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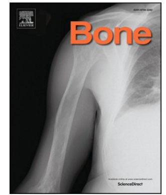

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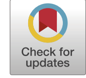

Human osteoclasts *in vitro* are dose dependently both inhibited and stimulated by cannabidiol (CBD) and Δ9-tetrahydrocannabinol (THC)

Simone S.R. Nielsen [a,b,1], Juliana A.Z. Pedersen [a,b,1,2], Neha Sharma [a,b,c], Pernille K. Wasehuus [d,3], Morten S. Hansen [e,f], Anaïs M.J. Møller [a,g,4], Xenia G. Borggaard [b,h], Alexander Rauch [e,f,i], Morten Frost [e,f,i], Teis E. Sondergaard [d], Kent Søe [a,b,c,*]

[a] Clinical Cell Biology, Pathology Research Unit, Department of Clinical Research, University of Southern Denmark, Campusvej 55, 5230 Odense M, Denmark
[b] Department of Pathology, Odense University Hospital, J.B. Winsløws Vej 15, 5000 Odense C, Denmark
[c] Department of Molecular Medicine, University of Southern Denmark, Campusvej 55, 5230 Odense M, Denmark
[d] Department of Chemistry and Bioscience, Aalborg University, Fredrik Bajers Vej 7H, 9220 Aalborg, Denmark
[e] Molecular Endocrinology Laboratory (KMEB), Department of Endocrinology, Odense University Hospital, J.B. Winsløws Vej 4, 5000 Odense C, Denmark
[f] Department of Clinical Research, University of Southern Denmark, Campusvej 55, 5230 Odense M, Denmark
[g] Department of Clinical Biochemistry and Immunology, Lillebaelt Hospital, University Hospital of Southern Denmark, Kabbeltoft 25, 7100 Vejle, Denmark
[h] Molecular Bone Histology, Pathology Research Unit, Department of Clinical Research, University of Southern Denmark, Campusvej 55, 5230 Odense M, Denmark
[i] Steno Diabetes Centre Odense, Odense University Hospital, Kløvervænget 10, 5000 Odense C, Denmark

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ABSTRACT

Legalized use of cannabis for medical or recreational use is becoming more and more common. With respect to potential side-effects on bone health only few clinical trials have been conducted – and with opposing results. Therefore, it seems that there is a need for more knowledge on the potential effects of cannabinoids on human bone cells. We studied the effect of cannabidiol (CBD) and Δ9-tetrahydrocannabinol (THC) (dose range from 0.3 to 30 μM) on human osteoclasts in mono- as well as in co-cultures with human osteoblast lineage cells. We have used $CD14^+$ monocytes from anonymous blood donors to differentiate into osteoclasts, and human osteoblast lineage cells from outgrowths of human trabecular bone. Our results show that THC and CBD have dose-dependent effects on both human osteoclast fusion and bone resorption. In the lower dose ranges of THC and CBD, osteoclast fusion was unaffected while bone resorption was increased. At higher doses, both osteoclast fusion and bone resorption were inhibited. In co-cultures, both osteoclastic bone resorption and alkaline phosphatase activity of the osteoblast lineage cells were inhibited. Finally, we observed that the cannabinoid receptor *CNR2* is more highly expressed than *CNR1* in $CD14^+$ monocytes and pre-osteoclasts, but also that differentiation to osteoclasts was coupled to a reduced expression of *CNR2*, in particular. Interestingly, under co-culture conditions, we only detected the expression of *CNR2* but not *CNR1* for both osteoclast as well as osteoblast lineage nuclei. In line with the existing literature on the effect of cannabinoids on bone cells, our current study shows both stimulatory and inhibitory effects. This highlights that potential unfavorable effects of cannabinoids on bone cells and bone health is a complex matter. The contradictory and lacking documentation for such potential unfavorable effects on bone health as well as other potential effects, should be taken into consideration when considering the use of cannabinoids for both medical and recreational use.

* Corresponding author at: University of Southern Denmark, Dept. of Clinical Research, Clinical Cell Biology, Pathology Research Unit, Campusvej, 5230 Odense M, Denmark.

E-mail addresses: Simone.Snitgard.Rosendal.Nielsen@rsyd.dk (S.S.R. Nielsen), juliana.pedersen@sund.ku.dk (J.A.Z. Pedersen), Neha.Sharma@rsyd.dk (N. Sharma), msshansen@health.sdu.dk (M.S. Hansen), ANAIMO@rm.dk (A.M.J. Møller), xborggaard@health.sdu.dk (X.G. Borggaard), arauch@health.sdu.dk (A. Rauch), mmfnielsen@health.sdu.dk (M. Frost), tes@bio.aau.dk (T.E. Sondergaard), Kent.soee@rsyd.dk (K. Søe).

[1] These authors contributed equally to this work.
[2] Present professional address: Dept. of Neuroscience, Faculty of Health, University of Copenhagen, Blegdamsvej 3, 2200 København, Denmark.
[3] Present professional address: DermaPharm, Europavej 10, 8990 Fårup, Denmark.
[4] Present professional address: Center for Gene and Cellular Therapy, Department of Clinical Immunology, Aarhus University Hospital, Palle Juul-Jensens Blvd. 99, 8200 Aarhus, Denmark.

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Abbreviations

CBD	cannabidiol
THC	Δ9-tetrahydrocannabinol
OC	osteoclast
OCs	osteclasts
OBs	osteoblasts
AEA	anandamide
2-AG	2-arachidonyl glycerol,
NAPE-PLD	N-acyl phosphatidyl ethanolamine-phospholipase D
FBS	fetal bovine serum
MCSF	macrophage colony-stimulating factor
RANKL	nuclear factor-kappaB ligand
TRAcP	Tartrate-Resistant acid Phosphatase
PBS	phosphate buffer solution
ALP	alkaline phosphatase
BMD	bone mineral density
OVX	ovariectomy
CTX	C-terminal telopeptide of type I collagen
PINP	procollagen type I N-propeptide

1. Introduction

The utilization of medical cannabis has a rich history dating back thousands of years. Over 5000 years ago in China, cannabis was employed as a medicinal remedy for various ailments such as pain, fatigue, inflammatory diseases, and malaria [1–4]. During the nineteenth century, cannabis extracts were utilized to alleviate pain and nausea. However, due to the psychoactive side effects, the use of medical cannabis waned, and it became illegal [2,4,5]. This situation underwent a transformation in the 1990s with the burgeoning interest in cannabinoids, driven by the discovery of the endocannabinoid system in the brain. This discovery suggested that cannabinoids could be effective in treating conditions like chronic pain [1]. By 2021, medical cannabis had been legalized in 28 European countries, even in places where it was initially permitted only on a trial basis. Additionally, as of 2023, it had gained legal status in 41 states and territories in the United States [4,6–8]. Today, cannabinoids are employed for medical purposes to address various conditions, including the management of chronic pain associated with cancer and cancer-induced bone disease, as well as the alleviation of chemotherapy-induced nausea and vomiting [6,9,10]. However, its clinical use is limited due to uncertainties about potential short- and long-term effects [7,10–13].

Human bone health depends on a coordinated action of primarily two cell types, the osteoclasts (OCs) and the osteoblasts (OBs) [14–16]. The OC is a multinucleated cell formed through fusion of mononucleated precursors of the myeloid-lineage. Once multinucleated, the OC is able to resorb the bone matrix. The OBs are the bone forming cells and originate from the mesenchymal-lineage. OCs and OBs have been shown to express two endocannabinoid receptors, cannabinoid receptor 1 (CB1 encoded by the gene *CNR1*) and -2 (CB2 encoded by the gene *CNR2*), involved in the pathways of the endocannabinoid system. Bone cells and their precursors have been reported to express higher level of CB2 than CB1 [17]. Furthermore, over several decades a regulatory endocannabinoid system has been discovered in bone tissue [18], which highlight that CB1 and CB2 show different activities in OCs and OBs [19,20].

Two known endocannabinoid agonists for the receptors CB1 and CB2 are anandamide (AEA) and 2-arachidonyl glycerol (2-AG) [21,22], discovered in 1992 [23] and 1995 [24], respectively. In bone tissue, both 2-AG and AEA are present at concentrations of nmol/g and pmol/g, respectively. Interestingly, these concentrations are significantly lower than the concentrations of the two endocannabinoids found in blood, which may suggest that 2-AG and AEA are synthesized locally in the bone [18,25]. Both endocannabinoids are derivatives of arachidonic acid. Due to the content of N-acyl phosphatidyl ethanolamine-phospholipase D (NAPE-PLD) and arachidonic acid-enriched membrane phospholipids in OBs and OCs, these cells have the ability to produce AEA and 2-AG, respectively [26]. Inconsistency of reported effects of endocannabinoids on OC formation have been reported. Using cells from mice *in vitro*, Ofek et al. [20] reported inhibition of RANKL-induced OC formation by the CB2 selective endocannabinoid agonist HU308, while Idris et al. [19] revealed a stimulation of RANKL-induced OC formation due to HU308, 2-AG or AEA. It has been shown by Whyte et al. [27] that AEA both stimulates the ability of human OCs to form actin rings as well as their ability to resorb bone *in vitro* at concentrations of 100 nM to 1 μM. The effect of AEA on OCs is furthermore shown to be mediated through CB2. In addition, an enhanced bone resorption by human OCs *in vitro* as a response to both 2-AG as well as AEA has been shown by Idris et al. [28].

