

High Fat Diet (HFD)-Induced Obesity Augments the Deleterious Effects of Estrogen Deficiency on Bone: Evidence from ovariectomized Mice.

Journal:	<i>Aging Cell</i>
Manuscript ID	ACE-22-0053.R3
Wiley - Manuscript type:	Research Article
Date Submitted by the Author:	n/a
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Keywords:	Aging, senescence, accelerated aging, obesity, Bone marrow adiposity, Menopause, Osteoporosis, Bone fragility



High Fat Diet (HFD)-Induced Obesity Augments the Deleterious Effects of Estrogen Deficiency on Bone: Evidence from ovariectomized Mice.

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Abstract

Several epidemiological studies have suggested that obesity complicated with insulin resistance and type 2 diabetes, exerts deleterious effects on the skeleton. While obesity coexists with estrogen deficiency in postmenopausal women, their combined effects on the skeleton are poorly studied. Thus, we investigated the impact of high-fat diet (HFD) on bone and metabolism of ovariectomized (OVX) female mice (C57BL/6J). OVX or sham operated mice were fed either HFD (60%fat) or normal diet (ND) (10%fat) for 12 weeks. HFD-OVX group exhibited pronounced increase in body weight ($\sim 86\%$ in HFD and $\sim 122\%$ in HFD-OVX, $p < 0.0005$) and impaired glucose tolerance. Bone microCT-scanning revealed a pronounced decrease in trabecular bone volume/total volume (BV/TV) ($-15.6 \pm 0.48\%$ in HFD and $-37.5 \pm 0.235\%$ in HFD-OVX, $p < 0.005$) and expansion of bone marrow adipose tissue (BMAT) ($+60.7 \pm 9.9\%$ in HFD vs $+79.5 \pm 5.86\%$ in HFD-OVX, $p < 0.005$). Mechanistically, HFD-OVX treatment led to upregulation of genes markers of senescence, bone resorption, adipogenesis, inflammation, downregulation of gene markers of bone formation and bone development. Similarly, HFD- OVX treatment resulted in significant changes in bone tissue levels of Purine/Pyrimidine and Glutamate metabolisms, known to play a regulatory role in bone metabolism. Obesity and estrogen deficiency exert combined deleterious effects on bone resulting in accelerated cellular senescence, expansion of BMAT and impaired bone formation leading to decreased bone mass. Our results suggest that obesity may increase bone fragility in post-menopausal women.

Keywords: Menopause, Obesity, Osteoporosis, Bone Marrow Adiposity, Aging, Senescence, Bone Fragility, Accelerated Aging

Highlights

- Obesity combined with estrogen deficiency led to shift in differentiation of BMSCs toward adipogenesis and expansion of bone marrow adiposity.
- Obesity combined with estrogen deficiency accelerated accumulation of pro inflammatory markers, senescence associated phenotype markers and senescent cells.
- Obesity combined with estrogen deficiency downregulated anabolic bone formation pathways which led to osteoporotic bone phenotype and increased fracture risk.

For Peer Review

1. Introduction

Post-menopausal estrogen deficiency is a major risk factor for bone fragility and osteoporotic fractures ([Kassem and Marie 2011](#)). Additional risk factors for osteoporosis and fragility fractures include age, parental osteoporotic fractures, medications e.g. glucocorticoids ([Buckley et al. 2017](#)) and more recently recognized obesity and its metabolic complication of type 2 diabetes ([Kozakowski et al. 2017](#)). This seems paradoxical since obesity is usually associated with increased bone mass ([Reid 2006](#)). However, obesity leads to several complications including impaired macronutrient metabolism ([Singla, Bardoloi, and Parkash 2010](#)), cardiovascular diseases ([Van Gaal, Mertens, and De Block 2006](#)) and muscle atrophy that predispose to bone fragility fractures ([Proietto 2020](#)). The relative contribution of these factors cannot be resolved using human epidemiological studies and can be studied using experimental animal models.

We have previously demonstrated that diet-induced obesity in male mice resulted in decreased bone mass associated with accelerated senescence and impaired differentiation of skeletal stem cells (also known as marrow stromal cells, BMSCs) which we suggested as potential mechanism for obesity induced bone fragility ([Tencerova et al. 2018](#)). However, the effects of obesity on female animal skeleton are poorly studied. Only one previous study in mice, has reported that obesity was not protective against OVX-mediated bone loss, but the study was descriptive and did not provide a plausible mechanism ([Cao and Gregoire 2016](#)). In humans, post-menopausal state is associated with increased prevalence of obesity due to changes in energy metabolism (reviewed in ([Ko and Kim 2020](#))) and obesity complications of increased risk for type 2 diabetes and cardiovascular complications ([Tandon et al. 2010](#)). Human studies of the effect of obesity in the postmenopausal women on risk of fractures yielded variable results. Some studies reported that obese postmenopausal women have lower risk for osteoporotic fractures ([Migliaccio et al. 2011](#); [Heidari et al. 2015](#); [Andreoli et al. 2011](#); [Yanik et al. 2009](#); [Silva et al. 2007](#)), while other studies reported increased fracture risk ([Compston et al. 2011](#); [Greco et al. 2010](#); [Rikkonen et al. 2021](#)) reflecting the heterogeneity of the populations studied and the limitation of using epidemiological methods to correct for confounding factors.

