Therapeutic glucocorticoids mechanisms of actions in rheumatic diseases

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Therapeutic glucocorticoids: mechanisms of actions in rheumatic diseases

[Au: Shortened title OK? (as the character limit for titles is 90 characters, including spaces). Also changed to "rheumatic" to make the rheumatology aspect more clear]

Comment [MC1]: This is ok.

Rowan S. Hardy[1], Karim Raza[1] and Mark S. Cooper[2] **[Au: Author names OK - with the order and abbreviations as you would like to them appear on the article?]**

Comment [MC2]: Yes all ok and accurate

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[Au: Affiliations correct?]

Comment [MC3]: Correct

Abstract

Therapeutic glucocorticoids have been widely used in rheumatic diseases since they became available over 60 years ago. Despite the advent of more specific biologic therapies, a notable proportion of individuals with chronic rheumatic diseases continue to be treated with these drugs. Glucocorticoids are powerful, broad spectrum anti-inflammatory agents but their use is complicated by an equally broad range of adverse effects. The specific cellular mechanisms by which glucocorticoids have their therapeutic action have been difficult to identify, and attempts to develop more selective drugs on the basis of the action of **[Au: OK? is this what you meant? Or the structure?]** glucocorticoids have proven difficult. The actions of glucocorticoid seem to be highly cell type and context dependent. Despite emerging data on the effect of tissue-specific manipulation of glucocorticoid receptors in rodent **[Au: rodent meaning mouse?]** models of inflammation, the cell types and intracellular targets of glucocorticoids in rheumatic diseases have not been fully identified. Although showing some signs of decline, the use of systemic glucocorticoids in rheumatology is likely to continue to be widespread and careful consideration is required by rheumatologists to balance the beneficial effects and deleterious effects of these agents **[Au: OK?]**.

Comment [MC4]: This is ok. The structure is not important, it is the action that is relevant

Comment [MC5]: Yes. All the models have used mice rather than rats. I would be happy with the term mouse instead of rodent.

Comment [MC6]: Yes, ok with me.

[Au: For your information, H1 and H2 refer to the level of heading and will be removed before proofs are made. H1 subheadings can have max 38 characters including spaces. H2 subheadings can have a max 80 characters including spaces. Subheads have been edited to fit these limits, where indicated]

[H1] Introduction

The introduction of glucocorticoids and their notable effects in the treatment of patients with rheumatoid arthritis (RA) led to the award of the Nobel Prize for Physiology or Medicine in 1950[1]. Subsequently, systemic glucocorticoid therapy has been employed in a range of rheumatic diseases. For many of these conditions, the evidence for glucocorticoid therapy remains based on clinical experience rather than rigorous clinical trials. Despite the introduction of biologic drugs, which have a much greater specificity for components of the immune system than glucocorticoids, systemic glucocorticoid therapy continues to be widely used[2].

In the general population, ~1% of individuals are treated with oral glucocorticoids on a long-term basis, and this figure rises to around 3% in elderly individuals[3,4]. In individuals with RA, oral glucocorticoid usage continues to be widespread, although is potentially declining **[Au: OK?]**[2]. For some conditions (such as systemic vasculitis, systemic lupus erythematosus and polymyalgia rheumatica), the use of glucocorticoids for long periods of time remains an importnat part of current treatment approaches.

The adverse effects of prolonged glucocorticoid therapy are well established and extremely common[5]. Glucocorticoids have effects on **[Au: OK? Is this what you meant?]** almost all tissues and bodily systems (the selected effects of glucocorticoids are represented in supplementary figure 1). Adverse effects of particular medical importance include osteoporosis and fracture, glucose intolerance and diabetes, central obesity, **[Au: What do you mean by central obesity?]** muscle wasting, increased risk of infection, depression and cataracts. **[Au: Please reference this statement.]** The effect of glucocorticoids on bone is related to the dose and duration of therapy but treatment durations beyond 3 months are associated with a 30% increase in overall fracture risk and at least a doubled risk of vertebral fracture[6]. The risk of developing diabetes is doubled in patients with RA taking prednisolone doses of 7.5mg or above[5]. Although adverse effects on bone can be mitigated pharmacologically[7] **[Au: With what therapies?]**, the effects on other tissues such as muscle wasting, skin thinning, obesity and increased risk of diabetes have no specific treatment. Patient perceptions of adverse effects often differ from those of their treating clinician[8,9] with features such as weight gain and insomnia rated very important by patients whereas clinicians focussed on features such as diabetes and infection risk (considered less important by patients). **[Au: Could you elaborate a little more (to clarify the relevance to glucocorticoid therapy specifically)?]**.

Comment [MC7]: Yes, this is what the data shows.

Comment [MC8]: Typo. Probably from the original version

Comment [MC9]: Yes this is ok.

Comment [MC10]: This is a common term in endocrinology but maybe not rheumatology. It refers to the accumulation of fat in the abdomen (and neck) rather than in the limbs. 'Visceral' obesity would be a synonym. Alternatively 'accumulation of fat in the abdomen' would be accurate.

Comment [MC11]: Much of this literature is classical but I would suggest: Strehl C, Bijlsma JW, de Wit M, Boers M, Caeyers N, Cutolo M, Dasgupta B, Dixon WG, Geenen R, Huizinga TW, Kent A, de Thurah AL, Listing J, Mariette X, Ray DW, Scherer HU, Seror R, Spies CM, Tarp S, Wiek D, Winthrop KL, Buttgereit F. Defining conditions where long-term glucocorticoid treatment has an acceptably low level of harm to facilitate implementation of existing recommendations: viewpoints from an EULAR task force. Ann Rheum Dis. 2016 Jun;75(6):952-7.

Comment [MC12]: This is discussed later but if needed the phrase 'with bisphosphonates or denosumab therapy' could be added.

Comment [MC13]: Attempted to do so.

Despite their widespread use over many decades the mechanisms by which glucocorticoids have their desired anti-inflammatory effects remain unclear. Many cell types and cellular pathways have been proposed as the key targets for these effects. Experimental evidence suggests that there is likely to be a diversity of cell types and pathways involved and these targets are likely to differ between different diseases treated. An improved understanding of these targets in specific disease states opens up the potential to design novel therapeutics that retain anti-inflammatory effects with less risk of adverse consequences.

[Au: So far you have introduced the use of glucocorticoids and their adverse effects, but haven't mentioned anything about mechanisms of action (until the paragraph below, describing what this Review will cover). Perhaps you could have a short paragraph here to outline the outstanding questions surrounding mechanisms of action of glucocorticoids and why understanding these mechanisms is useful (to help introduce the precise of this Review)]

Comment [MC14]: Completely agree. Paragraph added.

In this Review, we summarise the current understanding of the basis by which glucocorticoids have therapeutic effects in inflammatory, and in particular rheumatic, diseases. We consider the pharmacological properties that enable glucocorticoids to have useful effects in a wide range of conditions and the probable cellular targets of these actions. We also consider the molecular mechanisms underlying the adverse effects of glucocorticoids and assess the prospects for developing novel therapeutics that retain beneficial properties with reduced risks of adverse effects.

[H1] Glucocorticoids and their receptors [Au: OK?]

Comment [MC15]: Yes ok

Cortisol (referred to as hydrocortisone when used as a therapeutic) is the main endogenous glucocorticoid in humans. Cortisol secretion is essential to life **[Au: Is it possible to reference this statement?]**. This steroid hormone is released in a pronounced circadian rhythm (high in the morning before waking and very low around midnight) and its synthesis is considerably upregulated during states of stress. Cortisol is synthesised in the adrenal cortex from cholesterol and retains the cyclopentanoperhydrophenanthrene 'steroid' backbone structure. The synthetic glucocorticoids most commonly used to treat systemic inflammation in rheumatology (prednisolone, methylprednisolone and dexamethasone) are very similar in structure to cortisol, with only relatively modest modifications (figure 1) **[Au: OK?]**. These changes variously reduce enzymatic breakdown of the molecule to increase the ability of the steroid to bind to the glucocorticoid receptor **[Au: our journal style is to avoid 2-letter abbreviations (with some rare exceptions), and so I have removed the abbreviation GR throughout (unless referring to particular isoforms) OK?]**, and reduce or eliminate the intrinsic mineralocorticoid (salt retaining) activity. **[Au: Is there a reference you can provide for this background information on glucocorticoids?]**

Comment [MC16]: This is tricky. Addison described this condition in 1855 and it was well known from then on that if the adrenal glands failed death was inevitable. I could propose one of my previous references but this is quite general (and I'm aware that I've quoted my work in other areas already). The reference would be: Cooper MS1, Stewart PM. Corticosteroid insufficiency in acutely ill patients. N Engl J Med. 2003 Feb 20;348(8):727-34.