Medical cannabis contains cannabinoids derived from the cannabis plant, namely Δ9-tetrahydrocannabinol (THC) and cannabidiol (CBD), which act as exogenous analogues to AEA and 2-AG. The most commonly used cannabis plant in the western hemisphere is the *Cannabis sativa* plant, which contains the phytocannabinoids, THC and CBD [29–31]. THC is the predominant psychotropic cannabinoid, while CBD is the primary non-psychoactive cannabinoid in the *Cannabis sativa* plant [31,32]. THC is known to function as an agonist for both CB1 and CB2, while the pharmacodynamics of CBD are a subject of debate. CBD has been demonstrated to function as a non-competitive negative allosteric modulator for CB1 and CB2, but primarily for CB1 [3,33] and also a low affinity antagonist towards CB1 and CB2 in different studies, respectively [34,35].

Kogan et al. [36] have shown that CBD enhances the mechanical properties of a callus formed following a mid-femoral bone fracture in rats. They hypothesized that the effects of THC and CBD on bone tissue involves modifications to the organic part of the bone matrix, specifically the ability of collagen to cross-link. This hypothesis is based on a CBD-dependent increase in mRNA levels of lysyl hydroxylase, PLOD1, which plays a role in cross-link formation within the collagen structure. CBD was shown to stimulate PLOD1 mRNA levels at concentrations of 1 pM and 100 pM. Interestingly, higher concentrations of CBD reversed the observed stimulatory effect, indicating a dose-dependent effect on bone tissue. An increase in mRNA levels of PLOD2 was observed by THC at a concentration of 10 nM.

To our knowledge, a limited number of clinical studies have been conducted to investigate potential side effects of cannabis/cannabinoids on bone health. Sophocleous and coworkers [13] tested differences between long-term intermediate/heavy cannabis smokers and smokers of ordinary cigarettes. They found that heavy cannabis users had lower bone mineral density (BMD) and elevated biomarkers of bone turnover compared to cigarette smokers. Thus, their data suggest that cannabis use shifts the balance of bone turnover in favor of bone loss. A study by Bourne and co-workers found in a cross-sectional survey based study that there was no significant association between cannabis use and BMD when adjusting for potential confounders [37]. In contrast, Kulpa et al. [12] conducted a double-blind placebo-controlled clinical trial using controlled medical cannabis products of both CBD and THC on healthy subjects, and found that bone turnover was suppressed by both CBD and THC treatment. So these clinical studies give conflicting results.

Building upon a series of contradictory findings concerning the role of the endocannabinoid system in bone, our study seeks to give further insights into the impact of THC and CBD on bone remodeling. To achieve this, we investigate how human OCs respond in both mono- and co-cultures with reversal cell-like OBs when exposed to increasing concentrations of THC and CBD.

2. Materials and methods

2.1. Extraction of cannabinoids THC and CBD

Cannabis extracts were obtained from two distinct strains: "Blueberry Bliss," characterized by high THC and low CBD content, and "Finola," known for its low THC and high CBD levels (Finola®, Kuopio, Finland). The process involved drying trimmed and mature buds in darkness at room temperature. We utilized 20 g of "Blueberry Bliss" and 3.71 g of "Finola" for extraction. The plant material was flash-frozen using liquid nitrogen, pulverized, and then suspended in 500 mL and 100 mL of cooled 96 % ethanol, respectively. After 20 min of sonication, the samples underwent filtration through a 0.22 μm filter, and the solvent was subsequently evaporated. The resulting material was heated to 100 °C for 45 min and resuspended in 100 mL and 25 mL, respectively. To isolate the two cannabinoids, THC and CBD, from the extracts, we employed high-performance liquid chromatography (HPLC) using an Agilent Technologies 1260 Infinity system with a Luna® 5 μm C18(2) 100 Å column (250 × 10.0 mm) from Phenomenex® (Torrance, California, USA). For the purification of THC, the gradient began at 81 % methanol (0.005 % TFA) and 19 % water (0.005 % TFA), which was maintained for 20 min, followed by an increase to 100 % methanol (0.005 % TFA) over a 2-min interval. The flow rate was set at 4 mL/min, and the injection volume was 100 μL. In the case of CBD purification, the gradient started at 83 % methanol (0.005 % TFA) and 17 % water (0.005 % TFA) for 20 min, followed by a 2-min increase to 100 % methanol (0.005 % TFA), with the same flow rate and injection volume as THC. Fractions were collected between 12.4 min and 13.3 min. Subsequently, the samples were subjected to analysis by liquid chromatography-mass spectrometry (LC-MS) using an amide column (Ascentis® Express RP-amide (Sigma-Aldrich, St. Louis, Missouri, USA); 15 cm, 4.6 mm, 2.7 μm) with a gradient that began at 70 % methanol (0.1 % formic acid) and 30 % water (0.1 % formic acid), reaching 100 % methanol (0.1 % formic acid) after 15 min. Additionally, we performed analysis by nuclear magnetic resonance (NMR) using a Bruker AVIII-600 MHz instrument. The quantification of cannabinoids was carried out using a Pulse Length–based Concentration determination (PULCON) spectrum (refer to Fig. S1 for CBD and Fig. S2 for THC).

When testing the effect of pharmacologic compounds *in vitro*, it is of interest to test dose ranges that are within a pharmacologic range. In two parallel phase I studies, Peters and colleagues found that intermediate dosing of CBD resulted in C_{max} of 1.1 μM, while the highest dose resulted in 3.2 μM CBD and metabolites [38,39]. Another phase I study [40], also tested treatment with CBD and found C_{max} concentrations of CBD and metabolites ranging from 10.7 to 17.1 μM in blood. In all three studies, it was not possible to detect THC or most of its metabolites. Only the carboxylated form of THC could be detected, which reached C_{max} levels in the lower μM range. Based on these pharmacokinetic data, the dose range of 0.3 to 30 μM CBD or THC, used in our present study, are within or close to the reported pharmacological range.

2.2. Generation of human OCs in vitro

Human $CD14^+$ monocytes were purified from anonymous male blood donors above the age of 50. Anonymized buffy coats were used in accordance with Danish legislation and all donors provided written informed consent for the use of surplus material from the donation. $CD14^+$ monocytes were differentiated into OCs using published procedures [41]. In brief, blood was centrifuged using Ficoll-Paque (GE Healthcare, Chicago, Illinois, USA), and monocytes were isolated through the use of BD IMag™ Anti-Human CD14 Magnetic Particles – DM (BD Biosciences, Franklin Lakes, New Jersey, USA) according to the supplier's instructions. Isolated $CD14^+$ monocytes were seeded at a density of 5×10^6 cells per T75 culture flask (Greiner Bio-One GmbH, Frickenhausen, Germany) in α minimum essential medium (α-MEM; Gibco, Gaithersburg, Maryland, USA) containing 10 % fetal bovine serum (FBS; Sigma-Aldrich), 1 % Pen-Strep (Sigma, St. Louis, Missouri, USA) and 25 ng/mL human macrophage colony-stimulating factor (MCSF; R&D Systems, Minneapolis, Minnesota, USA). The cells were cultured in a humidified atmosphere at 37 °C and 5 % CO_2 for two days, followed by seven additional days of incubation exposed to both 25 ng/mL MCSF and 25 ng/mL receptor activator of nuclear factor-kappaB ligand (RANKL; R&D Systems) with medium change twice. After these seven days cells were considered to have matured into OCs.

2.3. Osteoclastic fusion assay

After $CD14^+$-purification and incubation for two days with 25 ng/mL MCSF, cells were harvested by accutase (Biowest, Nuaillé, France) treatment and reseeded on plastic in 96-well plates in cell culture medium and 25 ng/mL MCSF and 25 ng/mL RANKL at a density of 2.5×10^4 OCs per well. Cells were allowed to settle for 1 h in the incubator, whereafter different concentrations of CBD or THC were added and cells were cultured in the incubator for seven days. Media was refreshed twice containing the same concentrations of CBD and THC. Conditioned media was collected and stored at −20 °C for later Tartrate-Resistant acid Phosphatase (TRAcP)-activity analyses. Final DMSO concentration did not exceed 1.0 %. Following seven days of differentiation, cells were fixed and stained using Giemsa staining as previously described [42,43]. The extent of OC fusion was quantified using light microscopy (model: IX71, Olympus, Shinjuku, Tokyo, Japan) with a 20× objective. Five randomized wells (from a total of 8 wells) were counted per condition. The surface area of each well was divided into 14 fields of which seven were counted per well. A random number generator was used for the randomization of the fields to count per well. The number of nuclei per OC and the number of multinucleated (≥2 nuclei) OCs were counted. TRAcP-activity measurements were performed on conditioned media as previously described [44,45].

2.4. Osteoclastic bone resorption assay

Matured OCs were detached and harvested using accutase (Biowest). Cells were counted using trypan blue staining and a Countess™ automated cell counter (Invitrogen, Waltham, Massachusetts, USA). Subsequently, 0.5×10^5 viable cells were reseeded on 0.4 mm thick bovine cortical bone slices (Boneslices.com, Jelling, Denmark) in 96-well plates using culture medium containing 25 ng/mL MCSF and 25 ng/mL RANKL. Cells were allowed to settle for 1 h in a humidified incubator at 37 °C and 5 % CO_2, followed by addition of CBD or THC to reach final concentrations indicated in the relevant figure legends. Subsequently, plates were incubated for three days in an incubator with a humidified atmosphere at 37 °C and 5 % CO_2. CBD and THC were diluted in DMSO. All wells contained the same concentration of DMSO and never exceeded 0.5 %. After the three days of incubation, conditioned media was collected and stored at −20 °C for later TRAcP-activity analyses. Cell viability was measured using CellTiter-Blue Cell Viability Assay (Promega, Madison, WI, USA) according to the instructions by the supplier. Subsequently, OCs were lysed in ddH_2O, bone slices were polished with a cotton swap, washed in ddH_2O, dried, and stained with toluidine blue staining for visualization of resorption events. The extent of eroded bone surface was determined by light microscopy (BX53, Olympus) in a blinded random systematic count. The percentage eroded surface per bone surface was determined with a 10× objective and a 100-point grid (Pyser-SGI, Edenbridge, UK) and was sub-divided into cavities appearing as pits or trenches according to previously published definitions [44,46]. TRAcP activity measurements were performed on conditioned media as previously described [44,45].