There is an increasing interest in studying regulatory role of bone marrow adipose tissue (BMAT) on bone remodeling. and age-related expansion of BMAT has been proposed as a causative factor leading to age-related bone loss ([Nehlin et al. 2019](#)). Estrogen deficiency in postmenopausal period has been associated with increased BMAT ([Syed et al. 2010](#)). We and others have shown that diet-induced obesity in mice leads to expansion of BMAT in male mice ([Tencerova et al. 2018](#); [Scheller et al. 2016](#)). However, the combined effects of obesity and estrogen deficiency in female mice and humans are not known. The cellular mechanisms underlying BMAT expansion are thought to be caused by changes in the BMSCs niche leading to a shift in differentiation fate of BMSCs from osteoblasts (OB) to adipocytes (AD) ([Veldhuis-Vlug and Rosen 2018](#); [Tencerova and Kassem 2016](#)).

Cellular senescence is considered one of the fundamental mechanisms underlying aging ([Khosla et al. 2020](#)) and recently has been demonstrated to mediate age-related bone loss ([Farr et al. 2017](#)). Cellular senescence indicate that the cell has lost its proliferative potential leading to irreversible growth arrest and is the result of accumulation of molecular cellular damage ([Kumari and Jat 2021](#)). Despite cell cycle arrest, senescent cells remain viable, and they are capable for producing a unique secretome, termed the senescence-associated secretory phenotype (SASP), which is comprised of proinflammatory cytokines and chemokines, as well as extracellular matrix degrading enzymes that have deleterious effects on tissue functions ([Coppe et al. 2010](#)). While one previous study showed that estrogen deficiency induced by ovariectomy (OVX) leads to increased senescent cell burden ([Wu et al. 2020](#)), Farr et al. were not able to corroborate this finding. ([Farr et al. 2019](#)). On the other hand, we have previously demonstrated that HFD-induced obesity in male leads to increased burden of senescence cells in bone ([Figeac et al. 2022](#)) and in cultured cells ([Tencerova et al. 2019](#)) that may contribute to impaired fracture healing and bone fragility observed in obesity. The combined effects of obesity and estrogen deficiency on bone cellular senescence are not known.

In order to study the relative contribution of obesity and estrogen deficiency to changes in the skeleton, we employed an OVX-mouse model with HFD-induced obesity as a model for postmenopausal

women. Employing cellular, transcriptomic and metabolic investigations, our results demonstrated that obesity interacts with estrogen deficiency leading to expansion of BMAT and accelerated cellular senescence in the bone and bone marrow microenvironment, resulting in impaired bone formation and decreased bone mass.

2. Results

2.1 HFD-OVX reduces bone mass

Tibiae from animals belonging to the 4 experimental groups, ND-SHAM, ND-OVX, HFD-SHAM and HFD-OVX (**Fig. 1A**), were scanned by μ CT and changes in bone mass were determined at 8 weeks post-OVX and compared to ND-SHAM (**Fig. 1B-E**). The HFD-OVX group exhibited the most pronounced trabecular bone loss (-62.44% as compared to ND-SHAM, while -55.4% for ND-OVX and -32.15% for HFD-SHAM) while the cortical bone mass was unchanged (**Fig. 1D-E**). The observed trabecular bone phenotype at 8 weeks post-OVX was maintained at 12 weeks moreover at this time point we observed decreased cortical bone mass in the HFD-OVX group (**Supplementary Fig. 1A-D**). Overall, we observed that the combination of obesity and estrogen deficiency at 12 weeks post-OVX has a more detrimental effect on bone loss both cortical and trabecular (**Fig. 1B-E and Supplementary Figure 1A-D**). TRAcP evaluation of Osteoclast surface per bone surface (OcS/BS) and Osteoclasts number per bone perimeter (OcN/BPm) did not show significant difference between groups (**Supplementary Figure 1E**). Finally biochemical bone turnover markers showed a high bone turnover state following OVX ([Yoon et al. 2012](#)), a low bone turnover following HFD ([Tencerova et al. 2018](#)) and in HFD-OVX group an intermediary bone turnover (**Fig. 1F**).