Comment [MC17]: Yes ok

Comment [MC18]: Yes ok

Comment [MC19]: I would suggest ref: Fuller PJ, Lim-Tio SS, Brennan FE. Specificity in mineralocorticoid versus glucocorticoid action. Kidney Int. 2000 Apr;57(4):1256-64.

[Au: Paragraph break OK here?] Endogenous glucocorticoids can bind to the glucocorticoid receptor (encoded by *NR3C1*) and the mineralocorticoid receptor **[Au: encoded by? (to match the previous statement?)]**. The glucocorticoid receptor is expressed in most cells within the body and is thought to mediate most of the anti-inflammatory and negative consequences of therapeutic glucocorticoids. The glucocorticoid receptor contains various structural domains important for ligand binding, nuclear localisation, DNA binding and activation functions[10]. The un-bound glucocorticoid receptor is found within the cytoplasm but is transported to the nucleus after binding of the receptor by glucocorticoid. The molecular actions arising from the glucocorticoid-bound glucocorticoid receptor are discussed in a later section **[Au: OK?]**.

[Au: Paragraph break OK here?] The mineralocorticoid receptor is expressed primarily in cells that regulate salt and water balance such as the distal tubule of the kidney, the salivary and sweat glands and the colonic epithelium. Even though glucocorticoids such as cortisol and prednisolone have an affinity for the mineralocorticoid receptor, the interaction between these glucocorticoids and the mineralocorticoid receptor is prevented by the presence of an enzyme (corticosteroid 11β-dehydrogenase isozyme 2 **[Au: OK?]** (11βHSD2)); this enzyme inactivates these glucocorticoids **[Au: these glucocorticoids specifically, or glucocorticoids in general?]** in mineralocorticoid-sensitive cells[11]. Glucocorticoids such as dexamethasone and triamcinolone do not bind the mineralocorticoid receptor and thus have no mineralocorticoid activity.

[H1] Pharmacokinetics **[Au: Shortened title (to fit character limits) OK?]**

A fundamental structural property of therapeutic glucocorticoids is that they can pass through biological membranes to access intracellular receptors (figure 2). Glucocorticoids such as prednisone and prednisolone are efficiently absorbed through the gastrointestinal tract. Although poorly soluble in water owing to their lipophilic nature, glucocorticoids can be carried effectively in the circulation through their association with plasma proteins (primarily globulin and albumin **[Au: OK?]**). Orally or intravenously **[Au: OK?]** administered glucocorticoids can thus penetrate most tissues. Coupled with the almost universal distribution of glucocorticoid receptors within tissues, this high degree of penetration means that glucocorticoid therapy can target cells that mediate inflammation at a systemic level. This high bioavailability, however, comes at the price of considerable 'off target' exposure of tissues unrelated to the condition being treated. Some therapeutic glucocorticoids, such as cortisone and prednisone, lack intrinsic glucocorticoid receptor binding activity but when given orally **[Au: why orally and not intravenously?]** are converted to their active counterparts, cortisol and prednisolone, by 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1), an enzyme that is highly expressed in the liver (figure 2). Expression of 11β-HSD1 in various stromal and immune cells can also amplify the actions of

Comment [MC20]: Yes ok

Comment [MC21]: Encoded by NR3C2. Agree should be added

Comment [MC22]: Yes ok

Comment [MC23]: Yes ok

Comment [MC24]: I don't think this is ok. I have never seen this terminology in the medical literature or used it myself. This might be the term used by pure chemists (and Wikipedia!) but would prefer to stick with 11b-hydroxysteroid dehydrogenase type 2.

Comment [MC25]: These glucocorticoids is correct. The enzyme is selective for certain glucocorticoids so 'glucocorticoids' would be incorrect.

Comment [MC26]: Yes ok

Comment [MC27]: No. There is a specific type of 'globulin' than has high affinity for cortisol. It is termed 'corticosteroid binding globulin' so the phrase such be 'primarily corticosteroid binding globulin and albumin'

Comment [MC28]: Yes ok

Comment [MC29]: Cortisone and predisone are only available in oral formulations. The key point here is that these can be given orally because they are converted to active forms on first pass through the liver (most oral drugs have to go through the liver before getting into the circulation). The sentence could be changed to include the phrase 'by first pass metabolism in the liver' since 'first pass metabolism is the technical term for this phenomena.

these glucocorticoids locally through conversion of these glucocorticoids from their inactive to their active forms[12,13].

The pharmacokinetic properties of glucocorticoids have been successfully modified to improve their therapeutic properties. The development of depot injections for joint and muscle injection has proven to be highly successful[14]. These depot injections are formulated such that the glucocorticoid is released at a much slower rate than typical glucocorticoid preparations. Another approach to improving glucocorticoid effectiveness through changes in their pharmacokinetics is the development of timed release preparations of glucocorticoids designed to mimic the circadian rhythm of cortisol release[15]. A formulation of prednisone has been developed that involves drug encapsulation. The drug is taken at night and the tablet releases prednisone ~4 hours after ingestion (that is, at approximately 2am if given at 10pm, thus mimicking the pattern of release of endogenous glucocorticoids). The timed approach seems to particularly benefit the early morning stiffness that occurs in RA[16]. Furthermore, this treatment approach has the potential to minimise the adverse metabolic effects of glucocorticoids that seem to be greater when administered at times when the normal circadian levels of cortisol should be low[17,18]. In addition to the overall circadian rhythm, cortisol synthesis also follows an ultradian (minute to minute) rhythm. In human studies, the pattern of cortisol exposure seems important in determining the cognitive and emotional response to glucocorticoids[19]. Any such patterning will be absent during treatment with therapeutic glucocorticoids.

Considerable scope remains to further develop glucocorticoid-based therapies on the basis of manipulation of their pharmacokinetic properties such that these agents more selectively target specific tissues of interest. Examples of new strategies include the development and clinical evaluation of novel liposomal-based or nanoparticle-based treatments[20,21]. In these preparations, glucocorticoids are attached to or incorporated within molecules that can be selectively taken up by specific cell types such as macrophages or that have better penetration **[Au: Can I just check you don't mean better targeting to sites of inflammation? Or retention in site of inflammation? Or do you actually mean that have better penetrate into these sites?]** at sites of inflammation. One study in a mouse model of arthritis showed the feasibility of loading glucocorticoids into a hydrogel that was sensitive to breakdown by enzymes released into the joint during inflammation[22]. After injection into the joint, this hydrogel–glucocorticoid complex reduced arthritis whereas the equivalent dose of free glucocorticoid did not, presumably because free glucocorticoid was rapidly lost from the joint. Although these strategies offer new opportunities for local and systemic treatments, they are likely to only fulfil their potential when targeted to the cells that directly mediate the beneficial effects of glucocorticoids **[Au: OK?]** .

Comment [MC30]: Better penetration is what we were getting at. Liposomes are large structures that cannot escape the circulation except where there is a breakdown of the vascular endothelium. Such a breakdown occurs at sites of inflammation.
I agree that the sentence is a bit ambiguous as various approaches are used to try to get selectively greater action of glucocorticoids at specific tissues. None of them however involve retention.