2.5. Culturing of osteoblast-lineage cells

Human OB-lineage cells were generated from bone fragments collected from osteoarthritis patients undergoing hip replacement

surgery (approved by the local ethics committee, S-2011-0114. Informed written consent was obtained from all patients) as previously described [47,48]. After 35 days of culture, human OB-lineage cells were ready to use. Cells were cultured for a maximum of seven passages.

2.6. Bone resorption assay of OCs and osteoblast-lineage cells in co-culture

Mature OCs were harvested through accutase treatment, resuspended in co-culture media (α-MEM with 1 % Pen-Strep and a final concentration of 25 ng/mL MCSF). Cell viability was measured using trypan blue staining and Countess™ automated cell counter. Harvested OCs were reseeded on 0.4 mm bovine cortical bone slices at a density of 5×10^4 cells in a 96-well in co-culture media for 45 min in a humidified incubator at 37 °C and 5 % CO_2. Subsequently, CBD or THC were added to reach the final concentrations indicated in the figure legends. The 96-well plates were placed in the incubator for 4 h before addition of OB-lineage cells. OB-lineage cells were harvested through accutase treatment, resuspended in co-culture medium, and the cell viability was determined using trypan blue staining and Countess™ automated cell counter. OB-lineage cells were added to each bone slice at a density of 1.25×10^4 along with CBD or THC reaching the final concentrations indicated in the figure legends. Final concentration of DMSO was 0.34 %. The 96-well plates were incubated in a humidified incubator at 37 °C and 5 % CO_2 for three days. Conditioned media was collected and stored at −20 °C for later TRAcP-activity test. Subsequently, ALP-activity was measured in the cell layer using 200 μL reaction buffer (0.06 M Na_2CO_3, 0.04 M $NaHCO_3$, 0.1 % TritonX-100, 2 mM $MgSO_4$, 6 mM 4-NNP). The 96-well plates were kept in the dark and incubated for 30–40 min at 37 °C. The reaction was stopped by addition of 1 M NaOH to the reaction buffer in a 1:1 relation. Absorbance was determined at 405 nm using plate reader (BioTek, Winooski, Vermont, USA. Model: Synergy HT). Bone slices were stained using toluidine blue (as described in paragraph 2.4). TRAcP-activity measurements were performed on conditioned media as previously described [44,45].

2.7. Bulk RNA-sequencing

Cells were differentiated, lysed and analyzed as previously described [49]. In brief, human OC precursors and OCs, differentiated as described above, were lysed using 1200 μL TRIzol/mercaptoethanol (100:1) at the following differentiation stages (dependent on days of RANKL stimulation): 1) −2 days (CD14+ monocytes), 2) 0 days, 3) 3 days and 4) 7 days (mature OCs). Cells were differentiated and seeded as described above with the following exemptions on days −2 and 0: day 0 cells were stimulated with MCSF (25 ng/mL) for 4 h before cell media was removed from one T25 flask. Cells were then washed in 5 mL phosphate buffer solution (PBS) before cells were lysed. For cells lysed on day 0, MCSF and RANKL (25 ng/mL each) were added to the T25 flasks and incubated for 4 h. Subsequently, media was removed, and cells were washed in 5 mL PBS before cells were lysed. On days 3 and 7, αMEM was removed from the T25 flask and cells were washed in 5 mL PBS before being lysed as described above. Before cells were lysed at day 7, they were microscopically assessed for being multinucleated (≥2 nuclei per cell). A corresponded batch of cells simultaneously differentiated were loosened with accutase and seeded on bovine bone slices as described above to determine resorptive activity of the mature OCs. Following lysis, cells were stored at −80 °C and thawed once for RNA purification. RNA was purified using Econo Spin columns (Epoch Life Sciences, Texas, USA) and cells were stored at −80 °C before final analyses. RNA was purified using Econo Spin columns (Epoch Life Sciences). RNA-sequencing was performed according to manufacturer's instructions (TruSeq 2, Illumina) using 2 μg RNA for preparation of cDNA libraries. Sequencing reads were mapped to the human genome (hg19) using STAR [50], and tag counts were summarized at the gene level using HOMER [51]. Differential gene expression was analyzed using DESeq2 [52]. Normalized expression counts and statistics for differential expression analysis were extracted from supplementary files [49] of gene expression omnibus (GEO) data set GSE246769.

2.8. Single nuclei RNA-sequencing on co-culture

Mature OCs were co-cultured on bone slices together with OB-lineage cells as described above. A 96-well plate with mature OCs and OB-lineage cells was incubated for 72 h in a humidified incubator at 37 °C and 5 % CO_2. After incubation, bone slices were washed in PBS and lysed in Nuclei Preparation Buffer containing 10 mM HEPES, 1.5 mM MgCl2, 10 mM KCl, 250 mM Sucrose, 0.1 % IGEPAL CA-630 and 0.2 mM Dithiothreitol in sterile DEPC-treated water. Nuclei Preparation Buffer containing lysed cells was collected and nuclei were isolated through centrifugation. The nuclei-containing pellet was resuspended in Nuclei Resuspension Buffer containing 20 mM Tris, 40 mM NaCl, 90 mM KCl, 2 mM EDTA, 0.5 mM EGTA, 0.2 mM Dithiothreitol and 1 U/μL broad spectrum RNase inhibitor (RNasin N261B, Promega) in sterile DEPC-treated water. Isolated nuclei were frozen in Nuclei Resuspension Buffer containing 10 % DMSO and stored at −80 °C.

Isolated nuclei were prepared to single nuclei RNA-sequencing using the 10× Genomics platform and sequenced on Illumina NovaSeq 6000 (covering approximately 50,000 reads per nucleus). Sequencing reads were prepared and mapped to the human genome (GRCh38.p13) using zUMI [53]. Quality-control was performed in R following a process described by Sárvari and colleagues [54]. The quality control included removal of nuclei with a proportion of mitochondrial reads above 10 %, unique molecular identifiers (UMIs) below 500, number of detected genes below 200 and UMI/detected gene ratio above 2.5. Outliers were filtered out by principal component analysis using the Scater-package in Bioconductor [55]. Remaining nuclei were clustered using the Seurat package in R.

2.9. Statistics

Statistical analyses and graphs were generated using GraphPad Prism software (GraphPad Prism version 8, San Diego, CA, USA). Obtained data was tested for normal distribution with a Shapiro-Wilk normality test. If normally distributed a parametric one-way ANOVA test was performed, whereas the non-parametric Kruskal-Wallis significance test was applied if the data was not normally distributed. For comparison graphs, a paired *t*-test was performed for normally distributed data and a Wilcoxon test for data that did not show normal distribution. Furthermore, multiple comparison tests were used as post-tests. In the figure legends the specific statistical tests used along with the exact *p*-values are indicated. Statistical significance was defined as $p < 0.05$. For correlation graphs, a Spearman non-parametric correlation test was performed. Specific details regarding statistics are listed in all figure legends.

3. Results

3.1. Osteoclast fusion is inhibited by both THC and CBD

A representative experiment showing the effect of both THC and CBD on osteoclastic differentiation and fusion is shown in Fig. 1. The number of nuclei per OC is gradually decreased with increasing concentrations of THC (Fig. 1a and c), but only reaches significance at 10 μM or more (Fig. 1c). With respect to the number of OCs a decrease can also be observed at 10 μM, but only reaches significance at 30 μM (Fig. 1e). In order to get an impression of the overall effect of THC on osteoclastic fusion, we calculated the total number of nuclei in OCs minus 1 in all fields analyzed (7 fields) per well (5 wells per condition) (Fig. 1g). This analysis gives a measure of the total number of fusion events that have occurred as published in [56]. Using a curve fit we could estimate an IC50 of 5.4 μM THC (Fig. 1g). The accumulated TRAcP-activity