2.2 HFD-OVX leads to obesity and impaired glucose tolerance

During 12 weeks of observation, changes in body weight were dependent on the experimental condition with ~37.5% in ND-SHAM group, ~45% in ND-OVX group, ~88% in the HFD-SHAM group, and ~124.5% in the HFD-OVX group (**Fig. 2A**). Body fat mass percentage as evaluated by DEXA scanning exhibited significant difference between the experimental groups: 20.5% in ND-SHAM, 27.7% in ND-OVX, 43.4% in HFD-SHAM and 51.33% in HFD-OVX (**Fig. 2B, left panel**) while body lean mass was unchanged between groups (**Fig. 2B, right panel**). Fasting blood glucose (**Fig. 2C**) was increased in both HFD fed groups as compared to ND fed animals and changes in GTT calculated as AOC, revealed a progressive impairment in glucose tolerance from ND-OVX, HFD to HFD-OVX ($p < 0.005$) (**Fig. 2D-E**). To determine the mechanism, we quantified pancreatic β -cell mass that was significantly increased by ~28.86% in ND-OVX, ~54.36% in HFD-SHAM while decreased in the HFD-OVX compared to HFD-SHAM group up to the ND-SHAM level revealing a β -cell failure in this group to potentially compensate for a peripheral insulin resistance explaining why the glucose metabolism of this HFD-OVX group, as visualized by GTT, is the worst (**Fig. 2F**). Moreover, calculation of HOMA-IR (for insulin resistance) and HOMA- β (for pancreatic β -cell function) parameters showed a trend to increased HOMA-IR value in HFD-SHAM group that was accentuated in the HFD-OVX group (**Fig. 2G**) combined with an trend to impaired insulin secretion as evaluated by the HOMA β ratio, which was more pronounced in the HFD-OVX group as compared to the ND-SHAM group (**Fig. 2H**). Interestingly in our study, we did observe that the ND-OVX group glucose tolerance through a combination of increased β -cell area and function (**Fig. 2F-H**).

2.3 HFD-OVX leads to BMAT expansion, inflammation, and insulin resistance in bone

Both estrogen deficiency and obesity dependent bone loss have been associated with increase in marrow adiposity. Quantifying BMAT content using μ CT scanning (**Fig. 3A**) revealed an increase by $\approx 289\%$ in ND-OVX, $\approx 732\%$ in HFD-SHAM and $\approx 1352\%$ in HFD-OVX. Obesity induced insulin resistance in adipose tissue is known to affect adipocyte function in part through low-grade chronic inflammation (Reilly

and Saltiel 2017; Zatterale et al. 2019). We quantified mRNA levels of genes related to inflammation (*Tnf α* & *Il1 β*), related to insulin signaling (*Irs1*, *Irs2* & *Insr*) and related to adipogenesis (*Lep*, *Cebpa*, *Ppar γ 2*) in subcutaneous (SAT) and visceral adipose tissue (VAT) (**Fig. 3B**). Gene expression profile in VAT revealed a decrease expression of insulin receptor gene but not IRS1 and 2. However, we observed decreased gene expression of adipogenesis-related genes and increased gene expression of inflammation-related genes, in HFD-SHAM, ND-OVX & HFD-OVX groups (**Fig. 3B**). On contrary, genes related to insulin signaling showed increased expression in SAT (**Fig. 3B**). In line with previously reported data from our group (Tencerova et al. 2018), insulin signaling was maintained if not improved in isolated and cultured bone derived BMSCs from HFD-SHAM mice (**Fig. 3C**) as evidenced by the phosphoprotein levels of AKT presented as the ratio pAKT-S473/total AKT. Insulin sensitivity was lost in BMSCs isolated from HFD-ovariectomized mice (**Fig. 3C**).

2.4 HFD-OVX is associated with downregulated genetic pathways of bone formation in BMSCs

To identify molecular mechanisms mediating bone loss in HFD-OVX, we evaluated mRNA levels of selected osteoblastic, osteoclastic and adipocytic gene markers using mRNA isolated from whole bone (**Supplementary Fig. 2A-B**), or whole bone marrow (**Supplementary Fig. 2C-D**). HFD-OVX was associated with increase in adipocytic genes: *Adipoq*, *Cebpa*, *Lpl*, *Fabp4*, *Ppar γ 2* and in *Alpl* and *Tnfsf11* (encoding RANKL) mRNA levels (**Supplementary Fig. 2A-D**), which was less prominent in the HFD-SHAM and ND-OVX groups. Those increased transcript levels of osteoblast and osteoclast activity partly match the elevated bone turnover (**Fig. 1F**), however, the ND-OVX with the strongest increase in bone turnover showed the lowest induction levels of *Alpl* and *Tnfsf11*. We focused on isolated BMSCs to test whether the altered microenvironment in the bone of ND-OVX, HFD-SHAM or HFD-OVX mice induces epigenetic changes that maintain an altered expression profile linked to the deteriorated bone phenotype (**Supplementary Fig 3A-C**). Comparative overlaps shows that the three treatment conditions do not lead