Comment [MC31]: Yes ok

[H1] Mechanisms of action

[H2] Glucocorticoid– glucocorticoid receptor interactions ***[Au: OK?]***

The majority of the therapeutic actions of glucocorticoids are thought to occur through interaction of glucocorticoids with the glucocorticoid receptor. It is now clear ~~n recent years~~ **[Au: We prefer to avoid the word "recent" as it can be construed by different readers differently. What time frame are you referring to here? The last few years? The last decade?]** ~~it has become clear~~ that the glucocorticoid receptor can have a variety of forms **[Au: OK?]**, which can influence glucocorticoid signalling. The complexity of the mechanism of action of the glucocorticoid receptor **[Au: Is this what you meant?]** is still being clarified and a diverse array of glucocorticoid receptor isoforms (such as splice variants and isoforms with different translational start sites) can be present and differ between different tissues and between cells within the same tissue **[Au: OK?]** [23]. From the single *NR3C1* gene locus, two main transcriptional variants of the glucocorticoid receptor have been identified (termed GRα and GRβ **[Au: OK?]**)[24, 25] (figure 3a). GRα is the primary transcript in most cells and contains all the domains required for glucocorticoid receptor signalling. The GRβ transcript lacks the ability to bind endogenous glucocorticoids and is produced at a lower rate in most cells **[Au: lower than what? Meaning lower than the GRα isoform?]** but in certain contexts production can be upregulated. In vitro studies suggest that GRβ can have a dominant negative action on GRα through the formation of GRα–GRβ heterodimers or GRβ–GRβ homodimers rather than GRα–GRα homodimers. A function for GRβ in the development of resistance to glucocorticoid therapy in the clinical setting has been proposed[25, 26]. Although these studies are intriguing, a concrete link between GRβ and rheumatic disease pathophysiology or response to therapeutic glucocorticoids has yet to be established.

Additional glucocorticoid receptor diversity arises from variation in glucocorticoid receptor isoform protein translation. At least 8 translational variants can be produced from the GRα transcript (termed GRA, GRB, GRC1, GRC2, GRC3, GRD1, GRD2 and GRD3). Importantly, these glucocorticoid receptor variants seem to differ in their ability to regulate gene expression[27,28]. Although GRA is the classical glucocorticoid receptor isoform that has been extensively studied, and is the isoform discussed almost exclusively below, the other protein isoforms can also be expressed at notable levels and this expression considerably varies between tissues **[Au: OK?]**. The glucocorticoid receptor isoforms can undergo a range of post-translational modifications including phosphorylation, acetylation, sumoylation and ubiquitination, which also influence the function of the glucocorticoid receptor[29-31]. The implications of translational isoforms and post-translational modifications of the glucocorticoid receptor in rheumatic diseases has not been examined, although a role for translational isoforms has been identified in the immune response to lipopolysaccharide in mice[27].

Comment [MC32]: Yes ok

Comment [MC33]: In light of this point I have changed the text. There are two different aspects to the isoform issue with different time frames so it would be confusing to specify a time frame.

Comment [MC34]: Yes ok.

Comment [MC35]: Yes ok

Comment [MC36]: Yes ok

Comment [MC37]: Yes ok

Comment [MC38]: Yes the GRa isoform

Comment [MC39]: Yes ok

[H2] Glucocorticoid receptor signalling

The mechanisms by which glucocorticoid receptor complexes function are complex and still poorly understood. Although many variations of glucocorticoid receptor signalling mechanisms exist, these mechanisms can be broadly divided into transactivation and transrepression (Figure 3b). In transactivation, direct binding of the glucocorticoid receptor to specific DNA sequences, referred to as glucocorticoid response elements (GREs), causes an increase in gene transcription; this process generally occurs with glucocorticoid receptor dimers **[Au: OK?]**. In transrepression, monomeric glucocorticoid receptor 'tether' to specific factors in such a way that they cannot bind to DNA, interfering with downstream proinflammatory signalling pathways **[Au: OK?]**. As such, gene transcription is reduced without the glucocorticoid receptor directly interacting with the DNA. The term transrepression is also used for an additional mechanism in which glucocorticoid receptor homodimers bind to DNA GREs (so called negative GREs) such that gene transcription is inhibited **[Au: Is this non-classical transrepression, as later you refer to "classical negative GREs" and "classical GREs". Or later, when you refer to "classical negative GREs", are you simply referring to all negative GREs?]**. Other, less well characterised mechanisms by which glucocorticoids can signal include the release of chaperone proteins during binding of the glucocorticoid to the glucocorticoid receptor and through binding of glucocorticoids to cell membrane-associated glucocorticoid receptors[32, 33]. A schematic overview of some of the ways in which the glucocorticoid receptor can function at a cellular level is outlined in figure 4. **[Au: I moved this sentence down from the start of the paragraph so that figure 3 could be discussed fully before figure 4 is mentioned]**

The classical view of glucocorticoid receptor signalling was that the metabolic actions of glucocorticoids (such as those leading to induction of hepatic gluconeogenetic enzymes) were primarily mediated via transactivation through binding of glucocorticoid receptor homodimers to the promoter regions of target genes. By contrast, the anti-inflammatory actions of glucocorticoids were thought to be caused by transrepressive interactions of monomeric glucocorticoid receptors with components of proinflammatory signalling pathways (most importantly NF-κB and AP-1) in ways not generally involving DNA binding, such that these pathways were suppressed. This model of glucocorticoid receptor signalling led to the concept that glucocorticoid-like molecules could be developed with reduced transactivation potential (and thus reduced adverse effects) but with retained or increased transrepression activity. This class of drugs has been labelled selective glucocorticoid receptor agonists (SEGRAs) or 'dissociated' glucocorticoid agonists. **[Au: Please provide references for your description of this concept and class of drugs]** Although providing a framework for commercial drug development, a range of experimental findings have shown that this concept has limitations. For example it is now established that some important anti-inflammatory genes such as *DUSP1*, *SPHK1* and *ANXA1* **[Au: OK? Are you referring to genes in mice or humans here?]** rely on ~~classical~~ transactivation **[Au: What do you mean by "classical transactivation" (i.e. what would be non-classical transactivation"? I'm not sure**

Comment [MC40]: Yes ok

Comment [MC41]: Yes ok

Comment [MC42]: noted

Comment [MC43]: A recent review is: Cooper MS, Zhou H, Seibel MJ. Selective glucocorticoid receptor agonists: glucocorticoid therapy with no regrets? J Bone Miner Res. 2012 Nov;27(11):2238-41. doi: 10.1002/jbmr.1753.

Comment [MC44]: These would be mouse studies

Comment [MC45]: I suggest removing the term classical as it is too confusing

this has been introduced)] for glucocorticoid-mediated transcription **[Au: OK?]**[34-36]. Mice have been generated that have a targeted mutation of the glucocorticoid receptor such that the glucocorticoid receptor retains ligand-binding and DNA-binding capacity but has a reduced ability to form homodimers and thus transactivate gene promoters **[Au: This sentence was a little long so I've split into two, OK?]**. These mice, known as $GR^{dim/dim}$ mice **[Au: OK?]**, have been used to examine the probable contribution that transactivation and transrepression make to the actions of glucocorticoids[37]. $GR^{dim/dim}$ mice were less responsive **[Au: meaning less responsive than wild-type mice?]** to the protective effects of glucocorticoid therapy during experimental sepsis indicating a protective role for glucocorticoid receptor transactivation in systemic inflammatory illness[38]. Additionally, glucocorticoids still have detrimental effects on the bones of dimerization-deficient mice **[Au: Still referring to 'dim-dim' mice?],** suggesting that the adverse effects on bone are mediated through transrepression[39].

