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The adiponectin agonist AdipoRon accelerates osteoporosis development in two different models and modulates adipocyte differentiation

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Abstract

Osteoporosis is an increasing concern in the aging population worldwide, culminating in increased economic concerns and diminished quality of life. Similarly, disturbances of lipid metabolism and adipocytes accumulate more and more in western societies and need solutions. Adipocytes have recently attracted much interest in relation to their endocrine products, one of which is adiponectin, normally associated with beneficial effects on cardiovascular health, inflammation, and cancer. In this study, we have investigated the effect of AdipoRon, an adiponectin receptor agonist with reported anti-osteoclastic properties, on the development of osteoporosis in two different preclinical models. Contrasting to our initial hypothesis, AdipoRon treatment accelerated metabolic changes and bone loss in both models. However, AdipoRon rescued bone marrow adipocytes presence induced by glucocorticoids. Investigations on adipocyte differentiation revealed that AdipoRon potently changes adipocyte identity, by exerting opposite effects on adipocyte-gene induction depending on the time point and duration of stimulation. In conclusion, adipocyte-derived Adiponectin deserves further investigation as an autocrine mediator in musculoskeletal research.

Keywords: osteoporosis, adiponectin, adiporon, glucocorticoid, bone

Introduction

Osteoporosis (OP) is a multifaceted and factorial disease, with a strong association to sex, age and lifestyle. It displays an increased economic burden on the general aging population as well as a significant treatment gap [1,2]. OP is characterized as a chronic low-grade metabolic disease, and the majority of associated factors are also importantly linked to systemic energy metabolism. In the context of aging bone, multiple changes occur simultaneously and eventually lead to a decrease in bone mass; osteogenesis is attenuated due to a preferential differentiation bias of mesenchymal stem cells (MSCs) towards adipogenesis, which necessarily reduces the number of osteogenic progenitors and mature osteoblasts, resulting in the accumulation of bone marrow adiposity [3]. Importantly, the bias in mesenchymal differentiation altering osteoblast number and activity also necessarily affects the bone resorptive process carried out by osteoclasts (OCLs), which is indispensable to maintain organ homeostasis and integrity throughout life [4]. OCLs are the only cells capable of carrying out the complex multi-step process of bone resorption. In contrast to other bone cells, they arise from the myeloid lineage under specific stimulation and are primary players in the development and establishment of osteoporosis [5,6].

Adipocytes, in addition to their fat storage capacity, also act as endocrine organs by secretion of various adipokines. Amongst them, adiponectin is one important secreted protein that exerts effects both on local and systemic levels by regulation of e.g. glucose and lipid metabolism [7] and has reported anti-inflammatory and anti-atherosclerotic properties [8]. Adiponectin mainly acts *via* adiponectin receptors 1 and 2 (AdipoR1, 2), that are expressed ubiquitously and participate in energy metabolism [9]. Both receptors have divergent affinities for globular or full-length adiponectin, but are efficiently targeted by the synthetic agonist AdipoRon [10]. We and others have shown beneficial effects of AdipoRon treatment in improving metabolic disturbances in glucose metabolism and insulin secretion during genetic and glucocorticoid (GC) induced obesity and diabetes [10,11]. Additionally, AdipoRon has shown potential in alleviating GC-induced depression and behavioural changes [12], as well as in counteracting certain types of cancers [13].

In regard to bone, studies have described an inverse association between adiponectin serum levels and total, cortical and trabecular bone mineral density in postmenopausal women [14,15]. More recently, we have reported that adiponectin action is pivotal to limit osteoclastogenesis and bone resorption *in vivo* in lipotrophic mice (genetic ablation of adipocytes through Pparg knock-out or AZIP transgenic constructs) and in a model of adipocyte-specific absence of Pparg [16]. Moreover, Pparg deficient mice presented a dual bone phenotype due to increased osteoblastic activity and bone formation [17], leading to extended trabecularization into the diaphysis of long bones [16]; simultaneously, we found higher levels of osteoclastogenesis in the cortical bone area, provoking

severe cortical porosity in part related to AMPK activation [16]. Interestingly, the activation of the adiponectin receptors with AdipoRon, which is also reported to induce AMPK, restored OCL phenotype and function. Thus, we hypothesized that adiponectin signalling induced by AdipoRon supplementation *in vivo* might exert beneficial effects in murine models of osteoporosis, by potentially limiting osteoclastogenesis and the resulting bone destruction phenotype. For this purpose, we investigated the effect of AdipoRon supplementation on bone loss and OP development, comparing postmenopausal OP in females and GC-induced OP in males to cover eventual sex- and mechanism-related differences. Contrasting to our hypothesis, AdipoRon treatment further diminished trabecular bone areas and increased cortical porosity in both models. Finally, we found an impact of AdipoRon on adipocyte identity *in vitro* which can be linked to the bone phenotype. Future investigations should aim to further clarify differences and similarities in AdipoRon activity in various adipocyte-related organs, taking into consideration its autocrine and paracrine way of action on both adjacent and distant tissues as well as the divergent local environment.

Results

Murine postmenopausal osteoporosis development is aggravated by AdipoRon treatment

We hypothesized that OCL-driven bone loss occurring during OP would be ameliorated by AdipoRon treatment. To mimic primary, hormone-related osteoporosis, 11-week-old mice were ovariectomized (OVX) and randomly assigned into groups receiving *ad libitum* control diet or an AdipoRon supplementation diet (ASD) yielding a mean dose of 40 mg/day/kg body weight. This oral dose of AdipoRon has been shown effective in various situations [10,18–20]. ASD started from 10 days post-surgery and continued for 6 weeks (Figure 1A). Both groups increased in body weight typically associated to this model, however slightly more pronounced in OVX-ASD (Figure 1B). Consistent with a small but steady increase in food intake in this group (Figure 1C, week 6), these small differences seem to be cumulative in disturbing systemic lipid metabolism and storage, indicated by the increase in both visceral and subcutaneous white adipose tissue (WAT) mass (Figure 1D). These measurements are contrasting to reports of AdipoRon supplementation in obesity models [10]. To investigate systemic glucose uptake under the different diets during and at the end of the protocol, blood glycaemia was measured at early (after 3w) and later (5w) stages of AdipoRon supplementation, respectively. No differences were observed between OVX-ASD and OVX groups, suggesting that insulin sensitivity and control of glucose metabolism are not affected by AdipoRon supplementation. However, glycaemia was reduced in both groups over time, indicating that OP might affect the long-term clearance of glucose (Figure 1E).

Regarding the bone phenotype, μ CT analysis of the tibiae (Figure 1F) revealed an unexpected loss of cortical thickness and cortical bone surface/bone volume (BS/BV) in ASD mice as compared to the OVX condition alone (Figure 1G). In line, in the OVX-ASN group, bone volume/trabecular volume (BV/TV) as well as trabecular parameters (area, thickness and number) were significantly diminished, while bone surface/BV (BS/BV) was higher and separation did not differ (Figure 1H). Overall, these data indicate lowered bone mass is induced by ASD both cortical and trabecular compartments, which was not expected according to our initial hypothesis.

To further explore AdipoRon action in OP, we next investigated whether ASD could reverse an established OP phenotype, which is typically associated to increased OCL numbers due to accelerated maturation and activity [21]. For this purpose, OVX mice were put on ASD 6 weeks after surgery (Figure 2A). In this setup, weight gain was similar in both groups until 10 weeks post-surgery (Figure 2B). Food uptake was almost equal, as measured daily and depicted at week 9 (Figure 2C). No impact of short-term ASD was observed neither on visceral nor subcutaneous WAT depots (Figure 2D), and glycaemia was not affected between the groups. In contrast with the in the previous setting

(Figure 1E), ASD slightly increased glycaemia at the later time point (Figure 2E). Finally, short-term ASD only altered BS/BV and trabecular thickness (Figure 2G), whereas other cortical and trabecular parameters were not affected (Figure 2F, G). Although these differences did not reach statistical significance, altogether they also tended to display lower bone mass in presence of ASD.

Thus, we conclude that primary, hormone-related bone loss is accelerated by ASD, which is in strong contrast to our initial hypothesis of a protective effect of AdipoRon on bone remodelling; in addition, we did observe a systemic metabolic impact of AdipoRon on lipid storage over time.