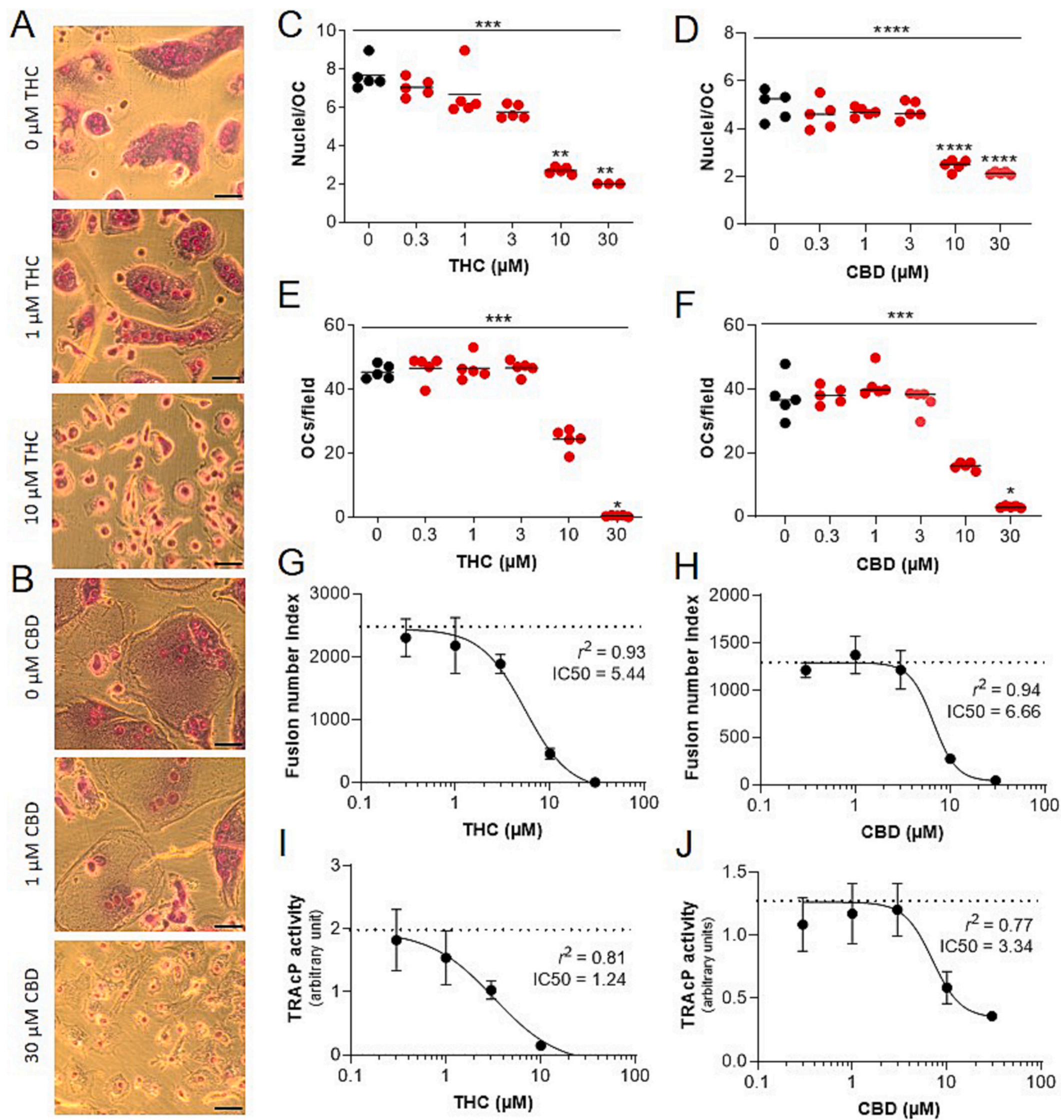

Fig. 1. Increasing doses of THC and CBD inhibit OC fusion – as exemplified from a full experiment using cells from one donor. (a) Examples of OCs treated with THC or (b) CBD. OCs were seeded on plastic and stained with Giemsa May-Grünwald. Scale bar = 50 μm. (c) Effect of increasing doses of THC and (d) CBD on the number of nuclei per OC. Statistics ($n = 5$): (c) Kruskal-Wallis test, ***$p = 0.0003$; Dunn's multiple comparisons test, **$p = 0.0025$ for 10 μM THC compared to 0 μM THC, **$p = 0.0012$ for 30 μM THC compared to 0 μM THC; (d) One-way ANOVA, ****$p < 0.0001$; Dunnett's multiple comparisons test, ****$p < 0.0001$ for 10 μM CBD compared to 0 μM CBD, ****$p < 0.0001$ for 30 μM CBD compared to 0 μM CBD; horizontal line indicates the mean. (e) Effect of increasing doses of THC and (f) CBD on the number of OCs per field. Statistics ($n = 5$): (e) Kruskal-Wallis test, ***$p = 0.0008$; Dunn's multiple comparisons test, *$p = 0.0253$ for 30 μM THC compared to 0 μM THC; (f) Kruskal-Wallis test, ***$p = 0.0003$; Dunn's multiple comparisons test, *$p = 0.0393$ for 30 μM CBD compared to 0 μM CBD; horizontal line indicates the mean. (g) Effect of increasing doses of THC and (h) CBD on the overall fusion potential of OCs as determined by total number of nuclei found in OCs (minus one) per condition of THC or CBD. Model: non-linear fit; baseline level represented by the dotted line, data points reflect the mean ($n = 5$) and error bars reflect SD. (i) Effect of increasing doses of THC and (j) CBD on the accumulated activity of TRAcP released into the media during OC differentiation. Model: non-linear fit; baseline level indicated by the dotted line, data points reflect the mean ($n = 8$) and error bars reflect SD.

measured in the conditioned media over the 10 days of differentiation at different concentrations of THC is shown in Fig. 1i. Using a curve fit, we could estimate an IC50 of 1.2 μM THC for TRAcP.

In the presence of CBD, the number of nuclei per OC was also gradually decreased with increasing concentrations (Fig. 1b and d), and just as for THC it reached significance at 10 μM or more (Fig. 1d). With respect to the number of OCs, a reduction can also be observed at 10 μM, but only reaches significance at 30 μM (Fig. 1f). With respect to the total number of fusion events, we could estimate an IC50 of 6.7 μM CBD (Fig. 1h) while it was 3.3 μM CBD for the TRAcP-activity (Fig. 1j).

Fig. 2 shows that the results shown in Fig. 1 are reproduced when repeating the analyses with six different donors for both THC (Fig. 2a and c) and CBD (Fig. 2b and d) and that the potency of THC and CBD is similar (Fig. 2e).

3.2. THC and CBD dose-dependently stimulate and inhibit osteoclastic bone resorption

In order to assess potential direct effects on osteoclastic bone resorption, we tested the sensitivity of matured OCs seeded on bovine cortical bone slices. Representative results from the OCs of a single human donor are shown for THC and CBD in Fig. 3. In contrast to the effects on differentiation and fusion, THC and CBD both stimulated bone resorption (Fig. 3a and b, respectively). For the total eroded surface a peak stimulation was obtained at 3 μM THC (Fig. 3a) and 10 μM CBD (Fig. 3b), for pit surface it peaked at 1 μM THC (Fig. 3c) and 10 μM CBD

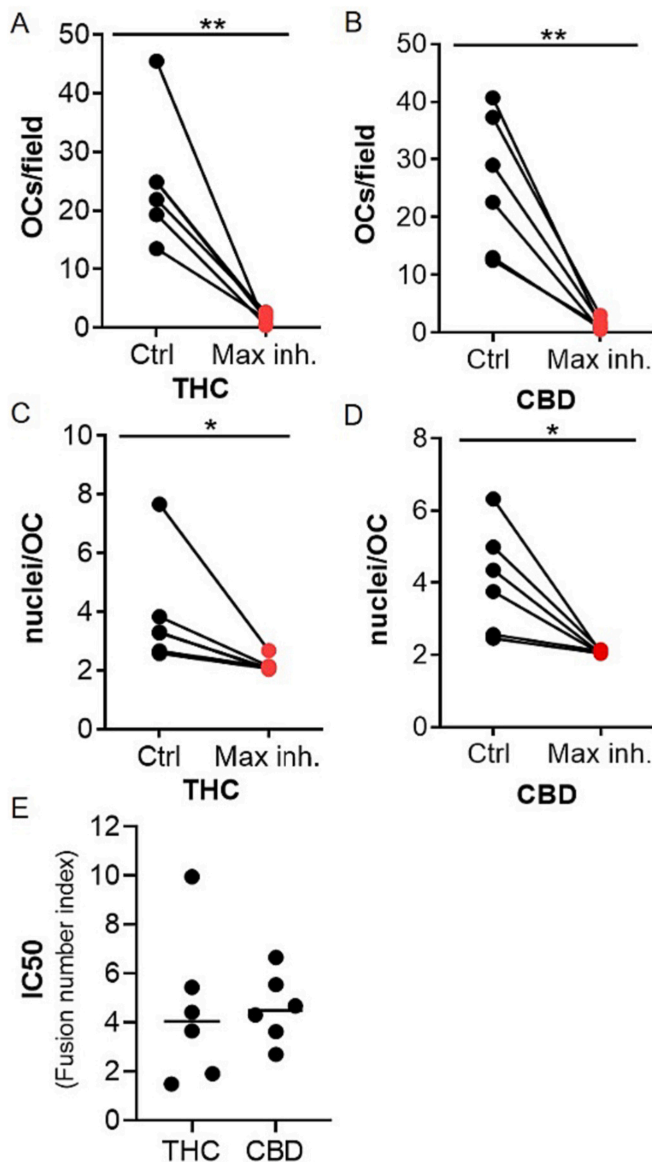

Fig. 2. THC and CBD inhibit OC fusion – summarized through comparison of results using cells from six different donors. (a) Maximum inhibition of OCs per field through THC and (b) CDB. Statistics: Paired *t*-test, two-tailed (a), $*p = 0.0038$; (b) $**p = 0.0035$. (c) Maximum inhibition of number of nuclei per OC by THC and (d) CBD. Statistics: (c) Wilcoxon test, two-tailed, $*p = 0.0313$; (d) $*p = 0.0313$. (e) Variation in IC50 for THC & CBD (μM) of the six different donors as determined through the fusion number index.

(Fig. 3d), and for trench surface at 3 μM THC (Fig. 3e) and 10 μM CBD (Fig. 3f). The stimulation of OCs making pits or trenches is equal since the percent trench surface per eroded surface is unaltered at the lower dose range for both THC and CBD (Fig. 3g and h, respectively).

However, while lower doses of THC and CBD stimulates bone resorption, this stimulation is lost at higher doses (Fig. 3a–f). This loss of stimulation or inhibition does not substantially alter the balance between pits and trenches (THC, Fig. 3g) (CBD, Fig. 3h).