Our understanding of the complexity of glucocorticoid receptor signalling has increased substantially with the advent of techniques that enable examination of in vivo glucocorticoid receptor binding across the whole genome. These techniques surprisingly show that the majority of glucocorticoid receptor DNA binding sites are not within ~~classical~~ predicted gene promoters and that monomeric glucocorticoid receptors commonly bind to DNA in association with other transcription factors; furthermore, many of these responses are through binding of ~~non-classical~~ negative GREs with structures unlike previously described negative GREs **[Au: As mentioned above, could you clarify what you mean by "non-classical" here (i.e. what would be a classical negative GRE")?]**[40]. During treatment with pharmacological levels of glucocorticoids **[Au: meaning that the affects you describe in the previous sentence are of non-pharmacological (/lower) levels of glucocorticoids?]**, there seems to be a shift from binding to these unexpected GREs **[Au: from what?]** to increased binding~~use~~ of expected~~classical~~ GREs by glucocorticoid receptor homodimers[41]. The ability of glucocorticoid receptors to bind to specific areas of DNA varies considerably between cell types and even within the cell type, depending on the developmental stage and chromatin organisation of the cell[42]. For example, in cells of the monocyte lineage, glucocorticoid treatment affects the expression of more mRNAs in monocytes than in differentiated macrophages[43]. Of the mRNAs affected in monocytes, the majority are related to cell differentiation. This finding is perhaps not a surprise given that the glucocorticoid receptor has to coordinate between determining cell lineage (glucocorticoids being important for the differentiation of many cell types) and regulating acute metabolic and stress responses **[Au: OK? Is this what you meant?]**.

Research in this area has been complicated by a reliance on experiments that involve cultured cells of mouse origin or transformed human cell lines. However, current studies are attempting to use primary cells isolated from humans, which should provide data that is generalizable and applicability to the clinical situation **[Au: OK?]** [44].

Comment [MC46]: Yes ok

Comment [MC47]: Yes ok

Comment [MC48]: Yes ok

Comment [MC49]: Yes, than wild type mice

Comment [MC50]: Yes these are the 'dim/dim' mice

Comment [MC51]: Yes. It might be made clear clearer by saying 'with higher levels of glucocorticoids in the pharmacological range'

Comment [MC52]: Yes it is

Comment [MC53]: Yes ok

[H2] Cellular targets of glucocorticoid signalling

This diversity of genomic targeting between cells is also paralleled by a diversity of transcriptional responses in various cell types. Glucocorticoids can affect the expression levels of up to 20% of all genes in immune cells[45]. However, the number of genes affected differs considerably between cell types. In 2019, one study examined the glucocorticoid-induced changes in the transcriptome of various human primary cells (that is, monocytes, CD4 T cells, B cells, neutrophils, fibroblasts, myoblasts, preadipocytes, osteoblasts and endothelial cells). The cells were treated with therapeutic levels of glucocorticoids for between 2 and 6 hours[44]. Notably, only a small number of genes were influenced by glucocorticoids in the same way across the different cell types. By contrast, each lineage had a distinct expression profile that only partially overlapped with the other cell types examined. This finding suggests that identifying some of the critical anti-inflammatory actions of glucocorticoids on specific cell types might lead to the development of more selectively targeted medications **[Au: OK?]**. However, these results also imply that therapeutic glucocorticoids in clinical practice target a range of cell types and cellular targets such that determining the exact mechanism by which glucocorticoids have beneficial effects, and testing these effects **[Au: OK? Is this what you meant? Or else, this what (i.e. "this" requires a noun)]** in adequately powered clinical trials, might prove extremely difficult.

Attempts to identify the exact target cells (for example, T cells, macrophages, dendritic cells and stromal cells **[Au: Are these examples of target cells, or examples of potential target cells?]**) of glucocorticoids in various rheumatic diseases has proven difficult. Even for cells that have been implicated in mediating glucocorticoid effects, identifying the specific cellular targets **[Au: meaning molecular targets?]** and/or consequences in these cells have proven difficult to dissect. In various contexts glucocorticoids have possible anti-inflammatory effects that can be mediated through changes in cellular proliferation, survival or differentiation, reduced expression of inflammatory mediators or increased expression of anti-inflammatory factors. Specific cellular factors, including nuclear factor-κB (NF-kB), AP-1, annexins, dual-specific phosphatases, glucocorticoid-induced leucine zipper and microRNAs, are considered important glucocorticoid targets in a variety of cell types[46]. None of these factors have proven to be a dominant mechanism by which glucocorticoids exert their anti-inflammatory action. Selected examples of molecular pathways and processes thought to be important targets of glucocorticoid actions in specific tissues are highlighted in Table 1.

[H2] Insights from mouse models

Comment [MC54]: Yes ok

Comment [MC55]: Yes exactly what is meant

Comment [MC56]: These are examples of potential targets. The actual targets are not known

Comment [MC57]: Yes molecular is better

Additional insights into the specific targets of glucocorticoids in inflammatory arthritis have come from genetically modified mice that have targeted alterations of glucocorticoid receptor expression or signalling capacity[47]. These studies (summarised in Table 2) have examined the critical target cells for therapeutic glucocorticoids in mouse **[Au: OK?]** models of acute and chronic polyarthritis. These inflammatory models include adjuvant-induced arthritis (AIA) and serum transfer induced arthritis (STIA). One study examined the effect of glucocorticoid receptor deletion in various cell types on the ability of glucocorticoids to suppress inflammation and joint swelling in the AIA model[48]. Deletion of the glucocorticoid receptor in T cells prevented the therapeutic effects of glucocorticoids but deletion of the glucocorticoid receptor in stromal cells (which includes synovial fibroblasts, chondrocytes and osteoblasts) did not. Notably, glucocorticoids suppressed T helper 17 (T_H17) type responses **[Au: OK? Referring to in the mice with T-cell specific GR deletion]**.

In 2018, the same group of researchers examined the mediators of glucocorticoid therapy in the STIA model.[49] Using a combination of approaches (including inducible gene deletion using Cre-*lox* technology and bone marrow chimeras) they demonstrated that the anti-inflammatory action of glucocorticoids in this model was not mediated via T cells or other cells of haematopoietic origin. Only when the glucocorticoid receptor was present in the stromal cell compartment was dexamethasone treatment able to reduce inflammation. As such, glucocorticoids seem to mediate different anti-inflammatory effects via two entirely separate cell types in two mouse models commonly used to model inflammatory arthritides such as RA. Further adding to the diversity of cellular targets of glucocorticoids, in a model of allergic dermatitis, the presence of glucocorticoid receptor in cells of the monocyte–macrophage or neutrophil lineages seemed essential for maintaining the beneficial effects of **[Au: OK? Is this what you meant?]** glucocorticoids[50]. These studies clearly show that the effects of glucocorticoids are probably mediated by different cell types in different diseases and that the exact targets cannot be reliably predicted without experimental testing using approaches such as those described above.

Although the anti-inflammatory actions of glucocorticoids are generally assumed to be mediated by effects on immune cells, other experiments also suggest a role for the stromal compartment in modulating inflammation. Glucocorticoid signalling in specific cell types can be blocked by ectopic expression of the 11βHSD2 enzyme. Artificial expression **[Au: expression or overexpression?]** of 11βHSD2 in chondrocytes resulted in increased levels of joint inflammation in the AIA and STIA mouse models[51]. This finding suggests that glucocorticoids can mediate anti-inflammatory effects via targeting chondrocytes. By contrast, 11βHSD2 expression in osteoblasts resulted in reduced joint inflammation in mice with STIA[52]. These results indicate that, paradoxically, glucocorticoid signalling in some stromal cells can have pro-inflammatory effects in arthritis. Glucocorticoids are also recognised to have pro-inflammatory effects in other cell types such as microglia[53].

Comment [MC58]: Yes ok

Comment [MC59]: No. Glucocorticoids suppressed responses in the wild type mice but not in the T cell specific GR deletion indicating that the GR in T cells regulated Th17 responses.

Comment [MC60]: Yes ok

Comment [MC61]: Expression. The enzyme is not normally present in chondrocytes so this is referring to the artificial expression of an enzyme that is not normally there

[H1] Mechanisms underlying adverse effects [Au: Shortened title OK?]

Comment [MC62]: Yes ok

Long term use of therapeutic glucocorticoids is associated with a range of adverse effects. The effects that have been studied most extensively are the actions of glucocorticoids on bone, muscle and glucose metabolism (figure 5).