to changes in opposing directions, but rather have similar changes or group specific regulation patterns (**Fig. 4A-B**). Clustering of the differentially expressed genes revealed patterns that are specific to HFD, OVX, as well as the combined treatment (**Supplementary Fig 4A-B**) and that differ in their enrichment for signaling pathways and gene ontology terms (**Supplementary Fig 4C-D**). GO pathway analysis revealed that downregulated genes from HFD-OVX BMSCs are associated with bone development, bone morphogenesis, ossification, osteoblast differentiation, and skeletal system morphogenesis (**Supplementary Fig 3C**) which is less prominent in ND-OVX and HFD-SHAM conditions (**Fig. 4C**). Comparing this group of genes across all experimental conditions, we observed that the strong downregulation in HFD-OVX is partly recapitulated by the single treatments but often due to an additive effect of both (**Fig. 4D**).

2.5 HFD-OVX leads to accelerated senescence in the bone and bone marrow microenvironment.

We have previously reported that in male mice and humans, obesity is associated with an accelerated senescence phenotype in cultured BMSCs ([Figeac et al. 2022](#); [Tencerova et al. 2019](#)). Thus, we investigated gene expression profile of senescence associated genes in bone and bone marrow samples. HFD-OVX exhibited higher expression levels of senescence associated genes (p16, p53 and p21) (**Fig. 5A&C**) and the senescence associated secretory phenotype (SASP) marker genes (**Fig. 5B&D**). In addition, RNA-seq of cultured BMSCs revealed enrichment for target genes of MAPK and JAK-STAT signaling in HFD-OVX (**Fig. 5E**), known to be associated with cellular senescence ([Anerillas, Abdelmohsen, and Gorospe 2020](#); [Ji et al. 2017](#); [Xu, Tchkonina, and Kirkland 2016](#); [Farr et al. 2017](#)) as well as a general increase of genes from the GO term “senescence pathway” (**Fig. 5F**). Finally, immunostaining for Lamin B1 (LMNB1) (a negative marker of cellular senescence) ([Baar et al. 2017](#); [Figeac et al. 2022](#)) performed on sections from proximal tibia showed increased number of senescent cells in HFD-SHAM and a more pronounced effect in the HFD-OVX (**Fig. 5G-H**). Interestingly, OVX alone did not result in increased number of senescent cells suggesting that obesity is the main driving force for the accelerated senescence phenotype.

2.6 Impact of HFD and estrogen deficiency on metabolic profiling of femur

Changes in intermediary metabolism provides a link between hormonal changes in microenvironment and cellular phenotype (Ferreira et al. 2020; Muller and Seitz 1984). We performed a global metabolomic analysis of crushed femurs obtained from ND-SHAM, ND-OVX, HFD-SHAM and HFD-OVX using liquid chromatography-mass spectrometry (LC-MS) (Fig. 6). While principal component analysis (PCA) was capable to detect variation based on diet but not surgery (Supplementary Fig. 5A), partial least square discriminant analysis (PLS-DA) (Fig. 6A) showed a clear separation of the treatment groups. The internal validity of the model was evaluated by estimating R^2 and Q^2 parameters that were both above the threshold for a significant biological model (Psihogios et al. 2008) (Supplementary Fig. 5B). From PLS-DA analysis, we also observed that 8 out of 15 top metabolites based on VIP scores that exhibited significant changes within experimental groups, followed gradual changes from ND-SHAM over ND-OVX and HFD-SHAM to HFD-OVX (Fig. 6B). ANOVA analysis of our dataset provided a list of 12 metabolites with significantly different concentration between the experimental groups (Supplementary Table 1) and revealed that purine/pyrimidine as well as carbohydrate and lipid metabolisms explain the observed differences among the experimental groups. The heatmap based on the comparison of the levels of metabolites in all the experimental groups, confirmed that the HFD was the driving force for the observed differences in metabolite concentration and metabolic pathways, among the experimental groups (Fig. 6C). Among those metabolites, many were linked to Purine/Pyrimidine as well as glutamate metabolism (Fig. 6D).

3. Discussion.

In this study we investigated the combined effects of HFD-induced obesity and estrogen deficiency on bone and BMAT in female mice as a model for obese postmenopausal women. We also determined the metabolic

and molecular mechanisms underlying the observed changes and we demonstrated that obesity and estrogen deficiency heighten the negative effects of obesity or estrogen deficiency alone, on bone and bone marrow microenvironment and led to severe deleterious effects on the skeleton.

