), BFR/BS - 52 % (all combined); fem: BV/TV: no diff, MS/BS - 39 % (comb), BFR/BS - 38% (comb)	BV/TV: no difference	aBMD: fem: - 8 %; L5: no diff	Axial compression (L5), 3-point-bend test (fem) Max Load: fem: - 7 % (no diff with lowest dose), L5: -22 %; Stiffness: fem:	(Lin *et al.* 2014)

									-17 % (no diff with lowest dose), L5: data not shown	
Rats, Wistar	F, 3, 6, 12 m	Pred, 28 days, daily SC inj., 2.0 and 20 mg/kg/d	+ 9 %, + 3 % No change in controls; +5%, no change, -8 % in high GC group (3, 6, 12-mth old mice, resp.)	Tib	pQCT (tib metaphysis and diaphysis)	NA	Trab vBMD higher/ lower/not diff (3/6/12-mth old), Cortical vBMD unchanged in either group (only %changes from baseline given)	NA	NA	(Ogoshi *et al.* 2008)
Rats, Sprague-Dawley	F, 6 m	MP, 30 days, thrice a week SC inj, 3.0 mg/kg/d	No change (no details shown)	Tib Total Body	Histomorph(tib)DXA (total body)	BV/TV - 11 %, MS/BS - 13%, BFR/BS - 18 %	NA	aBMD - 8 %	NA	(Dalle Carbonare *et al.* 2007)
Rats, Sprague-Dawley	F, 8 m	MP, 60 days, daily SC inj, 30 mg/kg/d	NA	Fem	DXA	NA	NA	aBMD - 9 %	3-point-bend test (femur) Max Load - 27 %	(Bitto *et al.* 2009)
Rats, Wistar	M, 8 m	MP, 42 days, weekly SC inj, 1.0 mg/kg/d	NA	Femur LS	Histomorph (distal fem); DXA (L2-L4 in vivo)	BV/TV -34 %	NA	aBMD -1% in controls,-10 % in GC (sig diff between gps)	NA	(Wimalawansa & Simmons 1998)
Rats, Sprague-Dawley	M, NA 200-225g	Dex, 19 days, continuous pump infusion, 16.3 ug/rat/d	+ 8 % in GC group, + 52 % in controls	Fem	Histomorph	BV/TV - 50 %	NA	NA	NA	(King *et al.* 1996)

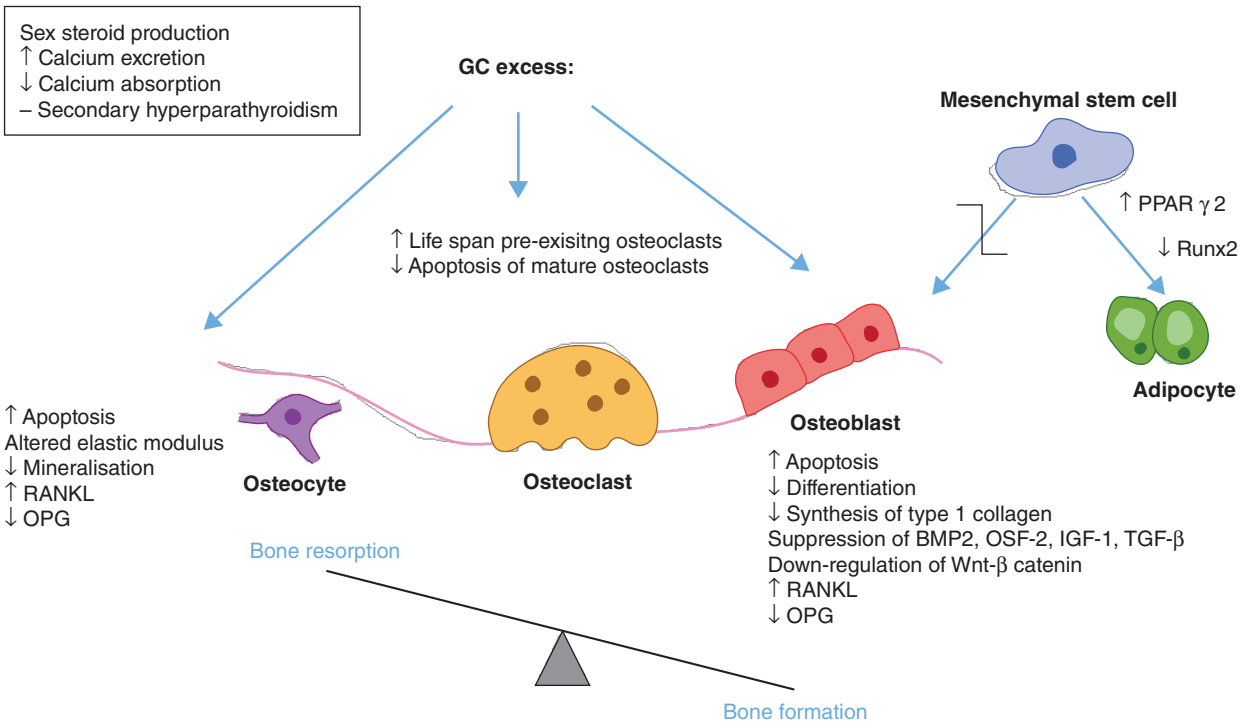
Abbreviations- aBMD: areal bone mineral density, BFR/BV: bone formation rate / bone surface, BV/TV: bone volume/tissue volume, Cort: corticosterone, Dex: dexamethasone, DXA: dual x-ray absorptiometry, F: female, Fem: femur, GC: glucocorticoid, Histomorph: histomorphometry, LS: lumbar spine, M: male, MAR: mineral apposition rate, MP: methylprednisolone, MS/BS: mineralizing surface / bone Surface, mths: months, NA: not available, Pred: prednisolone, pQCT: peripheral quantitative CT, QCT: quantitative CT, Tib: tibia, uCT: micro CT, vBMD: volumetric BMD, wks:weeeks