[H2] Effects of glucocorticoids on bone

An excess of glucocorticoids **[Au: OK? What would be considered "excess"? Or is this not quantifiable?]** is associated with reduced bone mineral density, impaired bone quality and an increased risk of fracture[7]. The relationship between glucocorticoids and bone is particularly complicated in rheumatic diseases as inflammation itself has a detrimental effect on bone[54]. It is likely that glucocorticoid suppression of inflammation has a net positive effect on bone metabolism but that the continued use of glucocorticoids when inflammation is low is detrimental. Glucocorticoids have effects on all the major cells involved in bone metabolism (the specific targets are highlighted in figure 5 and have been reviewed elsewhere[7, 54]). Glucocorticoids impair osteoblast proliferation and reduce their ability to produce bone matrix proteins[39]. High doses of glucocorticoids can cause apoptosis of osteocytes, the most abundant cell type within the skeleton and a critical mediator of the balance between bone resorption and formation[55]. Glucocorticoids also stimulate the activity of osteoclasts but during long term treatment the production of osteoclasts **[Au: by "production of osteoclasts" are you referring to osteoclast differentiation?]** is suppressed by glucocorticoids[55]. At a molecular level, the changes that occur in bone in response to glucocorticoids can be largely explained by the effects of glucocorticoids on inhibiting anabolic bone signalling in osteocytes and osteoblasts; in these cells **[Au: OK? Do glucocorticoids stimulate the next effects in both cells types? or just one of these cell types?]**, glucocorticoids stimulate the expression of inhibitors of anabolic bone signalling and thus reduce bone formation and increase the expression of the pro-osteoclastogenic factor receptor activator of NF-κB ligand (RANKL)[56]. In $GR^{dim/dim}$ mice, the adverse effects of glucocorticoids on the skeleton still occur, despite the reduced capacity of the glucocorticoid receptor to dimerise[39]. This finding suggests that these adverse effects on bone are mediated through glucocorticoid receptor transrepression **[Au:OK?]**.

Comment [MC63]: Good point. Glucocorticoids are normally present but if levels are too high from tumours or drugs then these problems develop. It might be phrased 'Exposure to supraphyiological levels of glucocorticoids (either endogenous or pharmacological is associated….'

Comment [MC64]: Glucocorticoids stimulate differentiation of mature osteoclasts but inhibit differentiation of very early precursors. It might be changed to 'long term treatment the differentiation of early precursors to mature osteoclasts'

Comment [MC65]:

Comment [MC66]: This applies to both cell types (the cell types are hard to tell apart in reality)

Comment [MC67]: Yes ok

[H2] Effects of glucocorticoids on muscle

Long term glucocorticoid use is associated with reduced muscle mass and strength **[Au: Could you please reference this statement?]**. In patients with RA receiving therapeutic glucocorticoids, muscle wasting is rapid, long lasting and is a considerable morbidity factor that increases the risk of subsequent falls and fractures[59, 62, 65-67] **[Au: I moved this highlighted sentence to here to improve the flow, OK?]**. The specific pathways involved in glucocorticoid-induced muscle wasting **[Au: OK?]** are highlighted in figure 5. In patients

Comment [MC68]: I would suggest original references 59 and 60 (Lofberg et al, wang R et al)

Comment [MC69]: Yes ok

Comment [MC70]: Yes ok

receiving therapeutic glucocorticoids, muscle wasting is mediated by both a robust reduction in anabolic protein synthesis and an increase in protein degradation **[Au:OK? (I split up this sentence as it was fairly long)]**. The reduction in anabolic protein synthesis is secondary to suppression of the PI3K–AKT–mTOR pathway and downstream targets ribosomal protein S6 kinase 1 (S6K1) and eukaryotic initiation factor 4E binding protein 1 (4EBP1, also known as eIF4EBP1) **[Au: OK? Do these targets mediate the anti-anabolic effects mentioned next? (or else, how to the two sentences connect?)]**. The anti-anabolic effects are mediated by a plethora of changes, including the suppression of anabolic signalling via insulin-like growth factor I (IGF1) and insulin receptor substrate 1 (IRS1), and the induction of the anti-anabolic factors myostatin and DNA damage-inducible transcript 4 protein (DDIT4, also known as REDD1) **[Au: Edits OK? I moved this sentence here to improve the flow, OK?]**[57, 60-62]. By contrast, increased protein degradation is mediated by E3-protein ligases, E3 ubiquitin-protein ligase TRIM63 and F-box only protein 32 **[Au:OK?]**, increased activity of the ubiquitin proteasomal degradation pathway, and increased autophagy[57-59]. The induction of catabolic proteosomal degradation and autophagy seem to be driven through an direct induction of forkhead box protein O1 (FOXO1) by glucocorticoids and an indirect induction **[Au: An indirect induction of what? FOXO1?]** secondary to suppression of the PI3K–AKT–mTOR pathway[63, 64].

[H2] Effects of glucocorticoids on glucose and lipid metabolism

Glucocorticoids have complex effects on the distribution of fat and the regulation of energy substrates at a systemic level[68, 69]. Glucocorticoids have effects on all the tissues involved in glucose and lipid metabolism (including the liver, muscle, adipose tissue and endocrine pancreas). A coordinated change in systemic energy metabolism is a feature of the stress response; hence, glucocorticoid-induced changes are probably simply a magnification of these changes. Given the diversity of targets of glucocorticoids, no single mechanism or cellular target within these tissues has been identified. Interestingly, some evidence from rodent **[Au: meaning mouse?]** studies suggest that glucocorticoid-induced changes in bone **[Au:OK?]** might mediate, at least in part, some of the effects of glucocorticoids on systemic energy metabolism[70]. Mice with ectopic expression of 11βHSD2 in osteoblasts and osteocytes, and thus an abrogation of glucocorticoid signalling selectively in these tissues, **[Au: By these tissues do you mean bone? Or do you mean these cells?]** do not develop insulin resistance and glucose intolerance in response to glucocorticoid therapy, unlike their wild-type counterparts. The osteoblast-specific protein osteocalcin **[Au: Meaning osteocalcin produced by osteoblasts?]** is a potential meditator between bone and energy metabolism **[Au: Ok? On the basis of what study(s)? Could you reference the study here?]**. In other contexts, osteocalcin can improve insulin sensitivity through a variety of actions on the liver and pancreas[71], but osteocalcin levels **[Au: meaning expression or activity?]** are notably inhibited by glucocorticoids. In support of this concept, mice with heterotopic

Comment [MC71]: Yes ok

Comment [MC72]: The other way around. The most downstream effects are on pi3k etc and these effects are secondary to changes in the pathways described next.

Comment [MC73]: ok

Comment [MC74]: ok

Comment [MC75]: should be 'a' rather than an

Comment [MC76]: yes FOXO1

Comment [MC77]: yes mouse

Comment [MC78]: yes ok

Comment [MC79]: cells is more accurate please change

Comment [MC80]: meaning that osteocalcin is only made by osteoblasts. It is not made anywhere else under normal circumstances

Comment [MC81]: please use reference 71 (lee et al.)

Comment [MC82]: actually this means the levels in the blood. Maybe 'the concentration of osteocalcin in the blood is notably' would be better

expression of osteocalcin in the liver are protected against the effects of glucocorticoids on metabolism[70].

[H1] Therapeutic implications [Au: H1 subheading OK?]

Comment [MC83]: yes ok

Although the mechanisms underlying the adverse effects of glucocorticoids are well defined (figure 5), this information has only resulted in approaches for reducing the effects of glucocorticoids on bone **[Au: OK? is this what you meant? Or did you mean in the context of bone diseases (in which case, which diseases are you referring to)?]** [7, 12, 70]. Notably, the cessation of therapeutic glucocorticoids results in a gradual recovery of bone mass and a return of normal anabolic bone formation over time[72]. By contrast, although further confirmatory studies are required, muscle loss associated with intra-muscular glucocorticoid injections seems to occur rapidly and has a limited capacity to return to pre-treatment levels [65]. Currently, anabolic and anti-catabolic treatments to manage muscle loss in this context are limited to exercise interventions, for which limited evidence of their efficacy in rhematic diseases is available **[Au: OK?]**. A hope for many years has been that SEGRAs might retain the anti-inflammatory activity of glucocorticoids but have reduced effects on metabolism. The development of systemically-administered SEGRAs has proven difficult and no SEGRA has yet made its way into clinical use in rheumatic diseases[73-75]. However, one potential SEGRA, fosdagrocorat, is currently being evaluated in the context of treatment of RA[75]. In the phase 2a study, fosdagrocorat **[Au: OK?]** treatment resulted in a reduction in disease activity **[Au: reduction compared with baseline, or reduction compared with a control group?]** with no notable adverse effects after 2 weeks. A follow up 12 weeks study in 323 patients with moderate to severe RA has compared various doses of fosdagrocorat and prednisone against a placebo, and has had encouraging results **[Au: Please reference this study here]**. At doses that gave equivalent ACR response rates **[Au: Which ACR response rate? ACR20?]** to prednisone **[Au: Prenidone at what dose - the standard recommended dose?]**, fosdagrocorat was associated with a reduction in levels of glycosylated haemoglobin ~~subunit β~~ **[Au: OK?]** whereas prednisone treatment was not **[Au: What is the relevance of reduced levels of glycosylated haemoglobin? Please clarify for the non-specialist reader (i.e. how this is linked to glycaemia)]**. This finding suggests that this drug has the potential to suppress inflammation (through repression) but has less effect on glycaemic control than currently-used glucocorticoids **[Au: OK? Or prednisone specifically?]** (owing to reduced transactivation). Further trials of this medication in RA and other conditions are awaited.

