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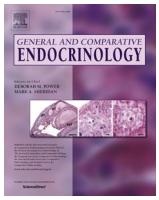

Review article

PFOA effects on osteoblast differentiation: Involvement of oxidative stress and endocannabinoid receptors☆

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ABSTRACT

Perfluorooctanoic acid (PFOA), a persistent perfluoroalkyl substance (PFAS), has been implicated in bone mineral density loss and defective osteogenesis. In this study, by employing human fetal osteoblast (hFOB1.19), we investigated whether PFOA interferes with osteoblast differentiation by altering the transcription of genes involved in osteogenesis, and protein levels of oxidative stress defense and cannabinoid receptors (CBs). The hFOB 1.19 were exposed to increasing PFOA concentrations (1–100 μM) for seven days, representing supra-environmental concentration commonly used in mechanistic *in vitro* studies. Osteogenic markers were then evaluated at transcriptional level together with matrix deposition, and the results were compared to an untreated control group. Exposure to PFOA at 10 μM increased the expression of osteocalcin (*BGLAP*) encoding for a protein involved in calcium deposition. Catalase (CAT) protein levels were upregulated at 1 μM PFOA, while superoxide dismutase (SOD1) did not change, suggesting a selective antioxidant response to oxidative perturbation. Notably, this increase in CAT correlated with a trend toward *RUNX2* upregulation, possibly representing a compensatory mechanism to preserve differentiation under oxidative stress. In addition, the highest concentration of PFOA modulated the endocannabinoid system (ECS), reducing CB1 and CB2 protein levels. Despite these molecular changes, Alizarin Red staining revealed a borderline and not statistically significant enhanced calcium deposition only at 50 μM PFOA, suggesting potentially aberrant mineralization. Overall, our findings suggest that PFOA perturbs osteoblast differentiation through oxidative stress-linked mechanisms and CBs modulation, with catalase emerging as a key protective mediator of osteogenic competence under environmental contaminants. Furthermore, the observed dysregulation of CB1 and CB2 receptors indicates that the ECS itself may represent a direct target of PFOA action in osteoblasts.

1. Introduction

The differentiation of osteoblasts from mesenchymal stem cells (MSCs), a process known as osteoblastogenesis, proceeds through three main phases: proliferation, extracellular matrix (ECM) maturation, and mineralization (Amarasekara et al., 2021; Lin et al., 2020; Zhu et al., 2024) Each of these stages is tightly regulated by specific transcription factors and signaling pathways. In this regard, one of the main "master gene" for the commitment of osteoblast (OBs) is the one encoding Runt related transcription factor 2 (*RUNX2)*, that lead the activation of some osteogenic gene expressions such as collagen 1 (*COL1A1),* osteopontin *(OPN),* and osteocalcin or bone gamma-Carboxyglutamate Protein (*BGLAP)* (Kim et al., 2020; Liu and Lee, 2012). In the complex interplay between OBs and osteoclasts (OCs) for the maintenance of bone homeostasis and bone remodeling, the OBs play a key role in forming new bone tissue producing and mineralizing fresh matrix (Blair et al., 2017; Lin et al., 2020). Within the bone matrix, OBs has been secreting a crucial set of collagenous and non-collagenous proteins that provide the

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scaffold for mineral lattice deposition through hydroxyapatite formation (Saito and Marumo, 2015; Sartori et al., 2015). This process is tightly regulated by alkaline phosphatase (ALP) activity and various mineralization modulators. Consequently, research has increasingly focused on OBs differentiation to better understand the mechanisms underlying bone formation.

Beyond mTOR signaling, recent studies suggest that the endocannabinoid system (ECS) also contributes to the regulation of bone cell activity and bone remodeling (Idris and Ralston, 2012; Saponaro et al., 2021). The main ECS receptors includes type 1 cannabinoid receptor (CB1), type 2 cannabinoid receptor (CB2) (Ho et al., 2015). In bone tissue, activation of CB2 promotes osteoblast proliferation and mineralization while inhibiting OC-mediated resorption (Fan et al., 2024; Hu et al., 2024). In contrast, CB1 exerts more complex, context-dependent roles. For example, the skeletal phenotype of CB1-deficient mice varies depending on the genetic background and the specific mutation introduced. In the *CB1*$^{-/-}$ line, in which the N-terminal 233 codons of the CB1 gene were deleted, males exhibit increased trabecular bone mass with normal bone formation, whereas females display only mild cortical expansion. Conversely, C57BL/6 *CB1*$^{-/-}$ mice, in which almost the entire protein-coding sequence was removed, presents low bone mass, elevated osteoclast numbers, and reduced bone formation rates. Moreover, mesenchymal stem cells from *CB1*$^{-/-}$ mice show impaired osteoblast differentiation but enhanced adipogenesis, driven by increased cAMP-dependent CREB phosphorylation and PPARγ expression, ultimately predisposing to age-related osteoporosis and marrow fat accumulation (Bab and Zimmer, 2008; Idris et al., 2009). In addition to cannabinoid receptors, several members of the transient receptor potential vanilloid (TRPV) family, including TRPV1 and TRPV4, are implicated in bone physiology. These channels act as polymodal, non-selective cation channels that regulate intracellular Ca^{2+} signaling and mechano-transduction processes involved in osteoblast differentiation and bone remodeling. TRPV1 influences osteoblast survival and differentiation, with knockout models showing increased bone density (He et al., 2017; Rossi et al., 2015), while TRPV4 functions as a mechanosensitive ion channel that mediates calcium influx in response to mechanical and osmotic stimuli and has been shown to regulate osteoblast activity, osteoclast differentiation, and bone mass homeostasis (Acharya et al., 2023; Lieben and Carmeliet, 2012; Suzuki et al., 2013; Wang et al., 2022). Dysregulation of ECS receptors and TRPV channels has thus been proposed as a contributor to impaired bone remodelling.