AdipoRon accelerates reduction of bone mass during GC treatment

A multitude of highly diverse factors contribute to the onset and progression of OP. In order to exclude the impact of the low-grade inflammatory state described in OVX-OP, we decided to investigate the effect of AdipoRon on a model of anti-inflammatory/immunosuppressive medication-induced OP and an established preclinical OP model.

OP is a frequent complication for patients receiving GCs, as such the GC-induced secondary OP is a frequently used and well reported model, displaying general changes in bone homeostasis and metabolism. The OP-like phenotype might differently affect trabecular and cortical bone regions [22]. This model acts independently of inflammatory processes, as corticoid treatment potently ablates inflammation and is in clinical use against multiple diseases to defeat pro-inflammatory processes. We used corticosterone, which has been reported to affect systemic metabolism, neurological functions as well as (bone) growth [11,12]. Five-week-old male mice were treated with corticosterone (35 mg/L) in drinking water to randomize timing and ensure a constant but rather low dose during 7 consecutive weeks. From 4 weeks after corticosterone initiation, AdipoRon was administered i.p. daily (ARN, 1 mg/kg/day) for 3 weeks on a randomly assigned part of both control (CTL) and GC groups (Figure 3A). This procedure has been already described to be effective to reverse behaviour and metabolic alterations in GC-model [11,12].

GC administration induced bone loss in comparison to the CTL group as indicated by decrease in cortical bone volume and area (Figure 3B) and trabecular thickness (Figure 3C). This was accompanied by an increase in number and a decrease in separation of trabeculae (Figure 3C), 2 parameters that normally tend towards the opposite during OP. Together, these results hint towards a general increase in bone turnover and remodelling in this state of GC-induced OP, rather than to a fully established OP phenotype. Interestingly, AdipoRon treatment induced trabecular bone loss in the CTL group and further accelerate bone loss in the GC group, respectively (Figure 3C). Moreover, in the CTL-ARN group, 3D reconstructive visualization of the femoral diaphysis revealed evidence that AdipoRon alone was sufficient to induce a porous phenotype specifically in the horizontal layers of

the central cortical midshaft, as shown in by bone volume and porosity/surface (Figure 3E). In fact, the effect of AdipoRon was highly comparable between CTL and GC groups, which points towards a possible mechanism of action of AdipoRon independently of the OP model; AdipoRon alone seemed to accelerate bone loss and OP establishment (indicated by slightly reduced bone area and trabecular parameters in Figure 3B and C) in addition to GC, which by itself was not sufficient here to induce severe OP.

Serum levels of RANKL, the master cytokine of osteoclastogenesis, was massively induced during GC-OP (Figure 3E). Contrasting to the bone phenotype, AdipoRon treatment consequenced a reduction of RANKL concentration in both groups and since OPG levels were not altered among the different conditions (data not shown), the RANKL/OPG ratio (a potential biomarker of ongoing osteoclastogenesis) displayed the same profile as RANKL.

Taken together, both models did not confirm our hypothesis, but rather support the notion of a possible detrimental effect of AdipoRon in feeding forward OP development in the 2 different models, independent of the hormonal/low-grade inflammatory or GC induced background. Thus, we decided to investigate the effect of AdipoRon on the local bone marrow environment.

Bone marrow adipocytes and OCLs are susceptible to both GC and AdipoRon treatment

Therefore, we investigated the effect of GC and AdipoRon on cells crucial for bone homeostasis, i.e., bone marrow adipocytes (BMAd) and osteoclasts. Histological analysis of decalcified bones revealed a severe effect of GC on BMAd content compared to CTL (Figure 5A), as reported previously [23–25]. Furthermore, and in exactly the same way as for epididymal WAT deposition and adipocyte hyperplasia [12], the BMAd compartment reacted oppositely to AdipoRon depending on GC or CTL conditions: under CTL conditions, AdipoRon caused a slight increase, while under GC-OP conditions, it significantly reduced BMAd (Figure 5A-B).

In regard to the OCLs, *in vitro* investigations revealed a reduction of mature OCLs after GC treatment, both during its presence throughout the whole differentiation (data not shown) as well as during the later fusion stages (treated from d4 of culture, Figure 4C and D). Importantly, we also confirmed the reported anti-osteoclastogenic effect of AdipoRon on OCLs *in vitro*, alone and in combination with GC presence (Figure 4C and D) [16]. In the same setting, we treated mature OCLs differentiated on bone mimetic surface and quantified their resorptive activity after 72h (Figure 4E and F). Quantification of the total resorbed area as well as the number of resorption pits did not show any effect of the GC dexamethasone (DXM), but again confirmed a strong inhibition of osteoclastic activity by AdipoRon (Figure 4D). Interestingly, when correlating the number of pits per well with the number of OCLs in the respective conditions, increased activity during AdipoRon

presence was observed, which was even more pronounced in the combination treatment (Figure 4G). In view of these findings, combining the results of our histological *in vivo* analyses between BMAds and *in vitro* assays of OCLs, it was concluded that OCLs are not the primary effector in the observed bone phenotype. We therefore sought to focus on adipocytes as mediators of the observed bone loss. We have previously reported that body weight gain was increased during GC treatment, and accompanied by increased food consumption as well as excess of leptin and insulin that had an impact on both bone development and adipose tissue distribution [11]. Thus, to explore the action of AdipoRon on systemic metabolism and how this might additionally affect the observed bone phenotype, we performed *in vitro* experiments to investigate GC-induced effects in presence of AdipoRon in adipocytes.

GC treatment impacts adipocyte maturation and phenotype, while the effect of AdipoRon is time-dependent

In our setting, as reported [12], GC treatment significantly altered fat mass and storage as GC-treated mice gained more weight than CTL mice, expanded epididymal WAT depots and adipocyte size, while AdipoRon treatment reversed both (Figure 3). Thus, we hypothesized that AdipoRon action on adipocyte differentiation, maturation and function might be sufficient to ablate the bone-sparing effect linked to its anti-osteoclastic potential [16].

For this purpose, the subcutaneous human stromal vascular fraction (hSVF, a heterogeneous fraction containing mesenchymal stem cells (MSC), fibroblasts, endothelial cells and different immune cell populations [26]) was cultured in presence of the DXM alone or in combination with AdipoRon to investigate the impact on early adipocyte induction capacity (d0-3). The effect of DXM +/- AdipoRon on full adipogenesis was investigated by addition of DXM for 12 days and subsequent AdipoRon stimulation from d3-12 (Figure 5A). For both experiments, transcript levels of typical adipocyte markers were measured by RT-qPCR at the end of the adipogenic induction by d12 (Figure 5B): *Ppar γ* is the master gene of adipocyte differentiation through regulation of fatty acid and glucose metabolism; Fatty acid binding protein 4 (*Fabp4*) is a transport molecule for fatty acids specifically expressed on mature adipocytes; *Perilipin 1* (*Pln1*) functions as a structural protein of lipid droplets. As already described [27], DXM rapidly induced *Ppar γ* as well as *Fabp4* and *Pln1* within the 3 days of stimulation (Figure 5B, dashed lines represent expression levels of no DXM control). Importantly, *Ppar γ* and *Fabp4* increase was strongly reduced by the concomitant treatment with AdipoRon, while *Pln1* levels were not influenced. In contrast, differentiating adipocytes cultured with DXM over the full period of 12 days showed highly similar levels of *Pln1* and *Fabp4*, whereas *Ppar γ* expression was significantly lower than in the short-term condition. Interestingly, during long-term DXM+AdipoRon co-stimulation, *Ppar γ* and *Fabp4* levels were significantly higher than with DXM

alone, while Pln1 expression remained unchanged. To conclude, DXM effect on early adipogenesis from hSVF-MSCs seems attenuated by AdipoRon exposure, while the latter upregulates the pro-adipogenic pathway through Ppar γ and Fabp4 over longer periods, provoking the exact opposite effect than DXM. We detected equivalent protein levels of perilipin (Figure 5C) in the 2 settings, and morphologic examination did not show major differences neither in differentiating (Figure 5D).