As expected, twelve weeks of HFD feeding led to obesity along with impairment of glucose metabolism in female mice and these changes were aggravated when combined with estrogen deficiency. Our findings in female mice are similar to previous studies in male mice that showed negative impact of HFD on glucose tolerance ([Tencerova et al. 2018](#); [Winzell and Ahrén 2004](#); [Gallou-Kabani et al. 2007](#)) and similar to the one study that combined HFD with OVX ([Gorres-Martens et al. 2018](#)). Interestingly, we observed that OVX mice maintained glucose tolerance by increasing β cell area and function. Similar findings were reported in a recent study on ovariectomized rats ([Chen et al. 2021](#)). Furthermore, the impaired glucose tolerance observed in HFD-SHAM was partially caused by diminished capacity of the β cell to secrete insulin even though β cell area was increased as evidenced by HOMA- β . This mechanism was more pronounced in the HFD-OVX where a combination of decreased β -cell area and β -cell function were observed in addition to the presence of insulin resistance suggesting that the compensatory mechanisms of enhanced β cell functions by OVX are compromised by HFD. Direct measurements of insulin levels and performing glucose-simulate-insulin-secretion test (GSIS) needs to be conducted to support these observations.

Like previous studies performed in male mice, the observed decreased bone mass caused by HFD alone in female mice was similar in direction but quantitatively milder ([Tencerova et al. 2018](#)). Our results are also similar to the findings of deteriorated bone microstructure, and increased bone fragility observed in male mice ([Fujita, Watanabe, and Maki 2012](#); [Devlin et al. 2018](#); [Shu et al. 2015](#); [Cao, Gregoire, and Gao 2009](#); [Patsch et al. 2011](#); [Scheller et al. 2016](#); [Gautam et al. 2014](#); [Kyung et al. 2009](#); [Tencerova et al. 2018](#)). We observed that combined HFD and OVX exerted a significant decreased both trabecular and cortical bone mass, compared to OVX or HFD alone which is similar to two studies where OVX performed

at age of 6-12 weeks and the mice received 10-18 weeks of HFD ([Cao and Gregoire 2016](#); [Núñez et al. 2007](#); [Ludgero-Correia et al. 2012](#)).

OVX-associated bone loss is a very well-studied phenomenon, and it is caused by multiple mechanisms but most importantly upregulation of pro-inflammatory cytokines including RANKL within the bone microenvironment leading to enhanced osteoclastogenesis ([Georgiou, Hui, and Xian 2012](#); [Khosla and Pacifici 2013](#)). We observed significant effects of estrogen deficiency on senescent cell formation only in combination with obesity. This applied to the number of senescent cells formed and the expression of SASP genes. Our findings corroborate previous results reported by Farr et al. that did not detect an independent contribution of estrogen deficiency on senescent cell formation in mice ([Farr et al. 2019](#)). However, the observed interaction between obesity and estrogen deficiency suggests that both conditions target senescence-associated genetic pathways. Cellular senescence is known to enhance osteoclastogenesis and impair osteoblastogenesis leading to bone loss ([Farr et al. 2016](#); [Farr et al. 2017](#)). We did not measure histologically the cellular activities of osteoclastic and osteoblastic cells. Alternatively, our genetic studies support this notion as they revealed an up-regulation of MAPK and JAK-STAT target genes which are two signaling pathways involved in the regulation of cellular senescence and bone cell functions. MAPK signaling activates expression of pro-inflammatory molecules and senescence associated factors in the p21/p53 and p16/RB pathways ([Anerillas, Abdelmohsen, and Gorospe 2020](#)) while JAK-STAT signaling regulate SASP production since many of the SASP genes contain JAK-STAT responsive elements ([Ji et al. 2017](#); [Xu, Tchkonian, and Kirkland 2016](#); [Farr et al. 2017](#)).

We observed increased BMAT mass in HFD obese mice which was more pronounced when combined with estrogen deficiency. Increased in BMAT in HFD mice has been reported previously in male mice ([Scheller et al. 2016](#); [Tencerova et al. 2018](#)) while estrogen deficiency has been reported to increase BMAT in mice ([Elbaz, Rivas, and Duque 2009](#); [Georgiou, Hui, and Xian 2012](#)) and humans ([Veldhuis-Vlug and Rosen 2018](#)). In our study, the increased BMAT was associated with decreased bone mass. However, Almeida et al. ([Almeida et al. 2020](#)) reported that increased BMAT was not associated with

appendicular bone loss in female mice. Other studies reported that increased BMAT in obese mice is associated with decreased bone mass ([Ambrosi et al. 2017](#)) ([Ambrosi et al. 2017](#)). It is plausible that the observed association between expansion of BMAT and decreased bone mass in HFD-OVX mice may be casual for the following reasons. First, expansion of BMAT is usually the result of changes in lineage allocation and a shift in the differentiation fate of BMSCs from osteoblasts (OB) to adipocytes (AD) which results in recruitment of fewer osteoblastic cells and impaired bone formation ([Nehlin et al. 2019](#)). Second, there exists a crosstalk between BMAT and extra-medullary adipose tissue depots that leads to bone loss. For example, a recent clinical study reported a positive relationship between BMAT and visceral fat and an inverse relation with bone formation and bone mass in post-menopausal women ([Bredella et al. 2011](#)). In our study, visceral obesity was associated with up-regulated expression of proinflammatory cytokines e.g. TNF α and IL1 β which leads to a state of chronic sterile inflammation and high expression of RANKL enhancing the formation and functions of osteoclastic cells and bone resorption ([Khosla 2001](#)). In one previous study, HFD-induced obesity has been reported to enhance ovariectomy-associated inflammatory response ([Ludgero-Correia et al. 2012](#)) which corroborates this hypothesis.