On the contrary, environmental contaminants are increasingly recognized as endocrine-disrupting chemicals (EDCs) that impair bone homeostasis and contribute to the onset of osteoporosis (Park et al., 2024; Smith et al., 2017; Turan, 2021). Among these, particular attention has been drawn to emerging per- and poly- fluoroalkyl substances (PFAS), such as perfluorooctanoic acid (PFOA), which persist in the environment and exhibit well-documented endocrine and metabolic toxicity affecting bone health (Beglarian et al., 2024; Blomberg et al., 2022; Khosrojerdi et al., 2024). Epidemiological studies have reported that PFAS exposure can reduce bone mineral density and increase risk of osteoporosis, while experimental models demonstrate impaired osteoblast differentiation, increased oxidative stress, and altered gene expression, such as *RUNX2* and *ALP* (Khalil et al., 2015; Koskela et al., 2017). Recently, PFOA has been consistently linked to an imbalance of antioxidant response leading to increased level of ROS and lipid peroxidation in human hepatocytes as well as in murine pancreatic cells (Feng et al., 2024; Kamendulis et al., 2014; Suh et al., 2017). In particular, PFOA can engage nuclear receptor signaling, including PPAR-dependent responses, and modulate antioxidant defense networks regulated by Nrf2, thereby altering the expression of antioxidant enzymes (Liu et al., 2015; Murase et al., 2023). These established oxidative stress-related mechanisms provide a rationale for examining catalase (CAT) as a protective mediator in osteoblasts and support the hypothesis that redox perturbations may intersect with ECS signaling (Lipina and Hundal, 2016).

One of the main PFAS studied and considered an emerging contaminant is the perfluorooctanoic acid (PFOA). Environmental contamination by PFOA results in measurable human exposure, as reflected by mean serum concentrations in the European population of approximately 1.9 ng/mL in adults and 3.3 ng/mL in children (Schrenk et al., 2020). These findings underscore the persistence and bioaccumulation potential of this compound. Concerning bone metabolism, several population-based studies have reported that higher circulating levels of PFOA are significantly correlated with decreased bone mineral density and content, particularly in postmenopausal women, who are more vulnerable to osteoporotic bone loss. Collectively, these observations suggest that PFOA may interfere with bone remodeling processes, potentially through endocrine or metabolic disruption mechanisms (Khalil et al., 2015; Koskela et al., 2016a; Liu et al., 2019). In this study, we hypothesize that PFOA interferes with osteoblast differentiation by altering osteogenic transcriptional programs, disrupting redox homeostasis, and modulating the cannabinoid receptors. To test this, we employed the hFOB1.19 cell line, a well-characterized human fetal osteoblast model that retains robust osteogenic capacity and a reproducible differentiation program under defined conditions (Marozin et al., 2021; Yen et al., 2007). Compared with primary human osteoblast-like cells (OBs), which can display substantial donor-to-donor variability and limited lifespan, hFOB1.19 provides higher experimental reproducibility and sensitivity for mechanistic studies of osteoblast differentiation (Bousch et al., 2025). Despite OBs are considered the "gold standard" for mimicking mature bone formation, hFOB cells represent a useful, immortalized model to study early-stage osteoblast differentiation and proliferation, which are sensible windows of xenobiotics exposure. After the exposure, we evaluated osteogenic markers at transcriptional and protein levels as well as assessed, the matrix deposition and mineralization.

2. Material and Methods

2.1. hFOB1.19 cell Culture

The hFOB 1.19 cells were cultured in Growth Medium (GIBCO), consisting of a 1:1 mixture of Ham's F12 Medium and Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 2.5 mM L-glutamine and without phenol red (Life Technologies Limited, Paisley, UK). The medium was further supplemented with 0.3 mg/mL G418/geneticin (Life Technologies Corporation, Grand Island, NY, USA) and 10% fetal bovine serum (FBS; Life Technologies Limited, Paisley, UK). Cells were maintained at 34 °C in a humidified atmosphere with 5% CO_2.

2.2. Differentiation of hFOB1.19 and PFOA treatment

To induce differentiation, hFOB1.19 cells were grown to confluency within 2 days after seeding and then switched to differentiation medium (Osteogenic Medium, OM), composed of 50 μg/mL ascorbic acid and 7.5 mM β-glycerophosphate. Differentiation was carried out at 34 °C in a 5% CO_2 atmosphere for a total of 7 days. The medium was refreshed every 3 days from the beginning of differentiation. The hFOB1.19 cell line is conditionally immortalized through a temperature-sensitive SV40 large T antigen, which promotes proliferation at higher temperatures but becomes inactive at lower temperatures, thereby allowing cells to exit the proliferative state and undergo osteogenic differentiation. To further confirm the suitability of this temperature for our experimental setup, preliminary experiments comparing differentiation conditions were performed, and the results are presented in Fig. S1 (Supplementary Fig. S1).

Cells were exposed for 7 days with Perfluorooctanoic acid (PFOA, 95%, 171468–256, Sigma-Aldrich, St. Louis, MO, USA) at various concentrations. PFOA was dissolved in dimethyl sulfoxide (DMSO) to a final DMSO concentration of 0.02%. According to previous reports (Pierozan

Table 1
List of primers indicating the Forward and Reverse sequences used for RT-qPCR and their optimal annealing temperature.