Fig. 4 shows a combined analyses of the results of OCs obtained from six different donors. In the case of THC, a significant stimulation of bone resorption at lower doses is seen (Fig. 4a), while there is a significant inhibition at higher doses (Fig. 4c). For three donors, the maximum stimulation was observed at 1 μM, two donors at 3 μM and one donor at 10 μM THC, while the lowest level of bone resorption was obtained at 30 μM THC for all donors (data not shown). Similarly, for CBD a significant stimulation of bone resorption was observed at lower doses for OCs from all six donors (Fig. 4b), but higher concentrations did not inhibit bone resorption compared to control level (Fig. 4d). For two donors maximum stimulation was observed at 1 μM, one donor at 3 μM, and three donors at 10 μM CBD, while the lowest level of bone resorption was obtained at 30 μM CBD for OCs from all donors (data not shown). Similar and reproducible results were obtained for both pit and trench surface per bone surface (data not shown).

3.3. Osteoclastic bone resorption is inhibited by both THC and CBD in co-cultures with OBs

Four biological replicates of co-cultures with OCs and reversal cell-like OBs were performed to observe the impact of THC and CBD under these conditions on OCs. Results from a single representative experiment are shown in Fig. 5. We found no stimulation of bone resorption with THC (Fig. 5a, c, e) or CBD (Fig. 5b, d, f). In contrast, bone resorption was inhibited with 10 μM THC for both total bone resorption (Fig. 5a) as well as pit surface per bone surface (Fig. 5c), while it for trench surface per bone surface only reached significance at 30 μM THC (Fig. 5e). In the case of CBD, inhibition set in already at 1 μM CBD for both pit and trench surface per bone surface (Fig. 5d and f, respectively). Although a reduction of eroded surface was also observed at 1 μM CBD it only reached significance at 10 μM CBD (Fig. 5b). Regarding the reversal cell-like OBs in the co-culture, significant reduction of ALP-activity was observed for both THC (Fig. 5g) and CBD (Fig. 5h). However, at 30 μM THC there was 50 % residual ALP-activity (Fig. 5g), while it at 30 μM CBD was 10 % (Fig. 5h), suggesting that the impact of CBD on the reversal cell-like OBs is more pronounced than for THC.

Fig. 6 shows a positive correlation between the extent of total eroded surface per bone surface and the corresponding ALP-activity of the reversal cell-like OBs in all four co-cultures conducted for both THC (Fig. 6a) and CBD (Fig. 6b).

3.4. CNR1 and CNR2, are both expressed in OCs during differentiation

From bulk RNAseq analyses of OCs during differentiation, obtained from 8 different healthy donors, we can confirm mRNA expression of both *CNR1* and *CNR2* (Fig. 7a). For both receptors, the maximum expression level is seen in the $CD14^+$ monocytes (day −2) directly after their purification from blood. Incubation with MCSF for two days (day 0) does not seem to alter the expression of *CNR2*, while the expression of *CNR1* is slightly reduced. However, addition of RANKL to the culture medium clearly reduces the expression level of both receptors, whereafter the expression is stabilized (days 2 and 7), and with a significantly higher expression of *CNR2* compared to *CNR1* on day 7 of RANKL exposure. It is also evident that the expression levels of both *CNR1* and *CNR2* are several orders of magnitude below the expression levels of typical OC-related genes such as ATPase H+ transporting V1 subunit D (ATP6V1D) and carbonic anhydrase 2 (CA2) (Fig. 7a).

3.5. During co-culture conditions, only expression of CNR2 can be detected for both OCs and reversal cell-like OBs

For the co-cultures, matured OCs were re-seeded on cortical bovine bone slices together with reversal cell-like OBs in the absence of serum and exogenous RANKL. The culture conditions were therefore different from the OC mono-culture conditions. We used single nuclei RNAseq to check for the expression of *CNR1* and *CNR2* in both OCs and reversal cell-like OBs under these culture conditions. We could confirm the expression of *CNR2* in both OC nuclei (positive for *ACP5*, *TNFRSF11A*, and *CTSK*) (Fig. 7b) and reversal cell-like OB nuclei (positive for *POSTN*, *GREM1*, and *RUNX2*) (Fig. 7c). *CNR2* was detected in 3.6 % of the OC nuclei and in 1.2 % of the OB nuclei. An expression of *CNR1* could not be detected.

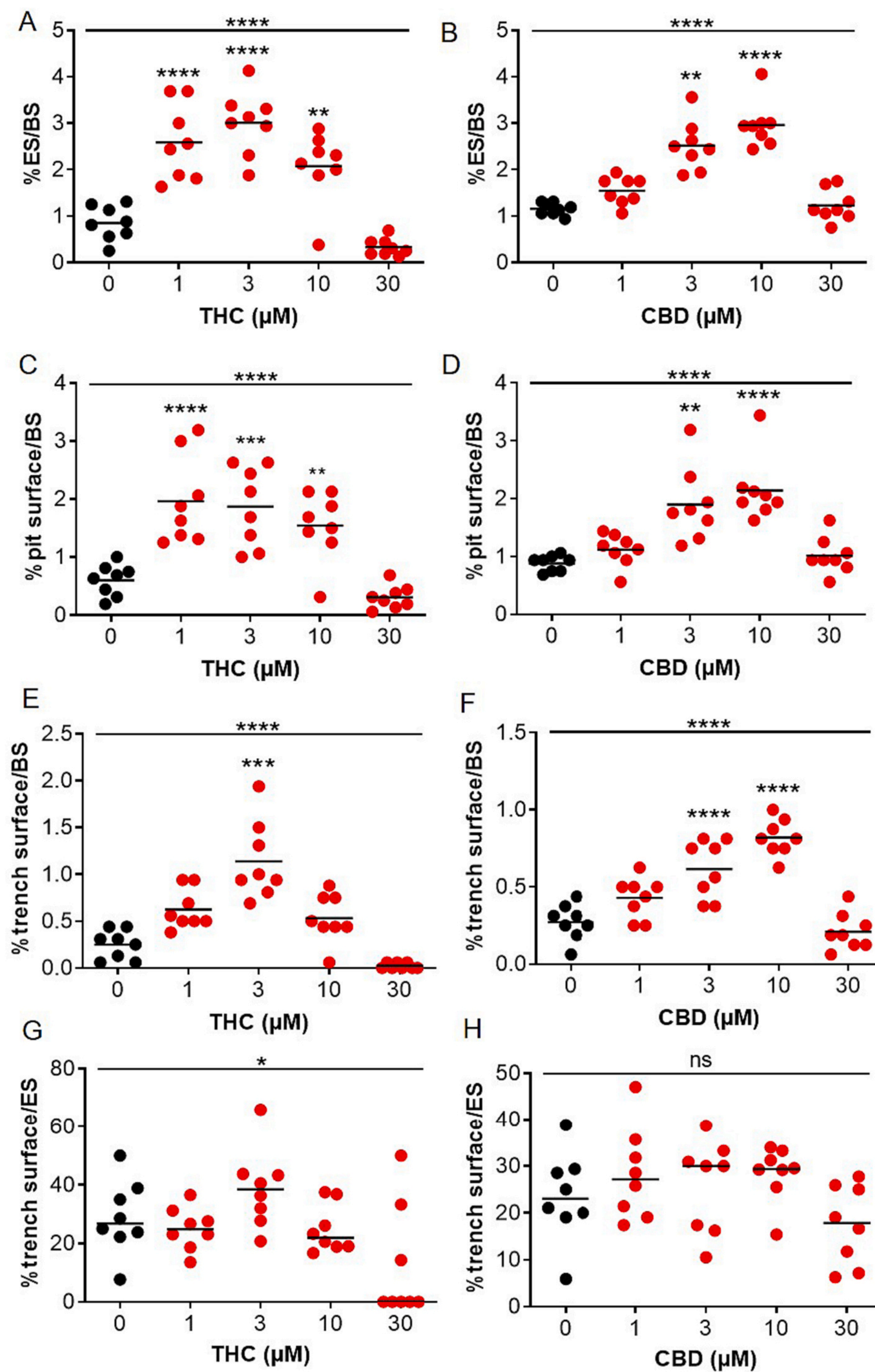

Fig. 3. THC and CBD dose dependently stimulate and inhibit OC bone resorption – as exemplified from a full experiment using cells from one donor. (a) Percentage of total eroded surface (ES) per bone surface (BS) in the presence of increasing concentrations of THC and (b) and CBD. Statistics: (a) Ordinary one-way ANOVA test, ****$p < 0.0001$; Dunnett's multiple comparisons test comparing to 0 µM THC, 1 and 3 µM THC ****p < 0.0001, 10 µM THC **$p = 0.0012$; (b) Kruskal-Wallis test, ****p < 0.0001; Dunn's multiple comparisons test comparing to 0 µM CBD, 3 µM CBD **$p = 0.0015$, 10 µM CBD ****$p < 0.0001$. (c) Percentage of pit surface per BS in the presence of increasing concentrations of THC and (d) CBD. Statistics: (c) Ordinary one-way ANOVA test, ****p < 0.0001; Dunnett's multiple comparisons test comparing to 0 µM THC, 1 µM THC ****p < 0.0001, 3 µM THC ***$p = 0.0002$, 10 µM THC **$p = 0.0056$; (d) Kruskal-Wallis test, ****p < 0.0001; Dunn's multiple comparisons test comparing to 0 µM CBD, 3 µM CBD **$p = 0.0014$, 10 µM ****p < 0.0001. (e) Percentage of trench surface per BS in the presence of increasing concentrations of THC and (f) and CBD. Statistics: (e) Kruskal-Wallis test, ****$p < 0.0001$; Dunn's multiple comparisons test comparing to 0 µM THC, 3 µM THC ***$p = 0.0008$; (f) Ordinary one-way ANOVA test, ****p < 0.0001; Dunnett's multiple comparisons test comparing to 0 µM CBD, 3 and 10 µM CBD ****$p = 0.0001$. (g) Percentage trench surface per ES in the presence of increasing concentrations of THC and (h) CBD. Statistics: (g) Kruskal-Wallis test, *$p = 0.0205$; (h) Kruskal-Wallis test, $p = 0.0991$, not significant (ns). Horizontal lines indicate the mean, $n = 8$.