Comment [MC84]: this is the correct meaning

Comment [MC85]: yes ok

Comment [MC86]: yes ok

Comment [MC87]: compared to baseline

Comment [MC88]: Buttgereit F, Strand V, Lee EB, Simon-Campos A, McCabe D, Genet A, Tammara B, Rojo R, Hey-Hadavi J. Fosdagrocorat (PF-04171327) versus prednisone or placebo in rheumatoid arthritis: a randomised, double-blind, multicentre, phase IIb study. RMD Open. 2019 Apr 16;5(1):e000889. doi: 10.1136/rmdopen-2018-000889. eCollection 2019.

Comment [MC89]: Yes ACR20

Comment [MC90]: The study was complex using various doses but for these analyses the prednisone dose was 10mg per day

Comment [MC91]: No. This is actually HbA1c so it would be the A chain. However, the standard term used is glycosylated haemoglobin or HbA1c.

Comment [MC92]: I think any clinician will be familiar with HbA1c etc. However, after haemoglobin you could add (an indicator of average blood glucose levels over time)

Comment [MC93]: I would stick with glucocorticoids since highly related forms such as prednisolone are used in some parts of the world.

Another notable adverse effect of glucocorticoid therapy is suppression of adrenal function. This effect is caused by feedback of glucocorticoids on the hypothalamus and pituitary glands, resulting in inhibition of corticotropin-releasing hormone and adrenocorticotropic hormone protein synthesis **[Au: Clarify what these enzymes are involved in (such as cortisol production) (for non-specialist readers)?]**. Up to one third of patients with RA treated with low-dose glucocorticoids have clinically notable suppression of adrenal

Comment [MC94]: This would be too complex. There are about 8 enzymes involved in the adrenal alone. The sites of negative feedback for CRH are complex involving lots of brain areas. Most clinicians know that glucocorticoids switch off the adrenals.

function[76]. Glucocorticoid feedback at the hypothalamus and the pituitary seem to depend on the transrepression function of glucocorticoids[77,78] and thus SEGRAs would be expected to also suppress endogenous cortisol production. In this situation, patients treated with SEGRAs would have no glucocorticoid available for transactivation functions. This effect could potentially lead to glucocorticoid deficiency in tissues that depend on transactivation for their normal metabolic function, the consequences of which are unclear **[Au: OK?]**.

A clinical problem that remains unaddressed is that of 'glucocorticoid resistance'. This term is generally used to describe situations in which particular diseases or inflammatory pathways that are usually responsive to glucocorticoids fail to respond to glucocorticoid treatment or lose sensitivity to glucocorticoids over time. The mechanistic basis for glucocorticoid resistance is unclear but possible mechanisms involve reduced glucocorticoid receptor expression in target tissues, upregulated expression of specific glucocorticoid receptor isoforms that are less effective in suppressing inflammation in the target tissues **[Au:OK?]**, disease-induced changes to chromatin structures that result in reduced access of glucocorticoid receptors to GREs or the switching on of inflammatory pathways that are intrinsically resistant to glucocorticoid suppression[79]. Unfortunately, attempts to reverse or overcome glucocorticoid resistance have been unsuccessful to date. One approach that has been attempted has been to pharmacologically open chromatin to enable access of the glucocorticoid receptor to otherwise hidden GREs in patients with chronic obstructive pulmonary disease **[Au: Could you please reference this statement?]**. This approach was tested on the basis of in vitro data indicating that low-dose theophylline (a methylxanthine drug currently used in the treatment of respiratory diseases **[Au: Addition OK?]**) could stimulate the activity of histone deacetylases important for promoting glucocorticoid sensitivity **[Au: OK?]**[80]. Unfortunately, in a large randomised controlled trial, theophylline did not improve the effectiveness of inhaled glucocorticoids in patients with chronic obstructive pulmonary disease[81].

Comment [MC95]: Yes ok

Comment [MC96]: Yes ok

Comment [MC97]: This would be the same as below ref 80 (Ito et al)

Comment [MC98]: Yes ok

Comment [MC99]: Yes ok

[H1] Conclusion

In the first two decades after the introduction of cortisol and cortisone, structural modification of glucocorticoids led to the successful introduction of oral and intravenous therapeutics that we still use today. These glucocorticoids have a high tissue penetration, a prolonged half-life and high affinity for the glucocorticoid receptor. Subsequent advances in modulating glucocorticoid properties in other medical disciplines have generally been on the basis of manipulating the pharmacokinetic properties of glucocorticoids to better target drugs to specific tissues (and thus limit systemic adverse effects) or to limit their activity to specific times of the day, rather than manipulating the molecular mechanisms of action of these drugs. This trend looks set to continue given the difficulty in unravelling the complex effects of glucocorticoids at a cellular level and the advances in development of novel drug delivery systems.

The actual cells and cellular targets most important to the action of glucocorticoids remain obscure for most rheumatic diseases. This lack of knowledge prevents the development of more specific therapeutic agents on the basis of how glucocorticoids work. Without these more specific agents, glucocorticoid use in these conditions is likely to continue. The research approaches most likely to lead to specific targets include genetically modified mice with tissue specific alteration of glucocorticoid sensitivity and transcriptome studies using primary human cells. **[Au: Could you perhaps comment on what the future direction is for this aspect of glucocorticoid therapy - i.e. are efforts still ongoing to understand these underlying mechanisms that might still be informative in future?]**

Comment [MC100]:

Comment [MC101]: Hopefully addressed this weakness

In the meantime, glucocorticoids will probably remain important drugs, particularly during initial disease management, for rapid control of disease flare and, for some people, for long term maintenance therapy at a low dose. In these situations, structured approaches to 'glucocorticoid stewardship' will be needed to ensure patients are treated with the minimum dose of glucocorticoids required to achieve the beneficial effects.

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Author contributions

The authors contributed equally to all aspects of the article.

Competing interests

The authors declare no competing interests. [Au:OK?]

Comment [MC102]: Yes ok

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Key points

- Therapeutic glucocorticoids are powerful, broad spectrum anti-inflammatory agents that are limited by a wide range of adverse effects.
- The specific mechanisms of action by which glucocorticoids mediate anti-inflammatory effects in rheumatic diseases are still unclear, hindering the development of novel therapeutic agents **[Au: OK (i.e. I merged the last bullet point you had to this one, as they were very similar)]**

Comment [MC103]: Yes ok

- Approaches to the study of glucocorticoid actions have been complicated by the widespread use of animal tissues and transformed cell lines rather than human primary cells.
- The development of novel glucocorticoids that 'dissociate' molecular transrepression from transactivation have proven difficult, however, one such dissociated glucocorticoid is undergoing clinical trials in patients with inflammatory arthritis.
- The use of genetically modified mice with altered glucocorticoid sensitivity in specific tissues and transcriptomic studies using primary human cells are the most promising approaches to define the most important cellular and molecular targets of glucocorticoids.