In order to investigate the effect on mature adipocytes, DXM was added for the last 3 days of the protocol and a subsequent addition of AdipoRon overnight (Figure 5A, days 14 to 17). Morphological examination as well as PLN1 protein level (Figure 5E and F) did not reveal any differences between the conditions. However, AdipoRon treatment significantly decreased lipolysis over control condition (measured as isoproterenol-stimulated release of glycerol, Figure 5G, left panel). In simultaneous presence of DXM, stimulated lipolysis was not significantly affected by AdipoRon in this setting (Figure 5G, right panel). Finally, gene expression was measured by RT-qPCR at d17 (Figure 5H), revealing a substantial impact of DXM alone on mature adipocyte identity. Briefly, DXM upregulated Pln1, Pgc1 α , Atgl, Fas and Fgf21, but downregulated Ppar γ , Fat/CD36, Lpl, and kept some transcripts unaffected (Fabp4, RIP140). In contrast of what would have been expected during GC-induced OP (Nicolas et al. 2018, Figure 1D, E), Fat/CD36 and Lpl expression, both involved in fatty acid uptake and lipid storage were reduced, while Atgl (triglyceride hydrolysis), Fas (de novo fatty acid synthesis) and Pln1 (triglyceride accumulation) were up-regulated. In addition, as expected for insulin-sensitive adipocytes [28], glucose uptake in adipocytes seemed to be coordinated through decrease in Glut1 but increase in Glut4 expression. Il1 β and Il6 expression was almost completely ablated, suggesting that the anti-inflammatory action of GCs also worked through the attenuation of adipocyte-produced inflammatory mediators.

In addition to DXM, AdipoRon only had minor effects on expression levels of most of the genes. However, while AdipoRon reversed DXM-induced increase of Fas and Fgf21 expression (an endocrine mediator regulating energy homeostasis), it further increased Pgc1 α levels (transcription coactivator regulating energy metabolism).

In summary, our data suggest that DXM treatment favours lipid turnover rather than storage, while AdipoRon itself does not seem to functionally impact mature adipocytes.

Discussion

The data presented here contradict our initial hypothesis of AdipoRon protection against OP development through osteoclastic inhibition. Instead, the results highlight the complexity of cellular interplays and cross talks, metabolic balance and mediators underlining the limitations of *in vitro* observations to reproduce the complexity of *in vivo* settings. Our two pre-clinical OP models allow to study onset and progression of bone loss in different conditions: while OVX mimics post-menopausal changes and thus female hormonal changes due to lack of oestrogen, the GC-induced model targets inflammatory processes as well as cellular and systemic metabolism. OVX mice develop bone loss simultaneously to the establishment of important systemic changes; these include weight gain and increased visceral fat depots due to metabolic changes as well as the establishment of a chronic low-grade inflammatory state, in part caused by an increase in intestinal permeability and microbial dysbiosis. Indeed, weight gain leads to inflammation with subsequent changes in hormonal balances and intestinal integrity, which is also strongly affected by oestrogen signalling [29–36]. In contrast, GC-induced OP strongly links systemic metabolism, especially aberrant glucose and lipid metabolism, as well as interference with different endocrine circuits to the development of the disease [37,38]. As reported earlier [12], ablation of circulating inflammatory mediators in the GC-ARN model was reversed by AdipoRon, which did not show any effect on the cytokine levels in the control group. Thus, in view of the effect of AdipoRon on the the bone phenotype even in control conditions, we suppose that the mechanism might be independent of the inflammatory environment affected by GC's.

Importantly, these two models also cover two different aspects of OP, namely primary OP as observed in postmenopausal females, as well as secondary OP induced by pharmacological treatment, or the persistence of secondary conditions linked to chronic inflammation [39]. Comparison of AdipoRon impact in these two models reveals variable effects on cortical parameters depending on the OP origin, at least under the herein tested conditions. To clearly conclude on this point, it might be of interest to increase the induction time in the GC model of young male mice to investigate a more severe OP phenotype. However, trabecular bone loss was rapidly and similarly established and accelerated by AdipoRon in both models, thus highlighting the important role of adiponectin signalling in the onset of trabecular osteoporosis, that temporarily precedes the cortical phenotype and thus hinting to spatial differences.

While enhanced osteoclastogenesis is the main driver of bone loss, our data suggests that this process is not the direct target of AdipoRon. Indeed, we observed that AdipoRon potently decreases OCLs formation *in vitro*, consistent with previous reports, while significantly increasing bone loss *in vivo*, suggesting that the regulation of bone remodelling by AdipoRon acts through other cells in a more complex regulation, that eventually favours bone loss indirectly. Moreover, this

indirect effect of AdipoRon on remodelling could explain the unexpected reduction in plasma RANKL levels.

Adiponectin & its systemic effects

AdipoRon (just as adiponectin) is an agonist of AdipoR1/2 that has been well described in obesity models including high fat diet. Adiponectin levels are correlated with sex, age, body mass index/fat mass (as indicators of obesity status), metabolic health status as well as bone mineral density; in line with our data, serum adiponectin has been inversely correlated with bone mass in human and mouse studies [14,15,40–42].

While AdipoRon was administered orally in the chow to OVX mice, intraperitoneal injections were performed in GC-treated animals in order to bypass the possible pharmacokinetical concerns related to its absorption and systemic distribution. In this regard, an important limitation in current investigations is the lack of comprehensive data on AdipoRon pharmacokinetics. According to the literature, it reaches its peak plasma concentration 30 min after intraperitoneal injection (in 2.5% DMSO) [12], and 2h after oral administration when dissolved in corn oil [43]. Both studies report a rapid clearance of detectable plasma AdipoRon (slightly over 3h versus 6h), indicating a rapid turnover, probably through multiple organ systems.

At the cellular level, we and others have shown that AdipoRon has an important impact on OCLs by regulating differentiation as well as resorptive activity through AMPK signalling [16,44]. In addition, it is well-described that GC provoke bone loss by changing the mesenchymal potential of osteogenic and adipogenic lineage differentiation from MSCs [45–47] by inducing apoptosis or transition in osteoblasts [48–50] as well as through OCL mediated mechanisms [51,52]. In humans, it is already described that BMAT is increased in osteoporotic conditions [53,54]; a recent study has further shown that GC-associated OP (induced by pharmacological GC treatment as well as due to an endogenous, persistent GC overload during Cushing Syndrome) comes together with increased adipocyte content, however, the extent might be different depending on the bone areas [25]. In the herein studied models, both OVX- and GC-induced OP come together with weight gain [12,21]. However, while combination of GC and AdipoRon reversed weight gain, epididymal WAT and adipocyte hyperplasia [12] as well as BMAd content in the bone, we could observe a significant increase in body weight as well as in adipose depot mass during ASD in OVX over time, which was not present in the short-term rescue approach. This is in strong contrast to previous studies on AdipoRon's impact on body mass [10,11,55,56], and raises the question whether the mode of administration impacts on the interaction of AdipoRon with adipocytes. Moreover, the different effect of AdipoRon on body weight in the OVX model compared to GC-induced OP might also be impacted by the ovariectomy-induced absence or changes in oestrogen receptor α signalling [57].

Interestingly, in a breast cancer study, adiponectin has shown divergent signalling depending on oestrogen receptor α expression: the authors described that cancer cell lines ER α ⁺ ablate AMPK activation but induce MAPK signalling and regulate the cell cycle progression [58].

Adiponectin & its effects on the MSC lineage

Adiponectin signals through AdipoR1/ 2 and subsequent AMPK or MAPK activation, respectively, thereby determining MSC differentiation [59–61]; adiponectin as well as its two receptors are expressed in all the cells that are present in the bone, including MSCs, osteoblasts, bone marrow cells, adipocytes, bone marrow-macrophages and osteoclasts [62–64]. The addition of adiponectin to BM-derived OBLs has been shown to significantly induce their RANKL expression [65]. In contrast, Liu et al. reported the pro-osteoblastic and anti-adipocytic effect of AdipoRon on MSCs from both bone marrow and adipose tissue origin; arising OBLs had reduced expression of pro-osteoclastic mediators (such as RANKL), adding to a diminished potential of macrophage progenitors to differentiate into OCLs [66]. While their findings on OCLs and reduced adipogenesis are in line with ours [11,16] and support our initial hypothesis of a protective effect of AdipoRon during OP, it is in contrast with our previous demonstration of lack of action on the growth retardation observed during GC treatment, a parameter that is strongly depending on osteoblastic differentiation and bone formation rate [11].