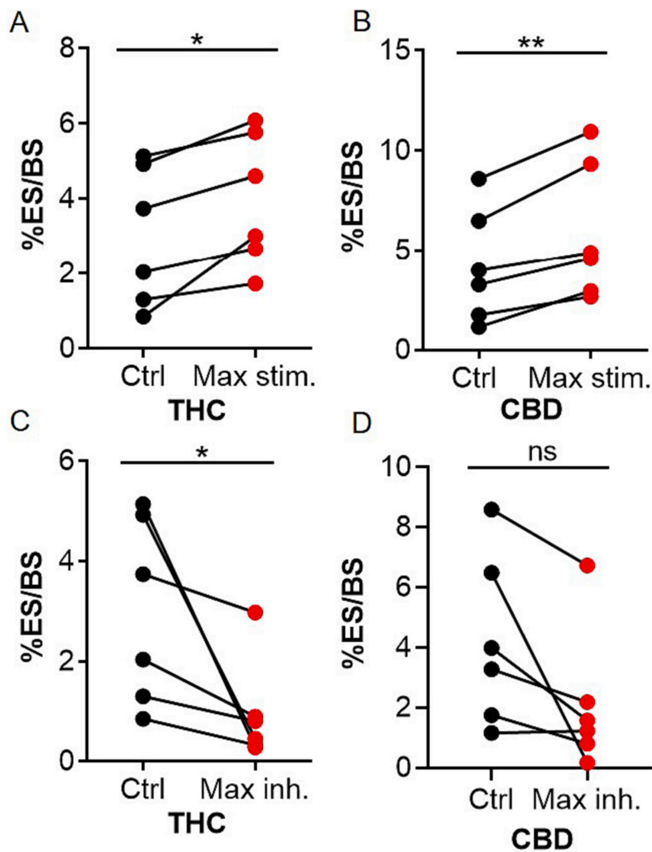

Fig. 4. THC and CBD inhibit OC bone resorption – summarized through comparison of results using cells from six different donors. (a) Maximum stimulation of THC and (b) CBD on percentage of total eroded surface (ES) per BS. (c) Maximum inhibition of THC or (d) CBD on percentage ES per BS. Statistics: (a) Paired *t*-test, two-tailed, $*p = 0.0123$; (b) Paired t-test, $**p = 0.0034$; (c) Wilcoxon, two-tailed, $*p = 0.0313$; (d) Paired t-test, two-tailed, $p = 0.0704$, not significant (ns).

4. Discussion

To our knowledge, this is the first time that a systematic analysis has been conducted to analyze the effects of both CBD and THC on human OCs alone and in co-culture conditions with human reversal cell-like OBs. We hope that the results of our study can improve our knowledge regarding potential effects of medical or recreational use of cannabis and cannabinoids. The current knowledge is rather limited and partly contradictory [8]. In our discussion, we will attempt to contextualize our findings within the context of the limited knowledge on the effect of exogenous cannabinoids on bone cells and the more elaborated (but still scarce) knowledge on the endocannabinoid system with respect to bone.

We show that both THC and CBD have dose-dependent differential effects on human OC differentiation/fusion and bone resorption. In the lower dose ranges of THC and CBD, OC fusion was unaffected while these doses stimulated bone resorption activity. At higher doses, an inhibition of both fusion and bone resorption was observed. In the co-culture setup with OCs and reversal cell-like OBs (absence of serum and exogenous RANKL), OC bone resorption was not stimulated but only inhibited, and an inhibitory effect on the reversal cell-like OBs was observed through a reduced ALP-activity. Interestingly, the level of inhibition of both OCs and reversal cell-like OBs seemed to be similar since there was a correlation between the level of bone resorption and the ALP-activity of the same culture wells across four independent experiments. Finally, we observed that *CNR2* is higher expressed in pre-OCs compared to *CNR1*, but also that differentiation to OCs was coupled to a reduced expression of *CNR2*, in particular. The expression of *CNR2* was more strongly affected than *CNR1* when RANKL was included in the media, but *CNR2* was still expressed at higher levels than *CNR1* during the differentiation to matured OCs. Interestingly, under co-culture conditions, we could only detect the expression of *CNR2* in OC as well as reversal cell-like OB nuclei.

Regarding the effects of THC and CBD on human OC differentiation and fusion, our findings seem to be comparable to the findings of Whyte et al. [27] and Smoum et al. [57]. They found that two synthetic agonists of cannabinoid receptors, CP55940 and HU433, and the endocannabinoid AEA all inhibit OC differentiation [20,27,57]. However, this is in contradiction to the findings of Idris et al. [19], who found that the endocannabinoid agonists, 2-AG and AEA, had a stimulatory effect on OC differentiation *in vitro*. In contrast, they found that three antagonists of CB1 (AM251) and CB2 (SR144528, AM630) inhibited differentiation. While Idris et al. [19] suggest that the stimulatory effects on OC differentiation by AEA is mediated through CB1, Whyte et al. [27] suggest that the inhibitory effects are mediated primarily through CB2. However, the data of Whyte et al. [27] also suggest that AEA may be agonistic through CB1, but that this is overpowered by the antagonistic effects on CB2. Therefore, the findings of Idris et al. [19] and Whyte et al. [27] may not be so different after all.

Regarding the dual dose-dependent effect of both THC and CBD on bone resorption, some support of these findings can found in the study of Whyte et al. [27]. They reported that AEA in the μM range (or lower) specifically stimulated the bone resorptive activity of already matured human OCs and that this effect was primarily mediated through the CB2 receptor. In our case, we cannot distinguish whether the effects are mediated through CB2 or CB1, but we detect a higher expression level of *CNR2* compared to *CNR1*. Importantly, CB2 was reported to be linked to BMD in humans, since a SNP in the *CNR2* locus is linked to BMD [58]. However, when it comes to effects of genetic KO of *CNR1* and *CNR2* in mouse models, it is not conclusive whether one receptor or the other primarily mediates a bone-protective or bone-destructive effect upon binding to agonists. Some years ago, Raphael & Gabet [18] published a comprehensive review of the existing studies at that time (2016), but in 2017 Sophocleous and coauthors [59] published results showing that a global KO of both receptors in mice increased bone mass during growth, it attenuated age-related bone loss, and prevented OVX induced bone loss. The authors suggest that this is primarily caused by an inhibition of osteoclastic bone resorption. This suggests that, in mice, endocannabinoids overall have a stimulatory effect on OCs, which could explain why we observe that low doses of both CBD and THC can induce bone resorption while not significantly affecting fusion ($\leq$3 μM). Other studies have yielded contradictory findings [19,20], as reviewed by Raphael & Gabet [18]. However, some support can be found in certain studies: 1) Whyte and colleagues [27] found that the endogenous cannabinoid, AEA, stimulates OC resorption. This stimulation of resorption seemed primarily to be mediated through CB2. CP55940 also stimulated bone resorption of existing OCs, but here it seems that both CB1 and CB2 are needed to mediate this effect. 2) Sophocleous and coworkers [59] showed that a global KO of both *CNR1* and *CNR2* in mice can prevent age- and OVX-induced bone loss by primarily inhibiting OCs. This suggests that endocannabinoids in general stimulate osteoclastic bone resorption more than bone formation. 3) In a cross-sectional clinical study, Sophocleous and coworkers [13] found that heavy cannabis users ($n = 144$) had lower BMD values across skeletal sites and elevated CTX and PINP values compared to controls (cigarette smokers, $n = 114$). Although both biomarkers were elevated, the lower BMD values suggest that cannabinoids such as CBD and THC in general shift the balance between OCs and OBs in favor of osteoclastic bone resorption, therefore resulting in a net bone loss. Contrary to the study of Sophocleous et al. [13], who used cannabis users to investigate the effects of cannabinoids in humans, Kulpa et al. recently published the results from a double-blind placebo-controlled clinical trial with controlled medical cannabis products of both CBD and THC using 83