Abbreviation	GENE	FORWARD (5′- 3′) **REVERSE (5′ – 3′)**	Tm (°C)	GenBank Number
RUNX2	RUNX family transcription factor 2	F: GTGCCTAGGCGCATTTCA R: GCTCTTCTTACTGAGAGTGGAAGG	61.5	NM_001024630.4
RUNX3	RUNX family transcription factor 3	F: TCAGCACCACAAGCCACTT R: AATGGGTTCAGTTCCGAGGT	54	NM_001031680.2
ALPL	Alkaline phosphatase, biomineralization associated	F: GCTGTAAGGACATCGCCTACCA R: CCTGGCTTTCTCGTCACTCTCA	61.4	NM_001369803.2
BGLAP	Bone gamma-carboxyglutamate protein	F: TGAGAGCCCTCACACTCCTC R: ACCTTTGCTGGACTCTGCAC	52.7	NM_199173.6
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	F: GTGAAGGTCGGAGTCAACG R: GGTGAAGACGGCCAGTGGACTC	60	NM_001289746.2
RPLP0	Ribosomal protein lateral stalk subunit P0	F: CTGGAAAACAACCCAGCTCT R: GAGGTCCTCCTTGGTGAACA	60	NM_001002.4
RPLP18	Ribosomal protein lateral stalk subunit P18	F: TGCGAGTACTCAACACCAACA R: GCATATCTTCGGCCCACA	60	NM_022551.3

et al., 2018; Sun et al., 2019; Wang et al., 2024), the experimental groups were designed to include five different doses: OM control (DMSO 0.02% + OM; CTRL) and PFOA treatments (0.1 μM, 1 μM, 10 μM, 50 μM, and 100 μM). Stock PFOA was first diluted in 100% DMSO and then further diluted in differentiation medium (OM) to reach the final concentrations.

2.3. Cell viability Assay: MTT

The MTT assay (1 mg/mL of the tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was performed on days 1 and 7 post-treatment in depletion medium. After 2 h of incubation at 34 °C, formazan crystals were dissolved in isopropanol, and absorbance was measured at 570 nm using a microplate reader (Synergy, Biotek, Winooski, VT, USA).

2.4. Alizarin red s staining and alkaline phosphatase staining

On day 7 of differentiation, cells were fixed and stained with Alizarin (ALZ) Red S to assess mineralization, and alkaline phosphatase (ALP) activity was measured with a spectrophotometer as previously described in (Sella et al., 2025 in press). Representative images were captured using the Lionheart XF Automated Microscope in brightfield.

2.5. RNA Extraction, cDNA synthesis and PCR

Cells were collected from plates using a cell scraper and centrifuged at 1,000 × g for 5 min. The supernatant was discarded, and cells were resuspended in RNAzol and homogenized by repeatedly passing the lysate through a 22-gauge needle attached to a syringe. Total RNA was extracted following the manufacturer's protocol. RNA concentration (ng/μL) and purity were assessed with a NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA), and RNA integrity was confirmed by electrophoresis on a 1% agarose gel.

Possible genomic DNA contamination was removed by employing the DNase I (Sigma-Aldrich, Milan, Italy) and cDNA synthesis was performed using the iScript cDNA Synthesis Kit (Biorad, Milan, Italy) following the manufacturer's instruction.

qRT-PCR was performed using SYBR Green (Bio-Rad, Milan, Italy) on a CFX thermal cycler (Bio-Rad, Milan, Italy) as previously described (Lombó et al., 2025). The thermal cycling profile was: 3 min at 95 °C followed by 45 cycles of 20 s at 95 °C, 20 s at 60 °C, and 20 s at 72 °C. *GAPDH*, *RPS18*, and *RPLP0* were used as housekeeping genes to normalize mRNA levels. Data analysis was conducted using the iQ5 Optical System software version 2.1 (Bio-Rad, Milan, Italy), including the Genex Macro iQ5 Conversion and Genex Macro iQ5 files. Relative mRNA expression levels across experimental groups are reported.

The primers for differentiation-related genes (*RUNX2*, *RUNX3, ALPL* and *BGLAP*) are listed in Table 1.

Table 2
List of Secondary antibodies used in Western blot (WB) analysis.

Primary Antibody	Origin	Manufacturer primary antibodies	Catalogue number
Anti-CB1	Anti-rabbit	Abcam	Ab259323
Anti-CB2	Anti-mouse	Santa Cruz Biotecnology	sc-293188
Anti H4K12ac	Anti-rabbit	Abcam	Ab46983
Anti H4	Anti-mouse	Abcam	Ab31830
Anti-GAPDH	Anti-mouse	Proteintech	60004–1-Ig
Stress defense cocktail (CAT, SOD-1, TRX, b-ACT)	Anti-rabbit	Abcam	Ab179843
2Ab	Anti-mouse	Bethyl laboratories	A90-116P
2Ab	Anti-rabbit	Invitrogen	65–6120
2Ab	Anti-rabbit	Abcam	Ab150077

2.6. Western blot

Cells were lysed and protein extracts prepared as reported in CDD. The sample was separated on 12% polyacrylamide gels and transferred into nitrocellulose membranes. Membranes were blocked with Tris-buffered saline containing 0.1% Tween 20 (TBS-T) and 5% bovine serum albumin (BSA) in TBS-T for the detection of CAT, SOD1, CB1 and CB2. The secondary antibody was conjugated with horseradish peroxidase (HRP) and incubated at 37 °C for 1 h under gentle agitation. After three washes in TBS-T, the signal was developed using Clarity™ Western ECL Substrate (Bio-Rad, Hercules, CA, USA), prepared at a 1:1 ratio of Clarity and Luminol (ECL), and visualized using a ChemiDoc™ XRS Molecular Imager (Bio-Rad). The Primary and Secondary antibodies used are listed in Table 2.

2.7. Statistical analysis

Statistical analyses were performed using Graphpad 8.0. The normality of the data was checked through Shapiro-Wilk's test ($p > 0.05$). Differences between control and PFOA treatments were tested with a one-way ANOVA following Dunnett's multiple comparison, whereas, for non-parametric data, a Kruskal-Wallis test was applied following Dunn's multiple comparison. The significance level was set at $p < 0.05$ in all cases.