Is there a link between BMAT expansion and the creation of senescent micro-environment observed in combined obesity and estrogen deficiency? The observed increase in BMAT may be caused, by enhanced responsiveness of BMSCs to insulin leading to increased formation of adipocytes as shown in our studies ([Tencerova et al. 2018](#); [Tencerova et al. 2019](#)). In our previous study in male mice ([Tencerova et al. 2018](#)) and in the current study, obesity-associated enhanced insulin signaling in BMSCs created a state of cellular hypermetabolism with increased mitochondrial oxidative phosphorylation (OXPHOS), and levels of reactive oxygen species (ROS) that can lead to DNA damage and cellular senescence ([Tencerova et al. 2019](#)). Interestingly, we observed that OVX alone impairs insulin signaling and this effect persisted in combined OVX-HFD suggesting that OVX-associated enhancement of cellular senescence is related to an insulin-independent mechanism e.g. enhanced inflammatory responses ([Ren et al. 2009](#); [Freund et al. 2010](#)) or direct effects on senescence-associated genes ([Farr and Khosla 2019](#); [Li et al. 2020](#)).

Studying the metabolomic changes taking place in bone of HFD-OVX revealed additional mechanism that may explain the negative combined effects of obesity and estrogen deficiency on bone mass. However, it is to note that based on molecular biology and metabolomic analyses, obesity (HFD) seems to be the driving force of the observed cellular inflammation and senescence. The metabolomic profile present in bones of HFD-OVX showed significant changes in the metabolites belonging to purine and pyrimidine, carbohydrate and lipids and glutamate pathways. Particularly, the decrease in metabolites from purine and pyrimidine pathways, may explain the observed increased in cellular inflammation, as it has been reported that pyrimidine and its derivatives exert anti-inflammatory as well as antioxidant effects, and inhibit osteoclastogenesis and bone resorption via RANKL inhibition ([Ihn et al. 2017](#); [Mitsuya and Broder 1986](#); [Xie et al. 2009](#)). Also, we observed a decrease in metabolites from the glutamate pathway, known to be important for BMSCs stemness and bone homeostasis ([Zhou et al. 2019](#)).

Our study has some limitations. We started our HFD intervention at relatively younger age (8 weeks old mice). At this early age, the mice did not reach skeletal maturity which may explain the lower bone mass phenotype observed in these mice which is at variance with the observed normal or increased bone mass observed in obese adult humans ([Burghardt et al. 2010](#); [Reid 2006](#); [Starr et al. 2018](#)). Regarding the metabolic studies, our results are confounded by differences in the macronutrient composition and the source of macronutrients between ND and HFD with respect to protein and carbohydrates that may have affected our results and thus those findings are preliminary and should be confirmed in futures studies.

Our findings are relevant to human physiology as the metabolic and hormonal changes observed in HFD and OVX mice parallel known changes in humans ([Szmuilowicz, Stuenkel, and Seely 2009](#); [Zhu et al. 2013](#)) and provide insight into the mechanisms underlying the interaction between obesity and estrogen deficiency, an area of research that has been poorly studied. While our study results need to be validated in human females, it suggests a more aggressive intervention strategy in postmenopausal obese women to correct hormonal and metabolic changes in order to maintain and improve bone health.

4. Experimental procedures

4.1 Animal Model

Female C57BL/6J mice (Taconic, Rensselaer, NY, USA) at 8 weeks of age were assigned for either ovariectomy (OVX) or sham operation and afterwards fed a R-70 normal-diet (ND) (Lantmännen, Sweden) (containing Kcal%: protein 14.5%, carbohydrates 60%, and fat 4.5%) in which fat content was from Oatmeal, barley, wheat bran, wheat flour (diet details shown in Supplementary table 2), or high-fat diet (HFD) (Research Diet [New Brunswick, NJ, USA] D12492, containing Kcal%: protein 20%, carbohydrates 20%, and fat 60%) in which fat content was from soybean oil and lard (diet details shown in Supplementary table 3). Mice were anesthetized before ovariectomy by the dorsal approach using intraperitoneal injection of ketamine (100mg/kg) and xylazine (10mg/kg). Animals were fed ad libitum and housed (4 animals per cage) under standard conditions (21°C, 55% relative humidity) on a 12-hour light/dark cycle for a period of 12 weeks. All experimental procedures were approved by the Danish Animal Ethical committee (2017-15-0201-01210).