In this respect, DXM is known to shift MSC differentiation towards adipogenesis rather than osteogenesis [45], while the existing *Col1a1*⁺ osteoblastic lineage cells found in bone might simultaneously acquire an endothelial-like phenotype [50]. Thus, bone formation is reduced, as is survival of mature OBLs and osteocytes, through GC effect on cell cycle progression during lineage differentiation [47–49,67]. Simultaneously, GC have been shown to feed forward adipogenesis and osteoclastogenesis, and even to partially ablate OCL apoptosis induced by bisphosphonates [51,67,68], eventually affecting bone-coupling mechanisms [52]. Interestingly, in this study, AdipoRon supplementation readily prevented the GC-associated changes in systemic lipid and glucose metabolism, but failed to reverse the bone growth defect [11], pointing towards an effect via adipocytes. Existing literature describes that activation of AdipoR's by synthetic agonists is beneficial against pathological bone loss e.g. in diabetic periodontitis [69]. We can only speculate, but the reason why we did not observe a bone sparing effect of AdipoRon in OP might be dependent on or mediated by its effect on another predominant cell type. This notion is supported by the fact that BMAd content did not correlate with the observed bone loss, and cortical bone parameters normally change with a delay when compared to trabecular bone, which is subject to rapid remodelling and thus also to degradation during OP [70].

Adiponectin & adipocytes

Consequently, drawing attention to the adipocyte lineage, reports on adiponectin/AdipoRon impact on adipocyte differentiation, maturation and function are rather scarce. Here, we show an AdipoRon- and GC-dependent change in adipocyte content in the femoral bone marrow, in line with changes in adipocyte surface and epididymal adipose tissue weight [12]. In an adipocyte cell line, AdipoRon was shown to suppress adipogenesis through AMPK signalling [71]. Moreover, white and brown adipocytes were shown to respond differently to Adiponectin overexpression as well as AdipoRon stimulation, by changing their metabolic mitochondrial respiration and activity [72]. A recent study described the reducing effect of both adiponectin overexpression and AdipoRon addition on the CD34 expression profile in adipose tissue-derived mesenchymal progenitors, thus rendering the stem cell compartment more prone to giving rise to beige rather than brown or white adipocytes and therefore changing their intrinsic metabolic properties [72].

Adipocyte-derived adiponectin acts in both autocrine and paracrine manners; as such it is both a target gene as well as an inducer of PPAR γ , whose KO has shown both an increase in OBLs and OCLs [16], while studies of bone phenotype in adiponectin KO mice did not show clear results [63,65,73]. Adipocyte-specific overexpression of adiponectin recapitulates the phenotype of animals treated with PPAR γ agonists in terms of adipocyte differentiation [72,74], while PPAR γ insufficiency ablated adipogenesis [75]. In the current study, Fgf21 was the most importantly induced by DXM and regulated by AdipoRon of the transcripts investigated. Fgf21 is an important endocrine mediator of glucose and lipid metabolism [76], and a link between adiponectin/AdipoRon and PPAR γ synergy [77–79]. In this context, Fgf21 has also shown an inverse correlation with bone mineral density and mass [80,81]. As such, the multidirectional axes between adiponectin-Fgf21-PPAR γ actions, Fgf21 production by adipocytes might exert divergent autocrine effects depending on the anatomical location.

However, BMAdS are a unique cell type within the adipocyte family, in particular glucose uptake and insulin responsiveness, and can neither be considered white, brown or beige [82]. Moreover, they are indispensable in regulating the BM environment by impacting on OBL, OCL but also e.g. vessel differentiation and function [83,84], and are generally inversely correlated with bone mass in multiple conditions [85]. Thus, this leaves us to speculate, that despite the local BMAd content is similarly affected than WAT by metabolic changes in our models, we cannot rule out the possibility that these adipocytes, in concert with AdipoRon, might impact bone parameters in a yet unknown manner. These factors make the interpretation of this compartment on overall bone cell function difficult. In addition, the spatial location and different types of bones need to be considered. As an example, BMAdS have been found in high quantities in the proximal tibia, but not to the same extend in femurs of rodents [82], which is where we looked. This opens the subject of the necessity to perform μ CT and histologic analyses on multiple bones and regions to understand this

relationship. In line, GC receptor α is expressed in all bone cells [86], but differences in expression patterns (depending on the developmental stage, the type of bone and the location linked with the ossification type) suggest variable effect of GC throughout life and location in the skeleton [87]. In this study, we've measured bone parameters of the long bones, and observed differences of changes in bone mass between the cortical and trabecular areas. In line, it is well described that the location can dictate a lot of diverse biological processes such as inducing OCLs in cancer metastasis models [88,89], interaction of the innate and adaptive immune system in the bone marrow [90,91] or also the appearance of GC/Cushing syndrome-induced changes in the bone remodelling units [92]. BMAds in their environment have long been regarded as sole placeholders of void areas; however, it became clear in recent years that their location, their proliferative or hyperplastic reaction to environmental changes e.g. during obesity as well as their individual capacity to switch from initial phenotypes (whitening, browning, beige-ing) in turn also impacts on the bone, mainly driven by their impact on stem cell differentiation and expansion capacity [93]. Adipocyte type and function depends on the bone area, especially with respect to red or white bone marrow, which might also explain some of the differences in the observed bone loss phenotypes between trabecular and cortical bone. Therefore, we conclude that further investigations into adipocyte maturation, function and their spatial distribution, both within bone and other subcutaneous or visceral depots, are critical areas to address. This includes not only their endocrine and paracrine functions through adipokine production, but also the diverse adipocyte phenotypes depending on their anatomical location, responsiveness to metabolic changes and regulative capacity of the BM environment during OP-associated pathologies.

Material and methods

OVX-induced osteoporosis

Female C57BL/6 mice were randomly assigned to groups and bilaterally ovariectomized or Sham-operated by Charles River Laboratory at 11 weeks of age. Mice were closely monitored during the entire healing period of the incision. All animals had ad libitum access to food and tap water and were maintained under a 12-hr light/dark cycle. For the first approach, starting from 2 weeks after surgery, a randomly assigned subset of mice received chow diet containing ADR (=ASD, 330 mg ADR/kg of pellets). This allows an ingestion of 40 mg of ADR /day/kg of mouse body weight (according to mouse mean weight (25 g) and the average food consumption results which is around 3.235 g/day/mouse) until six weeks after surgery, when mice were killed. In the second approach, mice fed the same ASD 6 weeks after surgery, for 4 weeks until killing after 10 weeks. All experiments involving animals were approved by the Veterinary Office of the Canton Vaud (Switzerland authorization number VD3311.a) in accordance with the Federal Swiss Veterinary Office Guidelines and conform to the Commission Directive 2010/63/EU.

Glucocorticoid-induced osteoporosis

Male C57BL/6 J mice were obtained from Janvier (France). Five-week-old male mice were housed on a 12-hour light/dark cycle with chow ad libitum. Animals were assigned randomly into groups and had free access to drink either corticosterone (35 mg/l dissolved in tap water containing 3.5 g/l β -Cyclodextrin) or vehicle alone (tap water containing 3.5 g/l β -Cyclodextrin) for 7 consecutive weeks. To prevent possible degradation, both vehicle and corticosterone solutions were changed every 3 days. After 30 days of GC treatment, both groups were split into two groups, which received either 1mg of AdipoRon /day/kg of body weight (1 mg/ml solution dissolved in 2.5% DMSO) or vehicle (2.5% DMSO) through daily intraperitoneal injection. Injections were done every day at 6 p.m. for 20 consecutive days until sacrifice. All experiments were approved by the French Ministry of Health, Higher Education and Research (permission number 010344.01) and conducted in accordance with the Institutional Ethics Committee on Laboratory Animals (CIEPAL-Azur, Nice Sophia-Antipolis, France).

μ CT and bone parameters

Long bones of mice were fixed in 4% paraformaldehyde o/n. Bone microarchitecture analysis of OVX experiments using high-resolution μ CT was performed at the preclinical platform ECELLFRANCE (IRMB, Montpellier, France). Cortical and trabecular femora were imaged using high-resolution μ CT with a fixed isotropic voxel size of 18 μ m with X-ray energy of 50 kV, current of 500 mA, 0.5 mm aluminium filter, and 210 ms exposure time. Quantification of bone parameters was performed on the trabecular region of the proximal part of each femur (1.72 mm long) and on the cortical region (0.43 mm long region centered at the femoral midshaft) on CT Analyzer software (Bruker microCT,

Belgium). For generation of the GC-OP data set, fixed femora were scanned using high-resolution SkyScan-1173 X-ray micro-computed tomography system (Synergie 4, Bruker, France) set with the following parameters: 7.76 μ m pixel size, 45 kV, 1mm thick aluminium filter, 0.5° of rotation step. Quantification of total trabecular and cortical bone parameters were performed on the whole femurs and on the cortical diaphyseal region (64 μ m centered at the femoral midshaft) on CT Analyzer software (Bruker microCT, Belgium). For visual representation, 3D reconstructions were generated using CTvox software (Bruker μ CT, Belgium).