Table 2 Animal models of glucocorticoid-induced growth retardation

Species	Sex + age	GC duration, method, dose	Measurement	Bone site	Results	Ref
FVB Mice	F, 3 wks	Dex, 5 days a week for 4 wks, Daily SC inj, 0.2 μg, 2μg or 20μg/ animal/ day (approx. 0.02mg- 2mg /kg/day)	BW, snout-tail length under anaesthesia weekly After cull, organs weighed, tib dissected, length measured using digital caliper. Tib dissected- GP width.	tib	Dex at 2 and 20μg/ day caused reduction in: - wt of tib, humerus and lumbar vertebra (only vertebra sig) - wt of organs esp. liver/muscle - total width of GP (mainly due to reduction in proliferative zone). Tibia length only slightly affected. No change in hypertrophic zone.	(Rooman *et al.* 1999)
FVB mice	F, 3 wks	Dex, 7 days, Daily SC inj, 20μg/day (approx. 2mg /kg/day)	BW Nose-tail length Tibiae dissected- GP width and zones TUNEL assay	tib	Dex caused reduction in:- - total body weight (16.7 v 13.6g) - length gain (1.9 v 1.3cm) - tib GP width (dec in width of proliferative zone) - number of prolif chondrocytes Inc in number of apoptotic chondrocytes.	(Smink *et al.* 2003a)
BL6 and BL6 (P21-/-)mice	F, 4 wks	Dex, 7 days, Daily SC inj, 5mg/kg/day	Daily BW, nose-rump body length on days 1 and 7. Digital caliper measurement of tib and organ weights after dissection.GP zone widths. Calcein labelling to measure MAR.	tib	Dex treatment caused reduction in:- - BW by D3 and CRL by D7 (8.2 v 7.6 cm) - liver, spleen and tibia Wt - GP width (esp in PZ and HZ) - MAR.	(Owen *et al.* 2009)
Homozygous Bax-deficient and C57BL6 mice	Both, 30-32 days	Dex, 28 days, Daily SC inj, 2mg/kg	Body weight Bones measured weekly by X-ray BrdU histology, TUNEL assay	fem	Dex caused reduction in: - fem growth (by 47% in female, 50% in males - BW (only significant in males) - chondrocyte proliferation and chondrocyte column density. Inc no. apoptotic chondrocytes.	(Zaman *et al.* 2012)
Wistar rats	Both, 10 days	Budesonide 10 days, inhaled, 50 or 200mcg Fluticasone propionate 10 days, inhaled, 50 or 250mcg	BW change during study period Tib dissected-GP zone widths, proliferation and apoptosis rates using Ki-67 and Tdt markers		Lowest weight gain in high dose fluticasone group All GP zone widths lower than controls (only significant at higher doses, more marked in high dose fluticasone than budesonide) Proliferative cell rates sig lower than controls Apoptosis in hypertophic zone of high dose fluticasone group almost doubled	(Kemer *et al.* 2015)
Sprague–Dawley rats	M, 23 days	Dex, 24 days, Daily intra-peritoneal inj, 40 μg/kg /day.	BW bi-weekly Nose-anal length prior to cull		Dex caused reduction in: - final BW (118 v 106g) - nose-anal length (18.5 v 17.8 cm) - growth rate (7.4 v 6.1g/day)	(Tulipano *et al.* 2007)
Long-Evans rats	M, 37 days	Cortisone, 4 days, Daily SC inj, 1 mg/25 g BW/ day	BW, tail length Right tib measured after cull, with calipers. GP Width measured	tib	Cortisone treatment showed reduction in: - tail + tib length - BW velocity Wider epiphyseal GP width seen.	(Mosier & Jansons 1989)