4.2 Glucose Tolerance Test (GTT)

Glucose tolerance test (GTT) was performed after 12 weeks of diet intervention. For GTT, overnight-fasted mice were injected with D-glucose 1 g/kg i.p. and blood glucose levels were measured from the tail tip at different timepoints using a CONTOUR® Glucometer (Tencerova et al. 2018).

4.3 Bone mass measurement via DEXA-scanning

Total body fat and lean mass percentage were evaluated using dual-energy X-ray absorptiometry (DEXA) by using a PIXImus2® (version 1.44; Lunar Cooperation, USA). DEXA scans were acquired after sedating mice with isoflurane at the end of the experiment.

4.4 Insulin ELISA

Mouse Insulin ELISA (Abcam: ab277390) kit was used to measure insulin level in serum samples from fasted animals at the end of the experiment.

4.5 Histology & Immunohistochemistry

Tibias and pancreases were fixed with 4% formalin for two days and tibias were demineralized in PBS-EDTA 12% for 14 days. Tissues were embedded in paraffin and sections were used for hematoxylin & eosin staining or immunostaining. In brief, paraffin tissue sections were deparaffinized, dehydrated and heat treated before incubation with primary antibody against Insulin (DAKO A0564) and Lamin B1 (abcam ab16048) followed by secondary antibody, chromogen visualization and counterstaining with hematoxylin. Insulin immunostaining was used to evaluate β -cell area per tissue area in at least 4 non-consecutive pancreas sections, and senescent cells in bone were counted as Lamin B1 negative cells in bone sections ([Baar et al. 2017](#); [Figeac et al. 2022](#)) (a minimum of 1500 cells were evaluated for each condition).

4.6 Micro-computed tomography scanning (μ CT)

Bone Parameters:

Proximal tibias of mice fed for 8 weeks and 12 weeks with HFD or with normal diet (ND) as control were scanned under isoflurane anesthesia (1.5-4.0%) with a high-resolution μ CT system (vivaCT40; Scanco Medical, Brüttisellen, Switzerland), according to the current guidelines ([Bouxsein et al. 2010](#)). Images with an isotropic voxel size of 10.5 μ m were acquired from 1000 projections done with an X-ray tube voltage of 70 kVp, a current of 114 μ A, and an integration time of 300 ms. Proximal tibiae were analyzed using a 700- μ m-high volume of interest (VOI) including trabecular bone beginning immediately below the most distal

part of the growth plate and a 500 μ m-high VOI for evaluation of the cortical parameters starting 1150 μ m under the growth plate.

BMAT evaluation:

For assessment of the BMAT in the tibiae, we used contrast-enhanced μ -CT (CE-CT). For this, tibiae were fixed in formalin for 24 hours, were transferred to phosphate buffered saline (PBS) and were thereafter transferred to a polyoxometalate (POM)-based contrast solution (35mg of Hafnium-Wells Dawson POM per 1 ml PBS). Prior to the POM-staining, the distal ends of the bones were removed to allow better diffusion of the contrast solution into the bone marrow compartment. Samples were incubated in the contrast solution, while shaking gently, for 48 hours prior to CE-CT imaging. We used a Phoenix NanoTom M (GE Measurement and Control solutions, Germany) at a voltage of 60 kV and a current of 140 μ A, and a 0.2 mm aluminum filter was used. The exposure time was 500 ms, and 1200 images were acquired over 360° (frame averaging = 3; image skip = 1). We scanned at a 2 μ m isotropic voxel size. The BMAT volume was assessed in the proximal metaphysis starting directly underneath growth plate and covering a height of 1.2mm distal to the growth plate using CTAn (Bruker MicroCT, Belgium), as previously described (Kerckhofs et al. 2018).

4.7 Biochemical markers of bone turnover assays

Mouse CTX-1 EIA for the quantitative determination of the C-telopeptide of type I collagen for bone resorption and Mouse P1NP EIA for the determination of the N-terminal propeptide of type I procollagen (P1NP) for bone formation (Immunodiagnostic Systems Nordic, Herlev, Denmark) were measured in serum samples from fasted animals at the end of the experiment. Two types of data were reported, bone turnover markers solely and a ratio: bone turnover markers over Bone Surface (mm^2) as determined by μ CT to normalize according to the drastic bone loss happening in some of the experimental groups.

4.8 RNA extraction and real time qPCR

RNA was extracted from cultured cells, flushed bone marrow or homogenized bone using Trizol combined with the Qiagen Rneasy Mini Kit (Qiagen) and reverse-transcribed using a Revert Aid H Minus First Strand cDNA Synthesis Kit (Thermo Scientific). Quantitative real-time PCR was performed with an Applied Biosystems 7500 Real-Time PCR System using Fast SYBR Green Master Mix (Applied Biosystems) with specific primers (Supplemental Table 1). 36B4 was used as a housekeeping gene for normalization of gene expression.