Glycaemia measurement

Blood was collected from the tail vein and glycaemia was evaluated by the use of a glucometer (Bayer Nreeze 2, Canada).

Serum ELISA measurements

Blood was sampled by intracardiac puncture from anesthetized animals and serum was separated from cellular fraction by centrifugation before storage at -80°C. RANKL and OPG were evaluated using Quantikine ELISA from R&D systems (#MTR00 and #MOP00 respectively) and following manufacturer's instructions. Limits of quantifications were 5 pg/ml for RANKL and 6.9 pg/ml for OPG, respectively.

Histological analysis

Femurs were collected and fixed in 4% PFA before decalcification in 10% EDTA for around 2.5 weeks and embedding in paraffin blocks. Blocks were cut in 5 μ m thick slices, dehydrated and stained with haematoxylin and eosin. Pictures were taken at a magnification of 10x at a Leica DM3000 LED microscope. BMAds were quantified per region of BM using Image J software, in one representative image of a sagittal image of the distal femur for 4-5 replicated per group.

Osteoclast assays

Primary bone marrow cells from long bones of mice were seeded with 30 ng/ml RANKL and 25 ng/ml M-CSF in α MEM (completed with 5% characterized SVF, 1% Penicillin-Streptomycin and 50 nM β -Mercaptoethanol) for 5 days, with a boost on d3. Respective treatments (1 μ M DXM, 10 μ M AdipoRon) were added from d0 or d4 of differentiation. Trap staining (according to manufacturer's instructions) was performed at d5. For functional assays, OCLs were differentiated on a bone mimetic surface and treated with DXM, AdipoRon or a combination from d4 for 72h. Resorbed area was quantified after counterstaining the matrix with 1% Alizarin red, using Image J software.

Adipocyte cultivation and treatment

Subcutaneous abdominal human adipose tissue primary progenitor cells were coming from a previous study [94]. Adipose progenitors were cultivated in DMEM containing 1 g/l Glucose, 25 μ g/ml Gentamycin and 10% FCS. Upon confluence, cell culture medium was supplemented with 1 μ M dexamethasone, 860 nM insulin and 500 μ M isobutyl methylxanthine for 2-3 days to induce

adipogenesis. Differentiation was continued with reduced concentration of 100nM insulin for differentiation and maturation for 8-10 days. When indicated, cells were treated with additional 1 μ M dexamethasone for certain time frames and/or 10 μ M AdipoRon, respectively.

RT-qPCR

Differentiating (D12) and mature (D17) adipocytes were treated with DXM and AdipoRon at different doses for different time slots. Total RNA was extracted with Trizol according to the manufacturer protocol. RNAs were reversely transcribed (Superscript II, Life Technologies), and RT-qPCR was performed using SYBR green and the primers indicated below. Results were normalized to the housekeeping gene Rplp0 with the 2 $^{-\Delta$ Ct method.

	FW	RV
Ppar γ	AGCCTCATGAAGAGCCTTCCA	TCCGGAAGAAACCCTTGCA
Fabp4	TGTGCAGAAATGGGATGGAAA	CAACGTCCCTTGGCTTATGCT
Pln1	ACCCCCTGAAAAGATTGCTT	GATGGGAACGCTGATGCTGT
Pgc1a	CTGTGTCACCACCAAATCCTTAT	TGTGTCGAGAAAAGGACCTTGA
Fat	TGATGATGAACAGCAGCAACA	AGACTGTGTTGTCCTCAGCG
Lpl	CGAGTCGTCTTCTCCTGATGAT	TTCTGGATTCCAATGCTTCG
Atgl	GGGAGAAGATCACGTCTCTGG	CTCCAGCAAGCAGATGGTGA
Glut1	ATACTCATGACCATCGCGCTAG	AAAGAAGGCCACAAAGCCAAAG
Glut4	CATTCCTTGGTTCATCGTG	ATAGCCTCCGCAACATAC
Fas	ACAGGGACAACCTGGAGTTCT	CTGTGGTCCCCTTGTGAGT
Il6	GGCACTGGCAGAAAACAACC	GCAAGTCTCCTATTGAATCC
Il1b	CTCTCTCCTTTCAGGGCCAA	GAGAGGCCTGGCTCAACAAA
Fgf21	GAGCCCCGAAAGTCTCCTG	TGGGCTTCGGACTGGTAAAC
Rip140	TTGGAGACAGACGAACACTGA	TCTACGCAAGGAGGAGGAGA
Rplp0	TGCATCAGTACCCATTCTATCAT	AGGCAGATGGATCAGCCAAGA

Western blot analysis

Cells were lysed in TNET lysis buffer (25 mM Tris-Cl (pH 7.4), 100 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.5% Nonidet P40, 1x protease inhibitor cocktail (Roche Diagnostics, Meylan, France)). Protein concentration was evaluated by BCA assay (PIERCE, Thermo Scientific, France) and blotted using SDS-PAGE basic protocol. Primary antibody incubation was performed overnight at 4°C (anti-Perilipin-1, Interchim #BP5015, dilution 1:10000; anti- β -actin, Sigma-Aldrich #A5441, dilution 1:2500; anti-

HSP90, Sigma-Aldrich 05-594, 1:1000) and then detected with HRP-conjugated anti-Guinea pig immunoglobulins (AcrisAntibodies, Herford, Germany). Detection was performed using Chemiluminescent HRP Substrate (Millipore, Molsheim, France). OD band intensities were evaluated using FIJI Software.

Lipolysis measurement

Adipocytes were treated with isoproterenol (1 μ M) for 4 hours. Media were collected for glycerol measurements (Free Glycerol reagent, Sigma) and cells used for protein quantification.

Statistical analysis

Statistics were prepared using Graph Pad Prism 9.2 software. Analyses for comparison of 2 groups were done using two-tailed, unpaired t-test assuming equal SD between the groups. For follow up time points, paired t-tests were performed. ANOVA with multiple comparison tests was used when comparing more than two groups. Statistical significance was considered at $p < 0.05$, and experimental values are presented as individual replicates, mean \pm SEM. Biological replicates were obtained from different mice and human samples.

Author contribution

JH - Formal analysis, Investigation, Methodology, Visualization, Writing – original draft, Writing – review and editing; SN - Investigation, Methodology, Validation, Writing – review and editing; FG - Investigation, Methodology, Validation, Writing – review and editing; CW - Investigation, Methodology, Validation, Writing – review and editing; MM - Investigation, Methodology; MS - Investigation, Methodology, Validation; JYJ - Writing – review and editing; CBW - Writing – review and editing; BD - Conceptualization, Funding acquisition, Supervision, Writing – review and editing; JC - Funding acquisition, Supervision, Writing – review and editing; DP – Conceptualization, Formal analysis, Funding acquisition, Investigation, Methodology, Resources, Supervision, Validation, Writing – original draft, review and editing; DM - Conceptualization, Formal analysis, Funding acquisition, Investigation, Methodology, Resources, Supervision, Validation, Writing – original draft, review and editing;

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Figure legends.

Figure 1: AdipoRon supplementation increases metabolic disturbance and stimulates bone loss during hormone-dependent osteoporosis development. Systemic changes of ASD on osteoporosis development. A) experimental setup; B) weight curves of OVX and OVX+AdipoRon supplemented mice; C) measurement of food intake; representative difference of food/mouse/day between 2 cages with 5 mice per group as shown for week 6; D) comparison of weight of subcutaneous (left) and visceral (right) fat depots between the 2 groups, normalized to body weight; E) glycaemic values during the early (3w) and late stage (5w) of osteoporosis development. F) representative 3D reconstituted μ CT distal femurs of the 2 groups; G) cortical and H) trabecular parameters of μ CT measurements; histograms display individual values and mean \pm SEM, statistical analyses were performed with two-tailed, unpaired t-test assuming equal SD between the groups (n=10), follow up graph E used paired t-test; * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$.