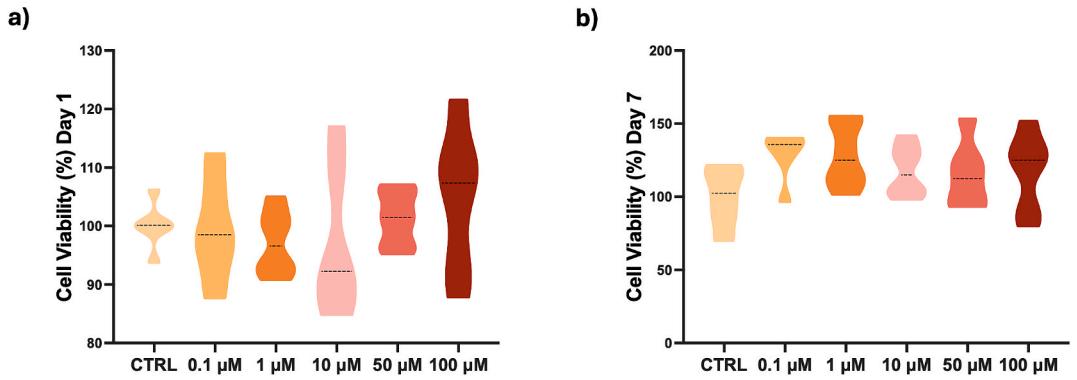

Fig. 1. Cell viability of hFOB 1.19 cells exposed to different concentrations of PFOA, assessed by MTT assay on day 1 and day 7 after the start of the differentiation treatment. Data are visualized as violin plot (n = 6).

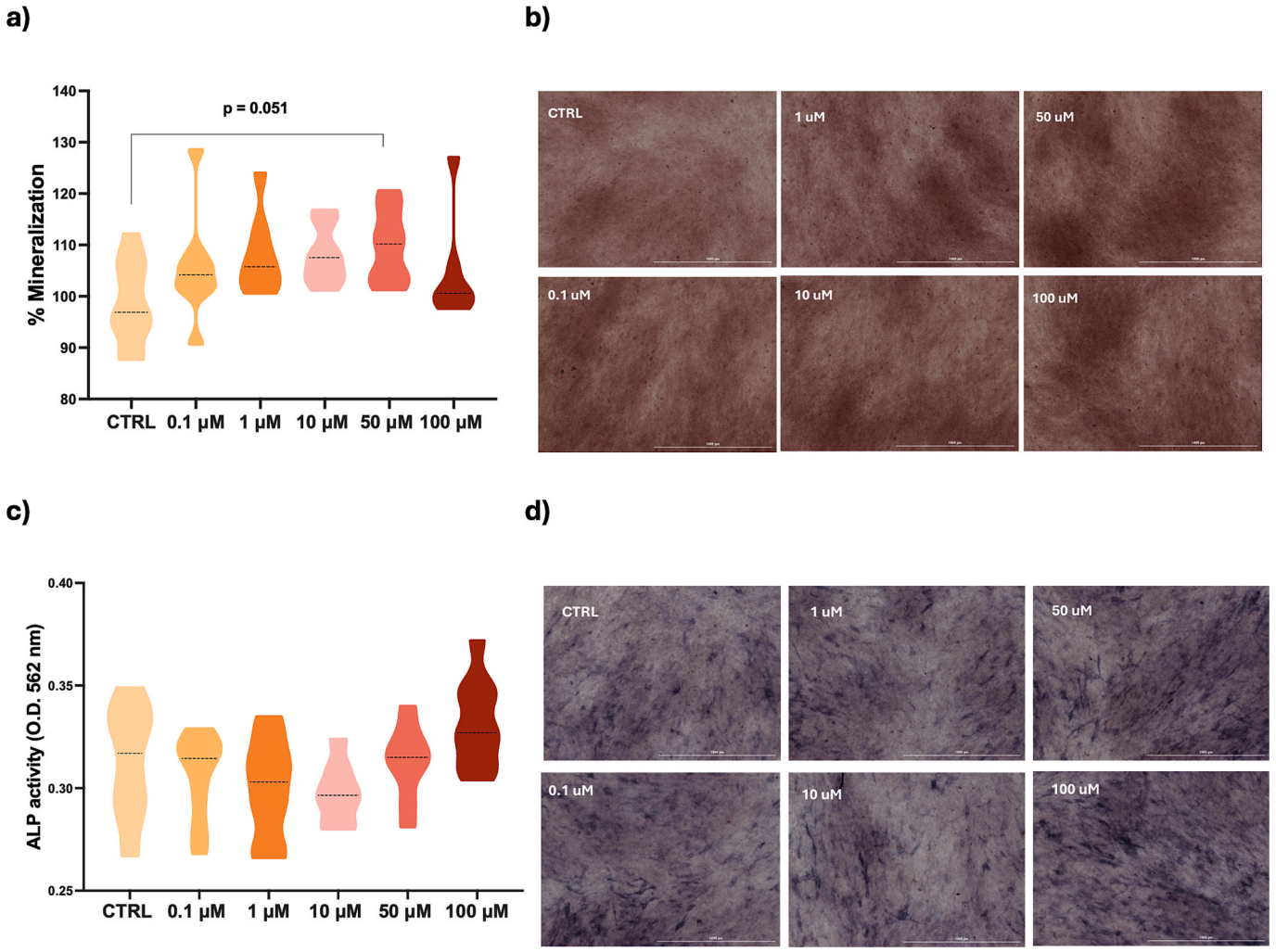

Fig. 2. ECM mineralization after PFOA treatment. a) Quantitative analysis of alizarin (ALZ) red staining in all groups, assessed by absorbance measurement via spectrophotometry at 550 nm. Data are visualized as violin plot (n = 8–10). b) Representative images of calcium deposition in the control and experimental groups treated with 0.1 µM, 1 µM, 10 µM, 50 µM, and 100 µM PFOA. Scale bar: 1000 µm. c) Quantitative analysis of alkaline phosphatase (ALP) activity in all groups, assessed by absorbance measurement via spectrophotometry at 562 nm. Data are visualized as violin plot (n = 8–10). d) Representative images of inorganic phosphate deposition in the control and experimental groups treated with 0.1 µM, 1 µM, 10 µM, 50 µM, and 100 µM PFOA. Scale bar: 1000 µm.