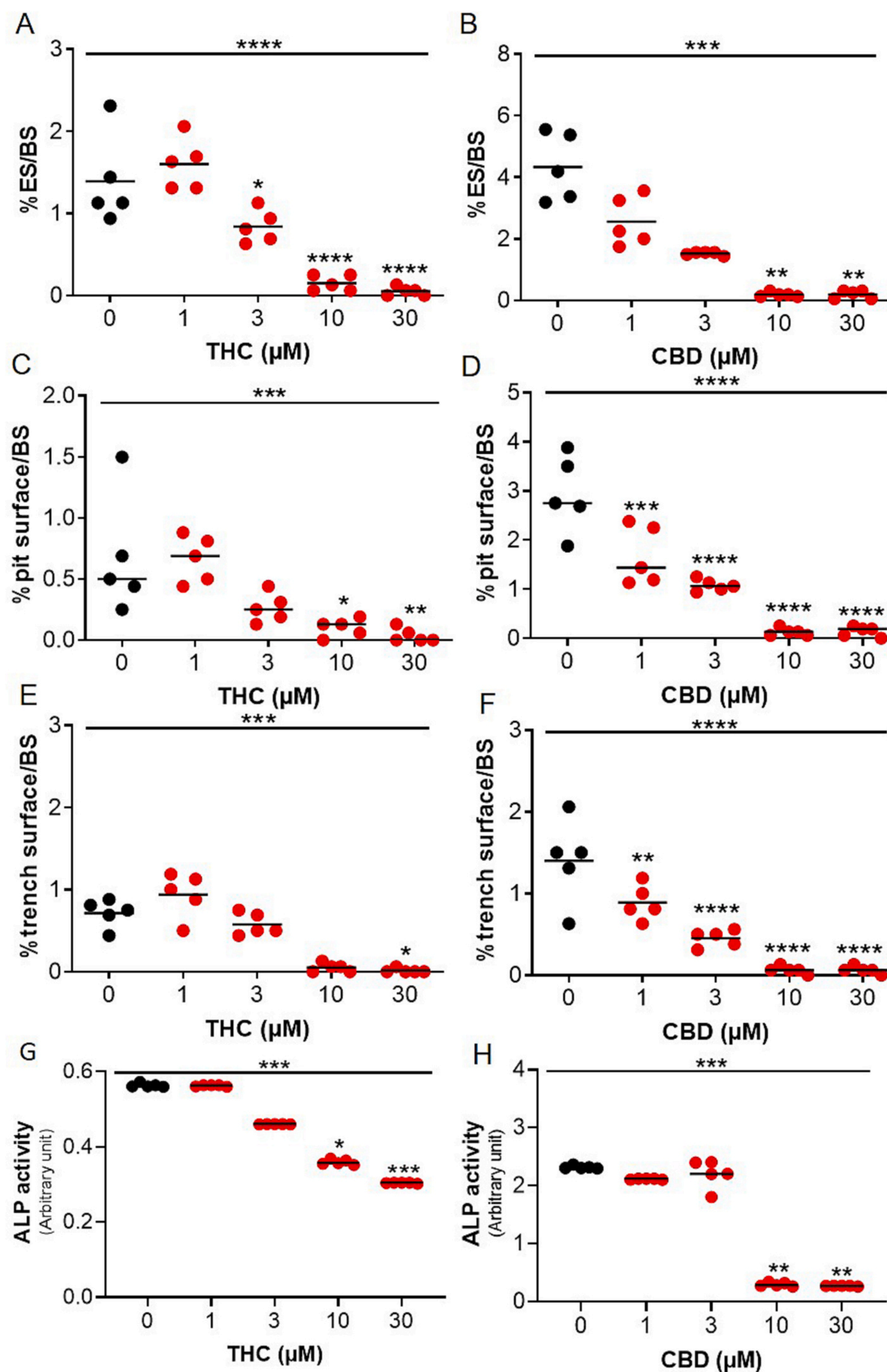

Fig. 5. High doses of both THC and CBD inhibit OC bone resorption activity in co-cultures with reversal cell-like OBs – as exemplified from a full experiment using cells from one OC and one OB donor. (a) Percentage of total eroded surface (ES) per BS in the presence of increasing concentrations of THC and (b) and CBD. Statistics: (a) Ordinary one-way ANOVA test, ****$p < 0.0001$; Holm-Sidak's multiple comparisons test comparing to 0 μM THC, 3 μM THC *$p = 0.0172$, 10 and 30 μM ****$p < 0.0001$; (b) Kruskal-Wallis test, ***$p = 0.0002$; Dunn's multiple comparisons test comparing to 0 μM CBD, 10 μM CBD **$p = 0.0010$, 30 μM CBD **$p = 0.0012$. (c) Percentage pit surface per BS in the presence of increasing concentrations of THC and (d) CBD. Statistics: (c) Kruskal-Wallis test, ***$p = 0.0006$; Dunn's multiple comparisons test comparing to 0 μM THC, 10 μM THC *$p = 0.0381$, 30 μM THC **$p = 0.0048$; (d) Ordinary one-way ANOVA test, ****$p < 0.0001$; Holm-Sidak's multiple comparisons test comparing to 0 μM CBD, 1 μM CBD ***$p = 0.0002$, 3, 10 and 30 μM CBD ****$p < 0.0001$. (e) Percentage of trench surface per BS in the presence of increasing concentrations of THC and (f) CBD. Statistics: (e) Kruskal-Wallis test, ***$p = 0.0005$; Dunn's multiple comparisons test comparing to 0 μM THC, 30 μM THC *$p = 0.0168$; (f) Ordinary one-way ANOVA, ****$p < 0.0001$; Holm-Sidak's multiple comparisons test comparing to 0 μM CBD, 1 μM CBD **$p = 0.0047$, 3, 10 and 30 μM CBD ****$p < 0.0001$. (g) ALP activity of OBs in co-cultures in the presence of increasing concentrations of THC and (h) CBD. Statistics: (g) Kruskal-Wallis test, ***$p = 0.0002$; Dunn's multiple comparisons test comparing to 0 μM THC, 10 μM THC *$p = 0.0298$, 30 μM THC ***$p = 0.0007$; (h) Kruskal-Wallis test, ***$p = 0.0005$; Dunn's multiple comparisons test comparing to 0 μM CBD, 10 μM CBD **$p = 0.0089$, 30 μM CBD **$p = 0.0012$. Horizontal lines indicate the mean.

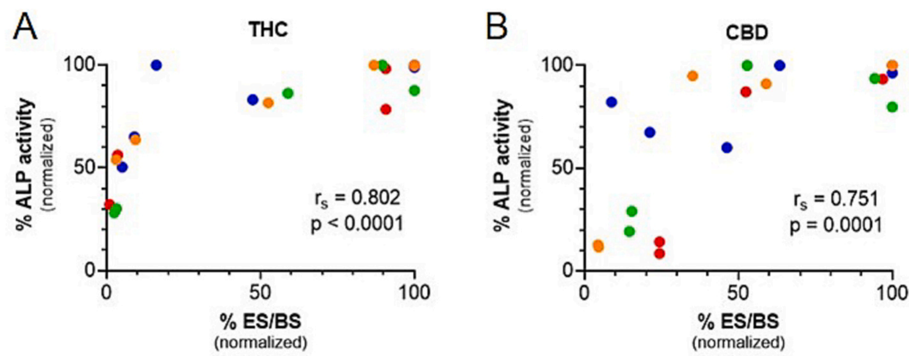

Fig. 6. ALP-activity of reversal cell-like OBs correlates with the ability of OCs to resorb bone (percentage eroded surface (ES) per bone surface (BS)) in response to treatment with (a) THC and (b) CBD. Statistics: Spearman correlation. Each point represents the mean value of five replicates for the following concentrations of cannabinoids: 0 μM, 1 μM, 3 μM, 10 μM and 30 μM for individual donors ($n = 4$ experiments using cells from different donors, indicated by different colors). For both % ALP activity and % ES/BS, data were normalized to the dose where the highest mean-value was obtained within the dose range (0 to 30 μM) for each donor.