Figure 2: AdipoRon supplementation marginally affects metabolic and bone parameters in established hormone-induced osteoporosis. Systemic effect of short-term AdipoRon supplementation diet (ASD) on osteoporosis rescue. A) experimental setup; B) weight curves of OVX and OVX+AdipoRon supplemented mice; C) measurement of food intake; difference of food intake/mouse/day between 2 cages with 5 mice per group as shown for week 9; D) comparison of weight of subcutaneous (left) and visceral (right) fat depots between the 2 groups, normalized to body weight; E) glycemic values before (5w) and during (7w) AdipoRon supplementation; F) cortical and G) trabecular parameters of μ CT measurements 10 weeks after surgery with or without 4 weeks of AdipoRon supplementation; histograms display individual values and mean \pm SEM, statistical analyses were performed with two-tailed, unpaired t-test assuming equal SD between the groups (n=10), follow up graph E used paired t-test; * p -value < 0.05

Figure 3: AdipoRon treatment accelerates the onset of GC-induced osteoporosis, and induces cortical porosity even in controls. Changes in bone phenotype during ARN treatment in GC-induced osteoporosis. A) experimental setup; B) cortical and C) trabecular parameters of μ CT measurements 10 weeks after OP induced with corticosterone, with or without 20 days of AdipoRon treatment; D) representative 3D reconstituted μ CT distal femurs of the 2 groups; E) representative images of 3D reconstitution of the cortical central diaphysis and respective values of cortical BV and porosity/surface, bars correspond to 500 μ m; E) ELISA serum measurements of osteoclastogenic cytokines; histograms display individual values and mean \pm SEM, statistical analyses between 2 groups (n=9-12) were performed with two-tailed, unpaired t-test assuming equal SD between the groups, ANOVA with multiple comparison test was used when comparing multiple groups (only selected significances are shown); * < 0.05 , ** < 0.01 , *** < 0.001 , **** < 0.0001 .

Figure 4: Histological and osteoclast-specific analysis of GC+/-ARN effect on the bone microenvironment. A) representative histological H&E stainings from the distal femurs, bar corresponds to 200 μ m; B) quantification of adipocytes per bone marrow area in the respective pictures from A); C) representative images of Trap-stained OCLs after in vitro differentiation from bone marrow progenitors in presence of DXM +/- AdipoRon; D) corresponding Trap+ OCL quantification; E) representative images of resorption assay of mature OCLs after 72h of DXM +/- AdipoRon presence; F) corresponding quantification of resorbed area/well (in %), left panel, and the number of pits/ well (right panel); G) resorptive activity as calculated by a ratio of pits per well to OCL numbers per well; histograms display individual values and mean \pm SEM, statistical analyses (n=3-5) were performed with ANOVA with multiple comparison test to compare the multiple groups, * < 0.05 , ** < 0.01 , *** < 0.001 , **** < 0.0001 .

Figure 5: Phenotypic analyses of adipocyte identity at different stages of differentiation during GC and AdipoRon stimulation reveal possible mechanistic insights. Changes in adipocyte phenotype during DXM (1 μ M) and ARN (10 μ M) stimulation. A) overview of experimental setup; B) RT-qPCR results of differentiating adipocytes from hSVF over 12 days, left bars from d0-3, right bars from d0-12, dashed lines represent expression levels of noDXM control; noDXM Fabp4 control level equals 0.001; C) protein level of Perilipin of differentiating adipocytes assessed by Western blot; D) Morphological appearance of differentiating adipocytes after indicated treatments with DXM and/or AdipoRon during early and late stages, scale bar represents 50 μ m; E) Morphological appearance of mature adipocytes after after DXM (d14-17) and AdipoRon stimulation (overnight), scale bar represents 50 μ m; F) corresponding PLN protein levels of E); G) functional investigation of the impact of DXM and AdipoRon presence on isoproterenol-stimulated glycerol release of mature adipocytes without (left graph) or after 1 μ M DXM stimulation; H) RT-qPCR results of mature adipocytes from hSVF at d17, after DMX from d14-17 and 10 μ M ARN overnight; histograms display individual values and mean \pm SEM, statistical analyses between 2 groups (n=3-4) were performed with ANOVA with multiple comparison test (only selected significances are shown); *<0.05, **<0.01, ***<0.001, ****<0.0001.

Authors declare no conflict of interest.

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Highlights

The adiponectin agonist AdipoRon accelerates osteoporosis development in two different models and modulates adipocyte differentiation

- **AdipoRon accelerates bone loss in hormone-related osteoporosis**
- **AdipoRon accelerates bone loss in glucocorticoid-induced osteoporosis**
- **AdipoRon-induced bone loss is related to adipocyte content**
- **AdipoRon does not appear to affect bone resorption through osteoclasts**

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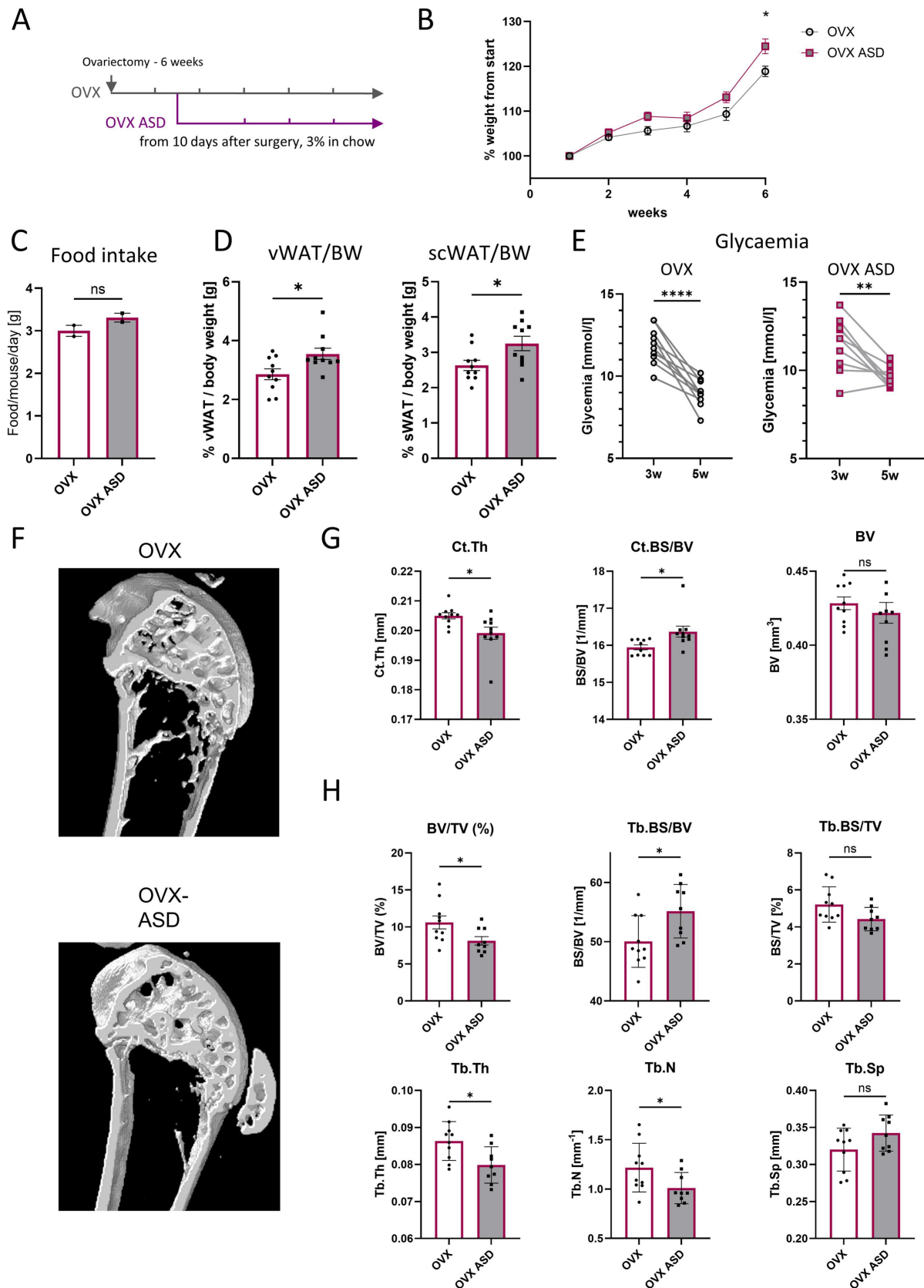