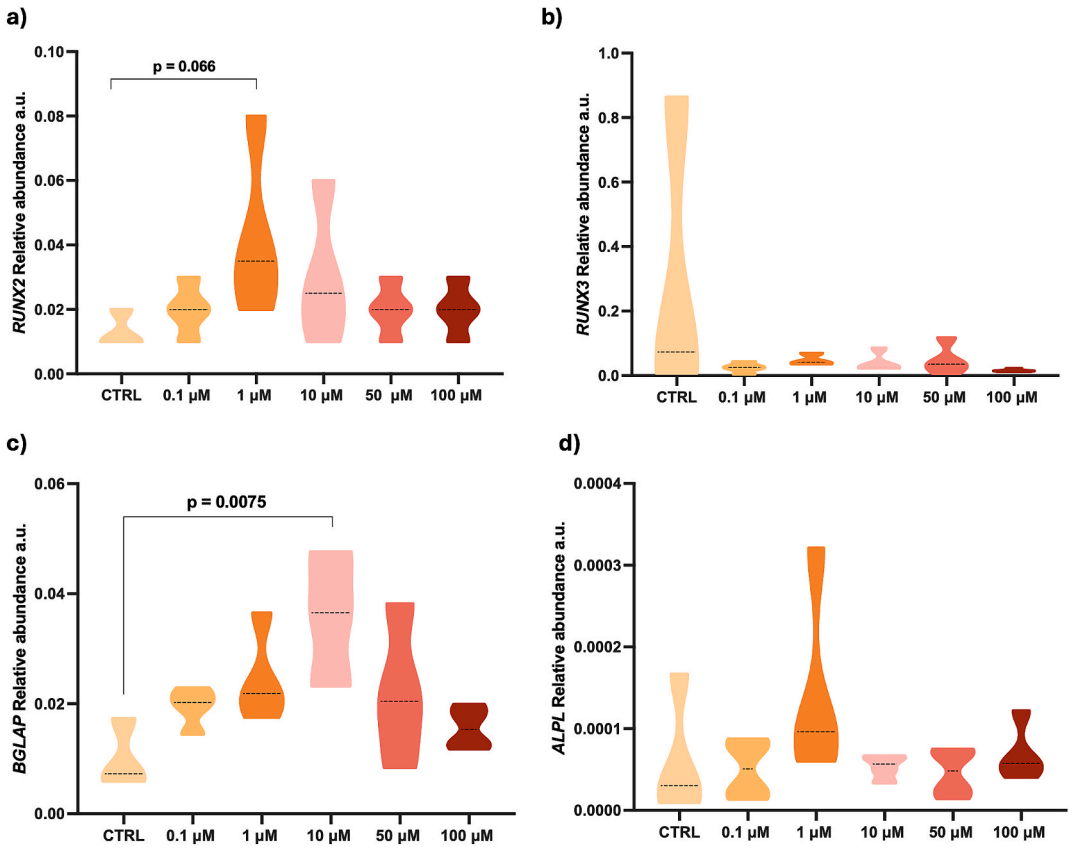

Fig. 3. Gene expression analysis of osteogenic markers in hFOB1.19 exposed to different concentrations of PFOA during differentiation. The violin plot shows median of transcripts abundance relative to the mean abundance of the internal controls (*GAPDH*, *RPS18*, and *RPLP0*) (n = 3–4).

3. Results

3.1. Effects of PFOA on the viability of hFOB 1.19 cells during the differentiation process

The enzymatic MTT assay performed on day 1 and day 7 (Fig. 1**a and 1b**) after the initiation of the differentiation treatment in hFOB 1.19 cells did not show significant changes in cell viability in each of PFOA-treated group compared the control. Therefore, all tested concentrations were carried forward for subsequent analyses.

3.2. Effects of PFOA exposure on ECM deposition

In osteoblast exposed to PFOA for 7 days, a slight increase in the Alizarin Red staining, suggesting the increase of calcium deposition, was observed in cells treated with 50 µM PFOA compared to the control (p = 0.051) (Fig. 2**a and 2b**). Conversely, data from ALP staining showed no significant differences across PFOA concentrations, indicating that PFOA does not affect early osteogenic differentiation or matrix maturation (Fig. 2**c and 2d**).

3.3. Impact of PFOA exposure on osteogenic and matrix deposition markers

To assess the effects of PFOA on osteoblast undergo differentiation, RT-qPCR analysis was performed on genes involved in osteogenesis and matrix deposition. *RUNX2b* transcript level showed a trend in increase in the 1 µM PFOA group with a p-value of 0.066, displaying a typical non-monotonic response commonly observed with EDCs when compared to control, suggesting a potential delay in osteoblast differentiation upon prolonged PFOA exposure (Fig. 3**a**). Notably, no changes in *RUNX3* and