[Au: Ideally, you should have 4-6 key points that mention the main aspects covered in this Review. Currently, these points only mentioned the unknown mechanisms and limitations of mouse models. Could you include some additional key points - perhaps one on glucocorticoid receptor signalling, one on cellular targets and one on therapeutic implications - to help give a nice coverage of the different aspects covered in this Review?]

Comment [MC104]: Hopefully addressed

Figure 1: Natural and synthetic glucocorticoids [Au: OK?]

Comment [MC105]: Yes ok

The molecular structures of the endogenous glucocorticoid cortisol and common synthetic glucocorticoid derivatives prednisolone, methylprednisolone, triamcinolone, dexamethasone and betamethasone. A hydroxyl group at position 11 of the steroid ring (highlighted in red) is critical to the activity of these glucocorticoids.

[Au: For part A, why is one of the "HO"s in red? Is this a key position shared by all the derivatives?]

Comment [MC106]: Hopefully addressed

Figure 2: Systemic and local metabolism and inactivation of circulating glucocorticoids.

Circulating glucocorticoids shuttle between their inactive form, mediated by dehydrogenase inactivation by corticosteroid 11β-dehydrogenase isozyme 2 (11βHSD2) in the kidneys, and their active form, mediated by oxoreductase activation by 11βHSD1 in the liver. Intracellular pre-receptor metabolism determines local activation and inactivation of glucocorticoids. Cells expressing 11βHSD1 increase local glucocorticoid activation and glucocorticoid receptor ligand binding. By contrast, cells expressing 11βHSD2 rapidly inactivate glucocorticoids, protecting the mineralocorticoid receptor from inappropriate glucocorticoid ligand binding and activation. 11keto and 11β-hydroxyl steroids are irreversibly 5α or 5β reduced to their inactive metabolites tetrahydrocortisone, 5α-tetrahydrocortisol and terahydrocortisol by the actions 5α and 5β reductase. Further metabolism by 20α and 20β reductase yields inactive α and β cortolones and cortols. THE, tetrahydrocortisone;

[Au: What does "ALDO" refer to in this figure?]

Comment [MC107]: ALDO is aldosterone

Figure 3: Glucocorticoid receptors

A) The structural domains of the glucocorticoid receptor isoforms glucocorticoid receptorα (GRα) and GRβ. B) During glucocorticoid receptor binding, homodimers of GRα bind to the glucocorticoid response element (GRE) to regulate gene expression whereas GRα–GRβ heterodimers function as dominant negative inhibitors, antagonising the activity of GRα. DBD (DNA binding domain); LBD (ligand binding domain)

Figure 4: Molecular mechanisms of glucocorticoid receptor signalling.

Glucocorticoid receptor signalling can involve transactivation (a), transpression (b) or other mechanisms (c). These mechanisms can involve either dimeric or monomeric receptors; can involve direct binding of these receptor complexes to DNA or indirect effects on other DNA binding factors; or can sometimes involve interactions in the cytoplasm or cell membrane. For direct dimer transactivation and transrepression, ligand-bound GRα homodimers bind to glucocorticoid response elements (GREs) to elicit either direct induction or suppression of downstream gene expression. For monomer signalling, ligand-bound monomeric GRα bind to GREs and recruit co-activators or co-repressors to influence secondary transcription factor regulation of gene expression (mediating transactivation or transrepression, respectively). For monomeric tethering, ligand-bound monomeric GRα bind directly to a secondary transcription factor to either positively or negatively regulate downstream gene expression (transactivation or transrepression, respectively). For cell membrane receptor signalling, glucocorticoids bind to cell membrane-bound receptors and mediate transmembrane activity resulting in non-genomic signalling. For chaperone protein signalling, the disassociation of chaperone proteins from the GRalpha on the binding of ligand liberates the chaperone proteins such they can influence ~~changes in~~ intracellular signalling pathways ~~mediated by unbound chaperone proteins secondary to their disassociation from ligand bound GRα~~ For PI3K competition, ligand bound GRa can sequestrate PI3K ~~modifying~~preventing its ability to activate AKT and regulate downstream AKT signalling ~~the ability of PI3K to influence AKT signalling~~. **[Au: Could you please clarify what you mean by this last sentence?]**.

[Au: Does "RE" (following XY and NKkB" refer to "response elements"?]

[Au: You've also mentioned "competition for PI3K" in the figure - could you briefly mention this aspect in the figure legend?]

Figure 5: The deleterious actions of glucocorticoids in muscle and bone.

Comment [RH(oMaSR108]: Mark. The figure for this implies that PI3K bound to GR can still signall. I don't think this is the case. Can you make a note for artistic editor that GR binding blocks PI3K signalling (Figure 4)

Comment [MC109]: Hopefully done

Comment [MC110]: This is not correct. It should be NF-kappaB with kappa as a symbol. It stands for nuclear factor kappa B.

Comment [MC111]: Yes RE is response element.

Comment [MC112]: Hopefully done

In muscle, glucocorticoids induce catabolic (E3 ligase and FOXO1) and anti-anabolic (Myostatin and DDIT4) signalling pathways and suppress anabolic signalling pathways (IGF-1, S6K1, 4E-BP, PI3K, AKT and mTOR), resulting in muscle wasting. In osteocytes, glucocorticoids directly induce the release of the anti-anabolic Wnt inhibitor sclerostin and induce osteocyte autophagy and apoptosis through increased BIM, ER stress and ATG7 signalling. Glucocorticoids suppress bone formation by inhibiting factors that regulate osteoblast differentiation and proliferation (Wnt, BMP, TGFβ **[Au: OK?]**, DKK1, sex steroids, AP-1) and inducing factors that induce osteoblast apoptosis and autophagy (PyK2, JNK, Bim, E4BP4, ER stress). Osteoclastic Bone resorption is directly upregulated by glucocorticoids~~via osteoclasts~~ **[Au: by glucocorticoids?]** through the direct suppression of OPG in combination with- induction of proteolytic enzymes in osteoclasts such as collagenase 3. Glucocorticoids can also have secondary effects on bone metabolism (green boxes). Briefly, glucocorticoids ~~directly suppress~~ lower OPG secondary to the reduction in mature osteoblasts and osteocyte numbers, whilst the reduction in muscle mass results in reduced loading and mechanosensing by osteoclasts, which further suppresses OPG production by these cells. . ~~suppression of osteoblast differentiation and maturation~~ **[Au: If they directly suppress OPG, isn't this a primary (rather than secondary) mechanism that should instead be mentioned above?]** ~~in osteocytes and osteoblasts, as well as indirectly suppress OPG in response to decreased mechanosensing in patients with muscle wasting~~ **[Au: Could you explain this mechanism a little more (I'm not sure I understand the link between muscle mass, mechanosensing and OPG production) - perhaps you could expand on this slightly for clarity?]**. This occurs in conjunction with the direct induction of RANKL in osteoblasts. Together these shift the OPG to RANKL ratio in favour of increased osteoclast maturation and activation and increased bone resorption. **Therapeutic interventions are able to prevent bone loss through their targeting of either anabolic or catabolic bone metabolism in osteoblasts and osteoclasts. The parathyroid hormone analogue t~~T~~eriparatide promotes anabolic bone formation in osteoclasts, promoting their differentiation and survival. Anti-catabolic agents such as b~~B~~isphosphonates directly promote cell death and apoptosis in osteoclasts, whilst d~~D~~enosumab blocks RANKL signalling in osteoclasts, preventing their maturation and activation.**

[Au: You also include therapeutic interventions in this figure (bisphosphates, denosumab and teriparatide). Could you mention these aspects in the figure legend?]