Figure 1

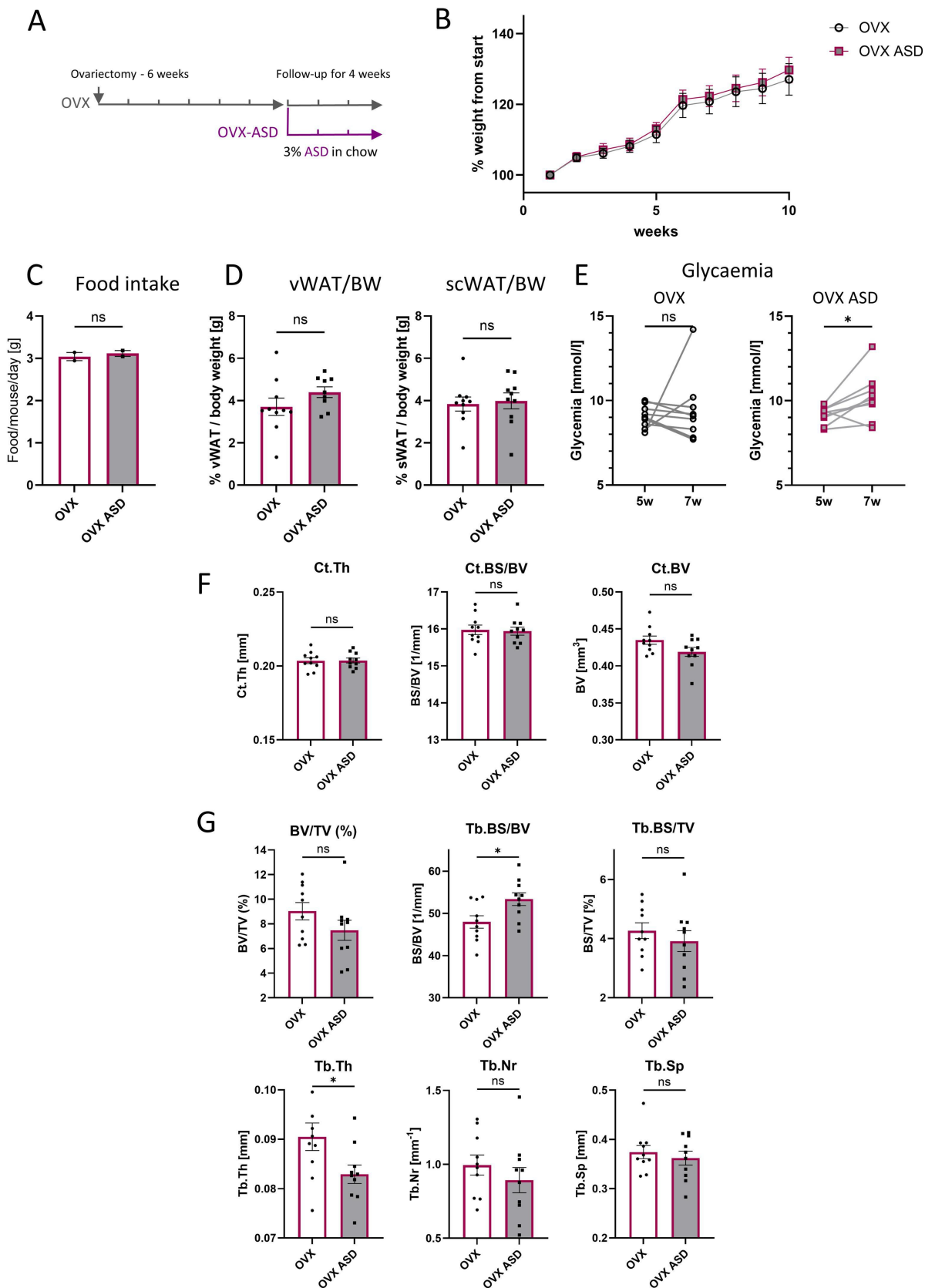


Figure 2

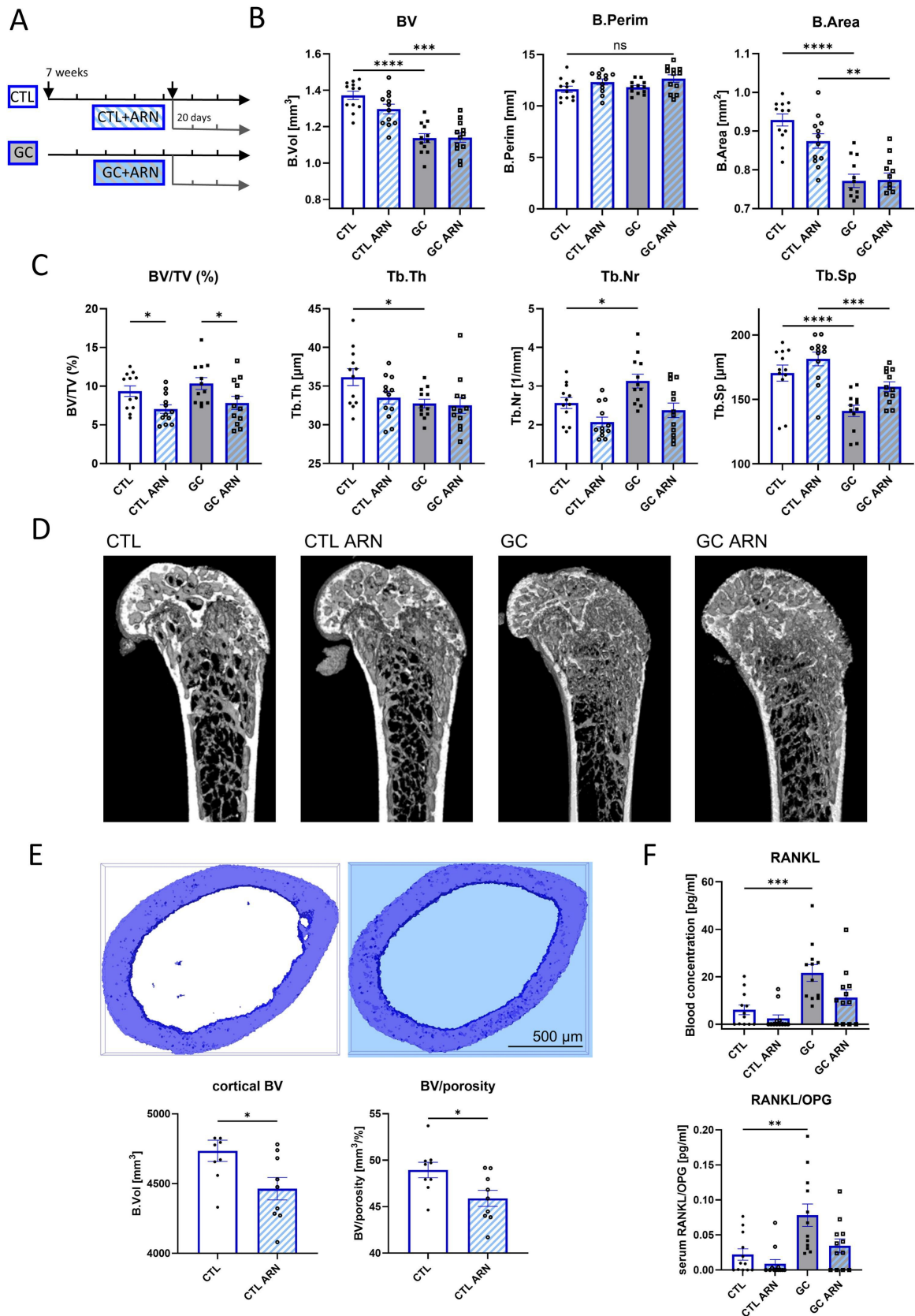


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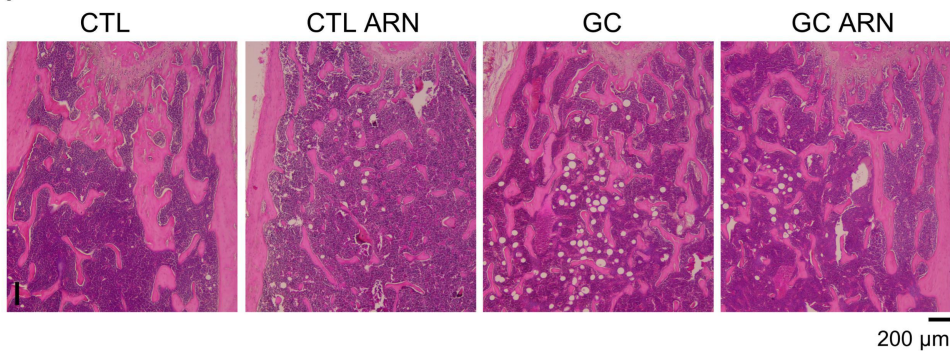
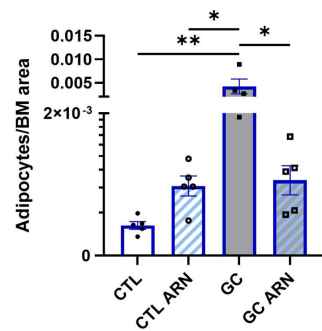
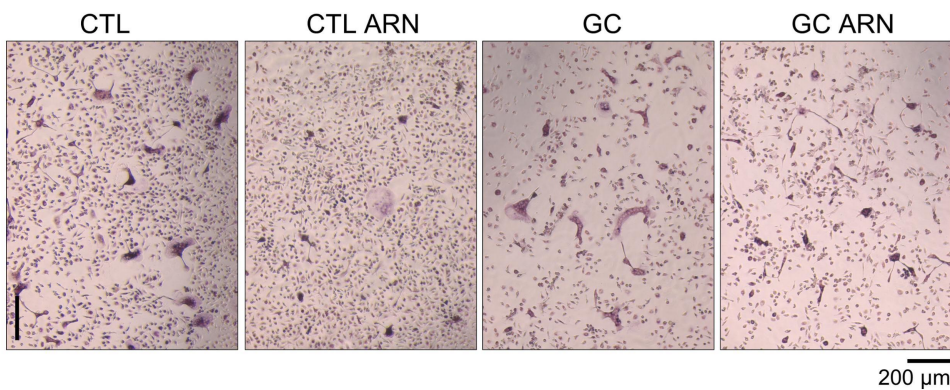