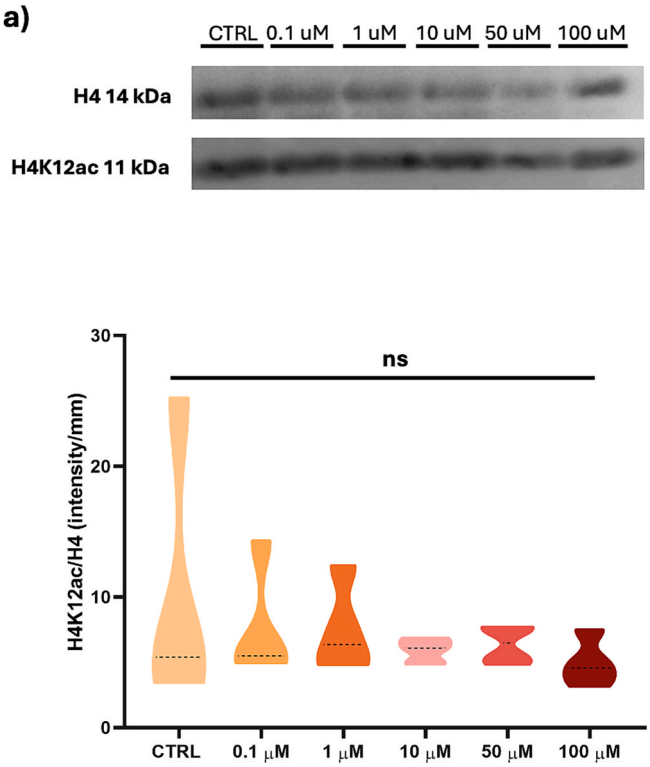

Fig. 4. Protein levels of osteogenic biomarkers: a) 11 kDa H4K12ac in hFOB1.19 cells. Representative blots (n = 4–5) were visualized by enhanced chemiluminescence. Data are visualized as violin plot.

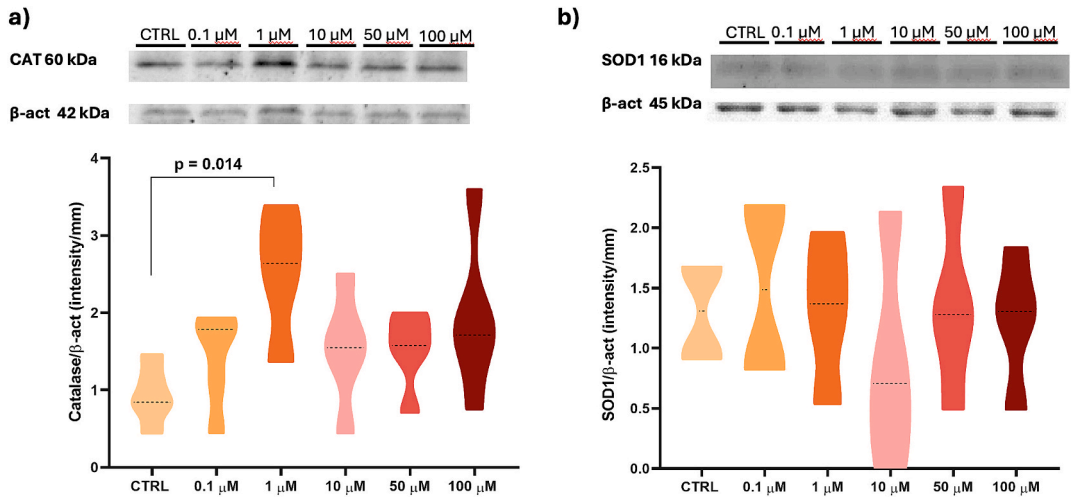

Fig. 5. Protein levels of antioxidant enzymes: a) 60 kDa CAT and b) 16 kDa SOD1 in hFOB1.19 cells. Representative blots (n = 4–5) were visualized by enhanced chemiluminescence. Data are visualized as violin plot.

ALPL transcript levels were observed under any treatment conditions (Fig. 3**b**). A significant increase in *BGLAP* expression was detected in cells exposed to 10 μM PFOA compared to controls ($p = 0.0075$) (Fig. 3**c**).

In the case of proteins involved in osteoblast differentiation, we examined also acetylation of lysine 12 in H4 (H4K12ac) levels, a marker of osteoblastogenesis (Sun et al., 2022), did not show significant changes after PFOA exposure during the differentiation phase (Fig. 4**a**).

3.4. Effects of PFOA exposure on oxidative stress defense markers

Western blot analysis revealed that catalase (CAT) protein levels were significantly increased in the cells exposed to 1 μM PFOA ($p = 0.014$) (Fig. 5**a**). As for the superoxide dismutase (SOD1) no significant changes were observed after 7 days of PFOA exposure (Fig. 5**b**).

3.5. Impact of PFOA exposure on ECS

As for the endocannabinoid system, CB1 and CB2 protein levels were evaluated after PFOA treatment. In the differentiation phase, the canonical 45 kDa CB2 levels remained largely unchanged in all the groups treated with PFOA (Fig. 6**b**). In contrast, the 55 kDa band showed a significant increase at 50 μM ($p = 0.043$) and 100 μM ($p = 0.012$) PFOA, while the 37 kDa band was not altered by PFOA exposure (Fig. 6**a and 6c**). On the contrary, CB1 showed a significant decrease at 100 μM PFOA ($p = 0.0061$), indicating that PFOA selectively affects CB1 receptor expression during osteoblast maturation (Fig. 6**d**).

4. Discussion

Modern research on the effects of PFAS on bone health and remodeling argued that contaminant exposure plays a key role in affecting stemness maintenance and differentiation of osteoblast (Blomberg et al., 2022; Garmo et al., 2025; Park et al., 2024; Wei et al., 2024). Recently, researchers have associated PFAS exposure with the insurgence of bone mineral density loss. In rodents, chronic exposure to PFOA (approximately 0.3 mg/kg/day) has been shown to alter bone microarchitecture and bone mineral density (Koskela et al., 2016). Moreover, epidemiological studies in humans have reported associations between circulating PFAS levels and reduced bone mineral density, particularly in populations at higher risk of osteoporosis (Khalil et al., 2015).

In that regard, we have studied the impact of PFOA exposure on early osteoblast differentiation over a 7-day period, with particular attention to different osteogenic markers as well as ECM deposition.