Sprague Dawley rats	M, 7 wks	Dex, 7 days, Daily SC inj, 5mg/kg/day	BW Growth rate by calcein labelling of tibia. TUNEL assay	tib	Dex caused reduction in: - BW (23% loss v 32% gain in controls. - growth rate (68 /207μm day) - chondrocyte column density 4-fold increase in apoptosis in THCs	(Chrysis *et al.* 2003)
Wistar rats	F, 2 mths	Methylpred, 90 days, Daily SC inj, Variable dose- 1,3,6 or 9 mg/kg/day	BW weekly. Nose-tail length, length of R lower extremity weekly for 4 wks, then fortnightly using sliding caliper. Calcein/ tetracycline labelling of GP sections from prox tib after dissection.	fem, tib	Dose-dependent decrease in: - weight gain - nose-tail length - fem and tib lengths (even at 1mg/kg/day) - growth at the prox epiphyseal GP - muscle mass Effect seen after 1 wk, persisted for study duration.	(Ortoft *et al.* 1998b)
Sprague– Dawley rats	M, 3 mths	Pred, 90 days, Oral gavage, Varied- 1.5/3.0/6.0 mg/kg/day	BW weekly Calcein/ tetracycline labelling to measure MAR and longitudinal growth rate	fem, tib	Pred caused dose dependent reduction in:- - BW (11.4, 14.7 and 19.2% with pred at 1.5/3.0/6.0 mg/kg/day respectively) - fem metaphyseal growth rate 6.0mg/kg/day caused reduction in periosteal MAR of tib cortex.	(Lin *et al.* 2014)
Wistar rats	M, 3 mths	Corticosterone, 3 wks, Daily SC inj, 10mg/day (approx 40mg/kg/day)	BW TUNEL assay Tib dissected- GP width.	tib	Corticosterone caused reduction in: - BW velocity - GP width Increased apoptosis of terminal hypertrophic chondrocytes.	(Silvestrini *et al.* 2000)
Wistar rats	F,105 days	Pred, 80 days, Daily SC inj, 5mg/kg/day (initially 10mg/kg/ day- dec due to s/e)	BW Height of L5 vertebrae	LS	Longitudinal bone growth of L5 arrested	(Ortoft *et al.* 1998a)
New Zealand white rabbits	Both, 3 wks	Dex, 8 wks, Eye drops, 20μl 10 times daily over 13 hr period. Gp 1- all doses, Group 2-alt doses. Ave daily dose 0.24 to 0.62 mg/kg/day.	BW and crown-rump length weekly Fem length measured after cull by micrometer	fem	Dex caused dose-dependent reduction in: - crown-rump length - fem length - BW gain	(Kugelberg *et al.* 2005)
New Zealand white rabbits	M, 4 wks	Dex, Local infusion into one proximal tibial GP, over 7 days, 80ng/μl, 1μl/hr	Serial radiographs of pinned tibia	tib	Dex caused reduction in: - epiphyseal growth rate compared with contralateral side. Most marked at days 5-8. Recovered by day 21.	(Baron *et al.* 1992)
New Zealand white rabbits	M, 5 wks	Dex, 5 wks, Daily SC inj, 0.5 mg/kg per day	Fem length measurement using digital caliper. Oxytetracycline labelling of longitudinal growth. Fem dissected- GP width/ zones. Chondrocyte prolif rate.	fem	Dex caused reduction in: - fem length - heights of the total GP, prolif and hypertrophic zones - BW gain	(Weise *et al.* 2001)
Large Polish White piglets	Both, 2 days	Dex, 12 days, IM inj every 2nd day, 0.5mg/kg of birthwt	BW at start and end of study Length of fem, hum (technique not specified)	fem, hum	Dex treatment caused reduction in: - BW - Femoral and humerus bone length (not significant)	(Śliwa *et al.* 2005)
Yorkshire piglets	M,4- 5 days	Dex, 15 days, bd by orogastric gavage, Tapering- 5 days each of 0.5, 0.3 and 0.2 mg/kg/d	Body weight, snout to rump length, fem length using single photon absorptiometry	fem	Dex caused reduction in: - length by day 6 and BW by day 11. Growth velocity reduction persisted only with 0.3 and 0.5mg/kg/day	(Ward *et al.* 1998)
Cross-bred piglets (Landrace x Yorkshire)	F, 6 wks	Pred, 5 days, oral, 5mg/kg/day	Tib dissected- GP width. TUNEL assay	tib	Pred caused reduction in: - total GP widths to 81% of controls,proliferative zone - trab bone length 7-fold inc in apoptotic chondrocytes in hypertrophic zone.	(Smink *et al.* 2003b)

Abbreviations- BD: twice daily, BW: birthweight, Dex: dexamethasone, F: female, Fem: femur, GP: Growth plate
IM: intramuscular, Inj:injection, LS: lumbar spine, M: male, MAR: mineral apposition rate, mths: months, Pred: prednisolone, SC:SC, S/E:side-effects, tib: tibia, Wks: weeks,


Sex steroid production
↑ Calcium excretion
↓ Calcium absorption
– Secondary hyperparathyroidism
GC excess:
↑ Life span pre-exisitng osteoclasts
↓ Apoptosis of mature osteoclasts
Mesenchymal stem cell
↑ PPAR γ 2
↓ Runx2
Adipocyte
↑ Apoptosis
Altered elastic modulus
↓ Mineralisation
↑ RANKL
↓ OPG
Osteocyte
Osteoclast
Osteoblast
↑ Apoptosis
↓ Differentiation
↓ Synthesis of type 1 collagen
Suppression of BMP2, OSF-2, IGF-1, TGF-β
Down-regulation of Wnt-β catenin
↑ RANKL
↓ OPG
Bone resorption
Bone formation