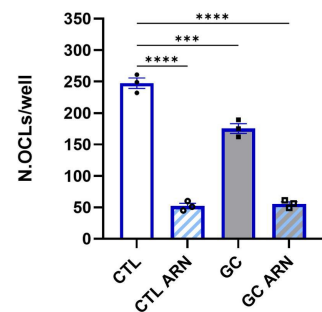
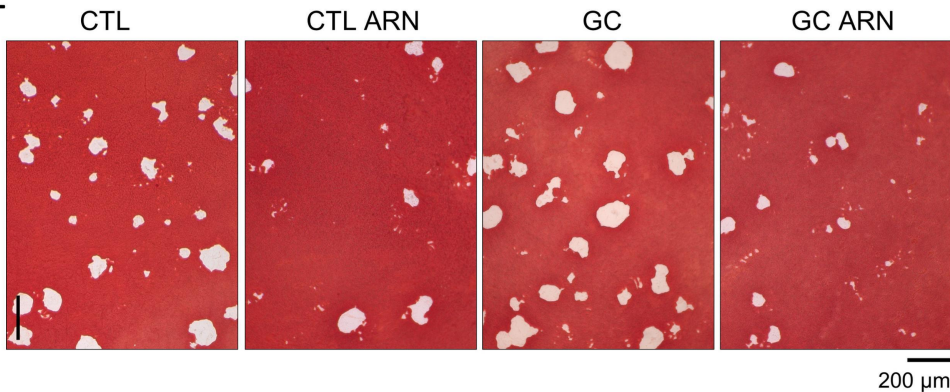
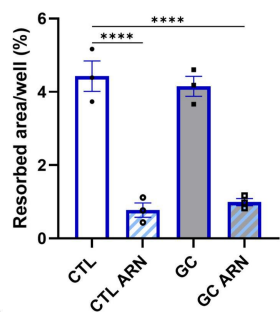
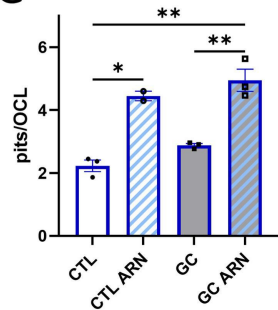
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Figure 4

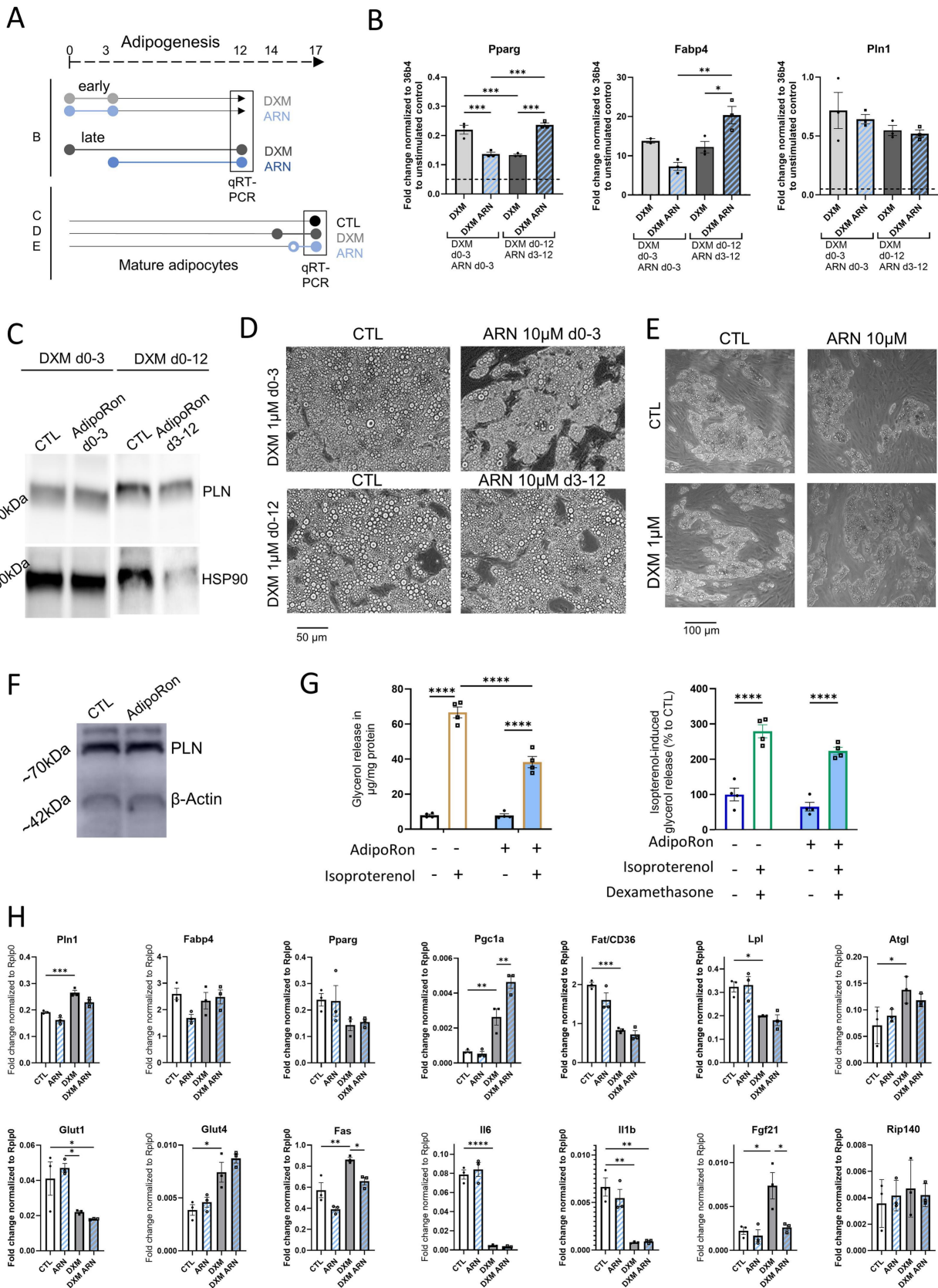


Figure 5