In the canonical differentiation pathway, an exit from proliferation is typically followed by upregulation of osteogenic master gene regulators such as *RUNX2* and downstream markers such as *BGLAP* (osteocalcin), ALP activity and eventual mineral deposition (Komori, 2019; Zoch et al., 2016). In our experiments, the gene expression of *ALPL* as well as its activity after PFOA exposure were like the control, while *BGLAP* was overexpressed in cells exposed to 10 μM PFOA. These findings are in contrast with *in vitro* studies conducting on another osteoblast line (MC3T3-E1) reporting that PFOA treatment (0.25–25 μM) led to downregulation of *ALPL* mRNA levels (Yang et al., 2025), while exposure to 100 and 200 μM PFOA for 7 days reduced the activity of ALP and the OCN levels (Wang et al., 2024). Studies investigating the interplay between osteogenesis and adipogenesis have revealed that inhibition of Wnt/β-catenin signaling, one of the main pathways for the expression or *ALP*, *BGLAP* and *RUNX3* (Zhu et al., 2024), increases the enrichment of H3K9Ac, H3K14Ac, and H4K12Ac (Li et al., 2021). In our study, the absence of significant changes in H4K12ac could explain why expression levels of *ALP and RUNX3* were not altered after PFOA exposure.

Regarding the possible involvement of oxidative stress in osteogenesis, it has been shown in rabbit calvaria osteoblast and murine preosteoblastic MC3T3-E1 cells that ROS could negatively affect osteoblastogenesis and bone matrix formation by activating MAPK signaling pathways (extracellular signal-regulated kinase, ERK and c-Jun N-terminal kinase, JNK), which reduce the expression of several osteogenic markers (ALP, OCN, COL1) (Drakou et al., 2025). Considering that PFOA has been demonstrated to alter the expression, translation and activity of antioxidant enzymes, therefore stimulating ROS production (Bonato et al., 2020; Yuan et al., 2017), we studied the protein levels of catalase and superoxide dismutase. In the present study, exposure to 1 μM PFOA significantly increased the protein level of CAT, which participates in osteogenic differentiation (Yang et al., 2022), while the level of SOD1 remains unchanged. This pattern suggests that CAT remains the primary enzymatic responder and target in osteoblasts to PFOA-driven oxidative perturbation, as we also demonstrated in the previous study on osteoblast exposed to PFOA during proliferation (Sella et al., 2025 in press). Importantly, recent mechanistic study links antioxidant capacity to RUNX2 stability and osteogenic competence: glutathione depletion leads to RUNX2 oxidation and degradation culminating in poor osteogenesis, whereas ROS scavenging (including catalase activity) stabilizes RUNX2 and rescues osteogenesis (Hu et al., 2023). Therefore, the increased catalase levels we observed in the cells exposed to 1 μM PFOA could be lying behind the upregulation of *RUNX2* in the same group, likely emerging as a compensatory and protective mechanism that attempts to preserve RUNX2 and differentiation in the face of oxidative