Table 1: Effects of therapeutic glucocorticoids on different cell types **[Au: Shortened title OK?]**

Cell type	Effect of therapeutic glucocorticoids on cellular responses	References **[Au: I've created row for each effect as we no longer have bullet points in our tables (and so this seemed to be the best way to display this information). However, I wasn't sure which reference referred to which effect - could you**

Comment [MC113]: yes

Comment [RH(oMaSR114]: addressed

Comment [RH(oMaSR115]: this has been expanded

Comment [RH(oMaSR116]: These have been included

Comment [MC117]: yes ok

		please move each to the correct row (making sure there is at least one reference for each)?]
Adaptive immune cells		
T helper cells	Decreased Th1 and Th17 differentiation and cytokine production~~Decrease cytokine production~~	Liberman AC et al. Cytokine Growth Factor Rev. (2007)[82] Franchimont D et al. J Immunol. (2000)[84]
	Increase apoptosis	Reichardt HM et al. Cell. (1998)[37]
	Decrease T cell signalling	Franchimont D et al. J Immunol. (2000)[84]
	Increased T_H2 differentiation and cytokine production ~~Increased T_H2 effects~~	Franchimont D et al. J Immunol. (2000)[84] Ramírez F et al. J Immunol. (1996)[83]
Cytotoxic T cells	Decreased cytokine production	Schleimer RP et al. J Immunol. (1984)[86]
	Increased apoptosis	Migliorati G et al. Pharmacol. Res. (1992)[85]
	Decreased T cell signalling	Schleimer RP et al. J Immunol. (1984)[86]
	Decreased cytotoxic capacity	Schleimer RP et al. J Immunol. (1984)[86]
B cells	Decreased B cell receptor signalling	Cupps TR et al. J Clin Invest. (1985)[88] Franco LM et al. J Exp Med. (2019)[44]
	Increased apoptosis	Lill-Elghanian D et al. Exp Biol Med. (2002)[87]
	Decreased TLR7 and BCR signalling	Franco LM et al. J Exp Med. (2019)[44]
	Upregulation of BLIMP1 and IL-10	Franco LM et al. J Exp Med. (2019)[44]
Innate immune cells		
Mast cells	Decreased Toll like receptor signalling	Zhou J et al. Allergy. (2008)[89]
	Increased histamine release	Zhou J et al. Allergy. (2008)[89]
Macrophages	Decreased pro-inflammatory cytokines	Franchimont D. Ann N Y Acad Sci. (2004)[92] Zhou JY et al. Br J Surg. (2010)[93]
	Increased pro-resolution cytokines	Barczyk K et al. Blood. (2010)[90] Franchimont D. Ann N Y Acad Sci. (2004)[92]
	Increased efferocytosis phagocytosis	McColl A et al. J Immunol. (2009)[91] Zhou JY et al. Br J Surg. (2010)[93]
	Increased M2 polarisation	Barczyk K et al. Blood. (2010)[90]
	Decreased Toll like receptor signalling	Franchimont D. Ann N Y Acad Sci. (2004)[92]
Neutrophils	Increased production	Cavalcanti DM et al. Pharmacol. (2007)[95]
	Decreased extravasation	Filep, JG et al. Circulation (1997)[94] Cavalcanti DM et al. Pharmacol. (2007)[95]
Basophils or Eosinophils	Decreased Toll like receptor signalling	Mogensen TH et al. Infect Immun. (2008)[98]
	Increased apoptosis	Sivertson KL et al. Cell Immunol. (2007)[97]
	Increased expression of CXCR4 and migration to the spleen, bone marrow and lymph	Khoury P et al. Allergy. (2018)[96]
Resident mesenchymal cells		
Osteoblasts or osteocytes	Decreased differentiation	Rauch A et al. Cell (2010)[39]
	Increased apoptosis	Chen F et al. Calcif Tissue Int. (2014)[99]
	Increased RANKL	Swanson C et al. Endocrinology (2006)[100]

Comment [RH(oMaSR118]: I have amended the headings and deleted one column to address the point on T cell function

		Humphrey EL et al. Bone. (2006)[101]
	Decreased OPG	Swanson C et al. Endocrinology (2006)[100] Humphrey EL et al. Bone. (2006)[101]
Chondrocytes	Increased MMP activity	Huang Y et al. J Steroid Biochem Mol Biol. (2018)[102]
	Decreased GAC production	Huang Y et al. J Steroid Biochem Mol Biol. (2018)[102]
Myoblasts and fused Myotubes ~~Myotubes~~ [Au: What cells are you referring to?]	Increased proteolysis	Braun TP et al. Front Physiol. (2015)[103]
	Increased autophagy	Troncoso R et al. Cell Cycle. (2014)[104]
Stromal fibroblasts	Decreased cytokine and chemokine production	Hardy RS et al, Arth Res Ther (2006)[105]
	Decreased invasiveness	Durmus M et al. Anesth Analg. (2003)[106]
	Decreased lymphocyte adhesion	Durmus M et al. Anesth Analg. (2003)[106]
	Decreased wound healing	Durmus M et al. Anesth Analg. (2003)[106]
Cells linking innate & adaptive immunity ~~**Innate/adaptive link immune cells**~~ **[Au: What do you mean by "adaptive link immune cells"?]**		
Natural killer cells	Increased activation	Pitzalis C et al. J Immunol. (1997)[107] Pitzalis C et al. Ann N Y Acad Sci. (2002)[108]
Dendritic cells	Decreased cytokine production	Cao Y et al. Blood. (2013)[28]
	Decreased maturation	Elftman MD et al. Immunology. (2007)[109] Cao Y et al. Blood. (2013)[28]
	Increased apoptosis	Cao Y et al. Blood. (2013)[28]
	Decreased antigen presentation	Elftman MD et al. Immunology. (2007)[109]

Comment [RH(oMaSR119]: Hopefully this clarifies. Blasts fuse to form multinucleate myotubes (muscle fibres)

Comment [RH(oMaSR120]: Clarified

Table 2: Effects of therapeutic glucocorticoids on mouse models of inflammatory arthritis **[Au: Shortened title OK?]**

Comment [MC121]: ok

Study	Inflammatory mouse model [Au: OK?]	Transgenic mouse	Treatment with dexamethasone investigated?	Inflammatory outcome
Koenen et al. Ann Rheum Dis, 2018[49]	STIA	Haematopoietic glucocorticoid receptor deletion	Yes	Normal response to glucocorticoid [Au: OK?]
		Stromal glucocorticoid receptor deletion	Yes	Resistant to glucocorticoid [Au: OK?]
Baschant et al. PNAS, 2011[48]	AIA	Macrophage glucocorticoid receptor deletion	Yes	Normal response to glucocorticoid
		Dendritic cell	Yes	Normal

Comment [MC122]: yes ok

Comment [MC123]: yes

Comment [MC124]: yes

		GR deletion		response to glucocorticoid
		B cell glucocorticoid receptor deletion	Yes	Normal response to glucocorticoid
		T cell glucocorticoid receptor deletion	Yes	Resistant to glucocorticoids
Tu et al. FASEB, 2018[51]	STIA	Chondrocyte blockade of glucocorticoid	No	Exaggerated inflammation and joint destruction **[Au: OK?]**
	AIA	Chondrocyte blockade of glucocorticoid	No	Exaggerated inflammation and joint destruction **[Au: OK?]**
Buttgereit et al. Arthritis Rheum, 2009[52]	STIA	Osteoblast blockade of glucocorticoid signalling	No	Attenuated inflammation and joint destruction **[Au: OK?]**
Hardy et al. J Autoimm, 2018[12]	TNF-transgenic model of arthritis **[Au: OK?]**	Global blockade of 11β-HSD1 glucocorticoid activation	No	Exaggerated inflammation and joint destruction **[Au: OK?]**
		Stromal blockade of 11β-HSD1 glucocorticoid activation	No	Attenuated inflammation and joint destruction **[Au: OK?]**
Coutinho et al. Endocrinology, 2012[13]	STIA	Global blockade of 11β-HSD1 glucocorticoid activation	No	Exaggerated inflammation and joint destruction

AIA, antigen-induced arthritis; STIA, Serum transfer induced arthritis

Comment [MC125]: yes

Comment [MC126]: yes

Comment [MC127]: yes ok

Comment [MC128]: yes ok

Comment [MC129]: yes ok

Comment [MC130]: yes ok

Table of contents blurb

Glucocorticoids are anti-inflammatory therapies commonly used in rheumatology but have wide-ranging adverse effects. Understanding the pharmacokinetic properties and

mechanisms of action of glucocorticoids could inform in the development of novel therapies with fewer adverse effects. **[Au: OK?]**

Comment [MC131]: yes