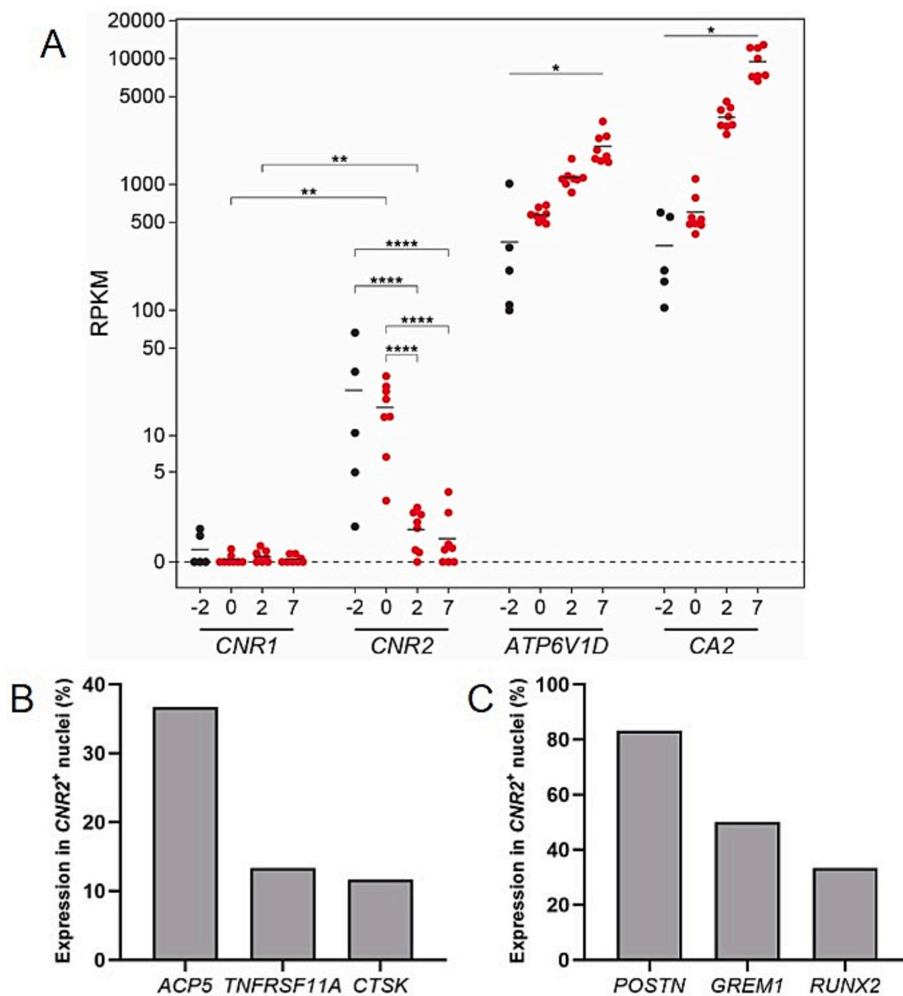

Fig. 7. Expression of CNR1 and CNR2 in OCs and reversal cell-like OBs. A) RNA-seq based gene expression (RPKM) of CNR1 and CNR2 as well as the osteoclastogenesis induced genes ATPase H+ transporting V1 subunit D (ATP6V1D) and carbonic anhydrase 2 (CA2) throughout human OC differentiation using cells from 5 to 8 human donors. Time points indicate days prior to and post initial RANKL stimulation. Horizontal lines represent the mean. Statistics: Paired students *t*-test comparing CNR1 and CNR2 expression, **<0.01. Adjusted *p*-values using a DESeq2 model with donor and time point variables to compare expression levels between time points, ****$<10^{-7}$. ATP6V1D and CA2 expression increases significantly from each time point to the next, *<0.05. B) Expression of osteoclastic markers in CNR2-positive nuclei within the OC cluster. RNA of TRAcP (ACP5), RANK (TNFRSF11A), and Cathepsin K (CTSK) were detected in parts of CNR2-positive nuclei after single nuclei sequencing. C) Expression of osteoblastic markers in CNR2-positive nuclei within the OB cluster. RNA of periostin (POSTN), gremlin-1 (GREM1), and Runx Family Transcription factor 2 (RUNX2) were detected in CNR2-positive nuclei after single nuclei sequencing. B & C show results from a single experiment.

healthy participants [12]. They found that both CTX and PINP were significantly suppressed by both CBD and THC treatment [12]. In another cross sectional study, Bourne et al. [37] investigated the effects of recreational cannabis use on BMD. They sub split users into never (n = 2162), former (n = 1933), light (n = 263), or heavy users (n = 385). Unadjusted models showed significantly lower BMD in users compared to controls. However, when adjusting for possible confounders (sex, age, and BMI) there was no longer a significant association with BMD [37]. So the human studies of Sophocleous et al. [13], Bourne et al. [37], and Kulpa et al. [12] seem to add to the number of conflicting results. Our monoculture approach both support inhibition (OC fusion and bone resorption) and stimulation (osteoclastic bone resorption), but our co-culture setup also support a primarily inhibitory effect.

Our tests with OCs in monoculture were all done under standard cell culture conditions with FBS, MCSF, and RANKL, but in our co-culture system, we employed another experimental design. This was designed to mimic the interdependency of OCs and reversal cells [47,48] as observed in human bone [16,60–63]. Using this cell cultural design, we no longer observed a stimulation of osteoclastic bone resorption. Instead, we observed that low concentrations had no apparent effect, while higher doses showed an inhibitory effect, just as we also observed under monoculture conditions. Interestingly, we could also observe an inhibition of the ALP-activity in the reversal cell-like OBs. In many studies, monocultures of OB-lineage cells have been used to test effects of various cannabinoids on these cell types. These studies were all done under standard culture conditions and not in a co-culture setup with OCs. However, they have primarily found that various cannabinoids have a stimulatory effect on OB-lineage cells [18,36,57,64]. We have not been able to reproduce this with our setup. It is important to highlight a major discrepancy between our study and already published studies, namely the culture conditions and the fact that we use primary human reversal cell-like OBs. An explanation for the discrepancy in the response could be that we have only used ALP-activity as a measure for osteoblastic activity, and that cannabinoids may require supportive molecules present in the bovine serum to have a stimulatory, but not an inhibitory effect. This latter point is also supported by the fact that the stimulation of bone resorption was also lost in the absence of serum. This raises an interesting speculation that circulating factors in the blood may be able to modulate the effect of CBD and THC on bone cells. As aforementioned, Sophocleous et al. [13], Bourne et al. [37], and Kulpa et al. [12] obtained contradicting results using human subjects, but while Kulpa and co-workers used healthy subjects and followed them over time, Sophocleous et al. and Bourne et al. investigated cannabis users in cross-sectional studies, which may not necessarily be compared to healthy participants. So is it possible that these very different cohorts could also have different levels of factors in their blood? Unknown factors, but which may be the same that could have a modulating effect *via* the bovine serum? Finally, an explanation for the strictly inhibitory effects of THC and CBD on both OCs and reversal cell-like OBs in co-cultures could also be related to the fact that we in this condition could only detect the expression of *CNR2*. Idris and co-workers [19] reported that *CNR1* is required for a stimulation of OCs, and Whyte et al. [27] reported that *CNR2* is mediating the inhibitory effect on OCs. Thus, the fact that *CNR1* is detected in OC monocultures, but not in co-culture conditions, may explain why the stimulation is lost in the co-culture.

Interestingly, in our co-culture setup we observed a coupled response of osteoclastic bone resorption and ALP-activity when treated with CBD or THC. We observed a strong positive correlation between the variations in bone resorption level (as a consequence of inhibition by both CBD and THC) and the ALP-activity level, suggesting some kind of coupled response. Such a coupled response was also suggested by Sophocleous and coworkers in their mouse double knockout model of *CNR1* and *CNR2* [59] and also in the clinical trials where Sophocleous et al. [13] saw an increase in both CTX and PINP, while Kulpa et al. [12] observed a reduction in both. In this regard, we cannot say if these coordinated effects on bone resorption and ALP-activity are due to a truly coupled response or if it is because OCs and reversal cell-like OBs simply show the same sensitivity and response towards CBD and THC. At the current stage, our knowledge on the potential biological meaning of these parallel effects on bone resorption and bone formation is simply too scarce to conclude. Therefore, more investigations in humans as well as *in vitro* model systems are needed.

When performing *in vitro* analyses to investigate potential effects of THC and CBD on bone cells, we acknowledge that such a model system will always have limitations. Humans are different in many aspects, for example due to genetics, age, and life-style. Although, we in some of our previous work have shown that the use of OCs generated from different individuals do reflect at least some of these variations [46,65,66], this will never be a perfect model. In order to ensure that the dosing of *e.g.* CBD and THC used to treat cells in culture is of pharmacological relevance, it is important to ensure that the dose range used is of pharmacological relevance. Three phase I studies have tested the peak concentrations of CBD and metabolites and found them to range between roughly 1 and 17 μM [38–40]. In addition, they found t_{max} to be about 5 h while $t_{½}$ varied from 5 to 40 h depending on the dose. Thus, a minimum of half-maximal concentration is maintained between 10 and 45 h [38–40]. In our experimental setup, we treated cells for 48 to 72 h with a dose range of 0.3 to 30 μM CBD or THC. The dose range seems to be within a comparable range, although 30 μM may be excessive, while the incubation times may be too high for a direct comparison. With respect to the incubation, it should also be considered that both CBD and THC are metabolized and inactivated, and we do not know to what extent this may occur in our cell culture models. It is also important to point out that THC and its metabolites are difficult to detect in blood [38,39]. Therefore, our *in vivo/in vitro* comparisons are restricted to knowledge on CBD. Thus, with some limitations, we find that our cell culture testing of CBD and THC mimics their pharmacological use. However, when considering the results from the pharmacokinetic studies, it is possible that the results obtained with the lower ranges of CBD and THC are of most pharmacological relevance. At lower doses ($\leq$10 μM), we observe no or only weak inhibition of OC differentiation while the same dose range stimulates bone resorption in OC-monocultures and inhibits bone resorption in OC and reversal cell-like OBs co-cultures.

5. Conclusions

In line with the existing literature on the effect of cannabinoids on bone cells, our current study shows both stimulatory and inhibitory effects. This highlights that potential side effects of cannabinoids on bone cells and bone health is a complex matter. The contradictory and lacking documentation for potential effects on bone health as well as other potential effects should be taken into consideration when considering the use of cannabinoids for both medical and recreational use. If reliable documentation for the safety of this usage is lacking more coordinated efforts in both basic and clinical research should be undertaken. Here it should also be considered whether dosing may give different results, something which is suggested by our results showing a stimulation of bone resorption at low doses and inhibition of both OCs and OBs at higher doses. Additionally, it should also be considered that the same dose of cannabinoids results in very different concentrations in the blood depending on the individual [38–40]. This could give opposite effects in individuals treated with the same dose and could therefore also contribute to inconclusive results in clinical trials. It would therefore be desirable to conduct thorough and systematic pharmacodynamic studies addressing both dosing and effect, something we presently have only very little knowledge about [67].

CRediT authorship contribution statement

Simone S.R. Nielsen: Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal

analysis. **Juliana A.Z. Pedersen:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis. **Neha Sharma:** Writing – review & editing, Methodology, Investigation, Formal analysis. **Pernille K. Wasehuus:** Writing – review & editing, Resources, Methodology, Investigation, Formal analysis. **Morten S. Hansen:** Writing – review & editing, Visualization, Resources, Investigation, Formal analysis. **Anaïs M.J. Møller:** Writing – review & editing, Investigation, Formal analysis. **Xenia G. Borggaard:** Writing – review & editing, Visualization, Methodology, Investigation, Formal analysis, Data curation, Funding acquisition. **Alexander Rauch:** Writing – review & editing, Validation, Resources, Methodology, Investigation, Formal analysis, Data curation. **Morten Frost:** Writing – review & editing, Resources, Methodology. **Teis E. Sondergaard:** Writing – review & editing, Visualization, Validation, Supervision, Resources, Methodology, Funding acquisition, Formal analysis, Conceptualization. **Kent Søe:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Project administration, Methodology, Funding acquisition, Formal analysis, Conceptualization.

Declaration of competing interest

Authors declare to have no conflict of interest.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bone.2024.117035.

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