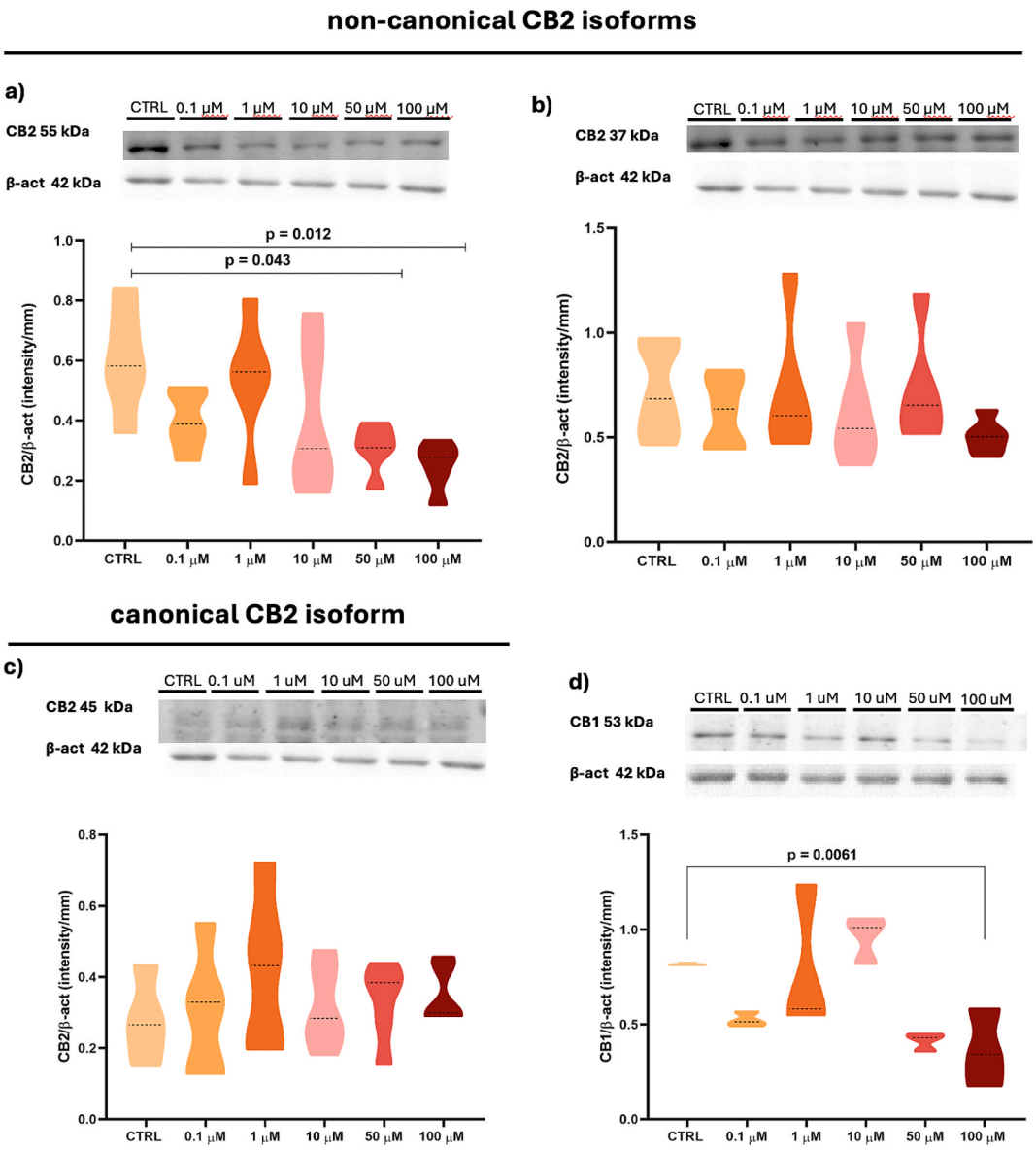

Fig. 6. Protein levels of endocannabinoid receptors: a) 55 kDa and b) 37 kDa non-canonical Cannabinoid Receptor 2 (CB2), c) 45 kDa canonical CB2, and d) 53 kDa Cannabinoid receptor 1 (CB1) protein levels in hFOB1.19 cells. Representative blots (n = 3–4) were visualized by enhanced chemiluminescence. Data are visualized as violin plot.

stress.

Given that ECS also plays an important role in bone metabolism (Saponaro et al., 2021), regulating bone mass and bone cell future and function we focused also on this system. Regarding CB1, previous study evidenced that *its* knockout in mouse MSCs had an impaired ability to differentiate into OBs, while they are more prone to differentiate into adipocytes (Idris et al., 2009). Furthermore, it was recently reviewed that the inactivation of CB1 by its inverse agonist AM251 in bone marrow-derived cells reduces the transcription of *RUNX2*, inhibiting OB differentiation(Rossi et al., 2019). In the present study, exposure of osteoblast to 100 μM PFOA reduced significantly CB1 protein levels, however these cells show *RUNX2* transcript levels similar to that of control group. On the other hand, CB2 is known to increase osteogenic markers such as *ALPL* in response to its agonist HU308 (Qian et al., 2010). Our results showed that its canonical 45 kDa form of CB2 was not altered by PFOA exposure, which may explain why key matrix components, such as *ALPL,* were not significantly affected by PFOA exposure. Previous findings in the MC3T3–E1 cell line, a model of mature osteoblasts, reported that CB2 stimulation not only induced osteoblast differentiation markers such as ALP but also enhanced matrix mineralization (Bab, 2005; Ofek et al., 2006). Although CB2 activation is known to promote osteoblast differentiation and matrix mineralization, our data suggest that PFOA exposure may trigger a distinct mechanism leading to increased calcium deposition. In that regard, we observed, using ALZ red staining, that hFOB 1.19 cells exposed to 50 μM PFOA for 7 days deposited more calcium than the control. This finding is consistent with previous studies investigating the effects of PFOA on MC3T3 osteoblasts (Koskela et al., 2016), where authors observed an increase in calcium deposition when exposing cells to 1 μM and 10 μM PFOA for 7 days suggesting a potential effect of low dose of PFOA on early osteogenic activity. Interestingly, additional CB2 bands were detected in our study. These additional bands likely reflect alternative protein processing and/or post-translational modifications, as previously reported (Cottone et al., 2013; Khan et al., 2018; Marchalant et al., 2014; Zhang et al., 2007). The altered levels in the 55 kDa form may suggest that PFOA exposure interferes with CB2 glycosylation processes, potentially affecting receptor folding, trafficking, and activity as also reported by other studies (Du et al., 2024, Du et al., 2023).

5. Limitations

Despite the mechanistic insights provided by this study, several limitations should be acknowledged. First, the concentrations of PFOA used in the present work fall within the micromolar range and therefore exceed typical human serum levels, which are generally reported in the low nanomolar range. However, such concentrations are commonly employed in *in vitro* systems to investigate mechanistic cellular responses. Second, osteoblast differentiation was performed at 34 °C, which is required for the temperature-sensitive hFOB1.19 cell line but differs from physiological temperature and may influence cellular responses to environmental contaminants.

Since this study aimed to provide an exploratory characterization of PFOA-induced alterations in osteoblast differentiation and ECS-related signaling, further validation experiments, using pharmacological modulators of the endocannabinoid system, were not performed. Direct ROS measurements and targeted modulation of antioxidant enzymes or cannabinoid receptors will be required in future studies to clarify these mechanisms.

6. Conclusion

Our data suggests that PFOA exposure perturbs osteoblast differentiation through selective oxidative stress and endocannabinoid signaling. Catalase modulation emerges as a reproducible and potentially protective response that correlates with RUNX2 regulation, supporting the concept that redox homeostasis is a pivotal mediator of osteoblast fate under environmental stress. On the other hand, the modulation of ECS receptors (CB1 and CB2) by PFOA could cause an aberrant mineralization. Considering that humans are typically exposed to mixtures of PFAS rather than single compounds, future research should also explore the effects of combined exposures and potential additive or synergistic effects among different PFAS. Integrating in vitro mechanistic studies with *in vivo* models and epidemiological data will be essential to better understand the contribution of environmental PFAS exposure to bone health and osteoporosis risk.

CRediT authorship contribution statement

Fiorenza Sella: Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **Christian Giommi:** Writing – review & editing, Methodology, Investigation, Formal analysis, Data curation. **Damiano Carbonari:** Resources. **Marta Lombó:** Writing – review & editing. **Oliana Carnevali:** Writing – review & editing, Resources, Project administration, Data curation, Conceptualization.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ygcen.2026.114932.

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