4.9 *In vitro* culture of mouse bone marrow skeletal stem cells (BMSCs)

Cells were isolated as previously reported ([Tencerova et al. 2018](#)). Briefly front and hind limbs were collected after 12 weeks of diet intervention, crushed and digested with 5% collagenase (StemCell). After filtration and washing steps with PBS/2%FBS, BMSCs were obtained as negative fraction from triple staining with antibodies against CD45, CD31 and Ter119 (Miltenyi Biotec) and passing through the magnetic columns. BMSCs were cultivated and expanded using standard tissue culture conditions and alpha-MEM supplemented with 20% FBS, 1% Penicilin-Streptomycin 1% Glutamax, non-essential amino acids (NEAA) and pyruvate sodium (Gibco). At passage 3 cells were used for the subsequent molecular and cellular analyses.

4.10 Western blot analysis

BMSCs cells were seeded and at 80-90% confluence and were starved in serum reduced medium (alpha-MEM with 0.5% BSA) for 4h prior to treatment with 100nM Insulin for 15minutes. Protein lysates were prepared using protein lysis buffer including protease inhibitors (Sigma-Aldrich). Protein concentration was measured using BCA assay (Thermo Scientific). Protein extracts (15 µg of protein) were examined by protein immunoblot analysis probing with antibodies against total AKT (#9272, Cell Signaling), pSer473AKT (#4051, Cell Signaling), β -Actin

(A2066, Sigma-Aldrich). Immunocomplexes were detected by enhanced chemiluminescence and analyzed by Image Lab software (BioRad).

4.11 Analyses of global gene expression by RNA-seq

RNA-seq was performed according to manufacturer's instructions (TruSeq 2, Illumina) using 1 µg RNA for preparation of cDNA libraries. Sequencing reads were mapped to the mouse genome (mm10) using STAR (Dobin et al. 2013) and tag counts were summarized at the gene level using HOMER (Heinz et al. 2010). TiCoNE (Wiwie et al. 2019) was used to cluster differentially expressed genes as determined by DESeq2 (Love, Huber, and Anders 2014), using a cut off of FDR < 0.05. Gene ontology analysis was performed using GSeq (Young et al. 2010) with all detected genes as background and pathway enrichment analysis was done using a hypergeometric test with gene sets from the SPEED pathway (Parikh et al. 2010).

4.12 Liquid chromatography-high resolution Mass Spectrometry (LC-MS) based Metabolomics

Metabolite extraction

Femur were collected, cleaned of soft tissue then flash frozen in liquid nitrogen. Each femur was crumbed and grounded into powder in liquid nitrogen. Metabolites were extracted in cold (−20°) with extraction solvent (50% methanol, 30% acetonitrile and 20% water), 10 µL per mg tissue. Samples were then homogenized by sonication in a Biorupter sonicator (10 cycles, 30 s, high power) and mixed in a thermomixer at 4 °C before a 30 min incubation at −20 °C. Cell debris and proteins were removed from the samples by 15 min centrifugation at 16.000g at 4 °C. At last supernatant were lyophilized and resuspended in 30 µL 1% formic acid of which 8 µL were transfers to a pooled (quality control) sample.

Liquid chromatography-high resolution Mass Spectrometry

The samples were overall analysed as in Dall *et al.* (Dall *et al.* 2021). In brief, 5 μ L was injected on a Vanquish Horizon UPLC (Thermo Fisher Scientific) where a flow of 400 μ L/min was used with the following composition of eluent A (0.1% formic acid) and eluent B (0.1% formic acid, acetonitrile): 3% B from 0 to 1.5 min, 3% to 40% B from 1.5 to 3 min, 40% to 95% B from 3 to 5 min, 95% B from 5 to 7.6 min and 95% to 3% B from 7.6 to 8 min before equilibration for 3.5 min with the initial conditions. The flow from the LC coupled to a Q Exactive HF mass spectrometer (Thermo Fisher Scientific) for mass spectrometric analysis in both positive and negative ion modes.

Data processing

The raw data were also processed as in Dall *et al.* (Dall *et al.* 2021) using MZmine (v 2.53) (Pluskal *et al.* 2010). Features were annotated by searching against NIST17 Tandem Mass Spectral Library, MoNA – MassBank of North America library and finally SIRUIS (v. 4.7.0 (Duhrkop *et al.* 2019)). Signals were finally corrected for drift in statTarget software (Luan *et al.* 2018).

4.13 Statistical analysis

Data were analyzed with graph pad Prism 8 software. Results are expressed as mean $\pm$ SEM. The differences between experimental groups (ND-SHAM, ND-OVX, HFD-SHAM and HFD-OVX) were determined by 1-way ANOVA followed by Tukey post-hoc test. P value < 0.05 was considered statistically significant.

Acknowledgments

The authors are grateful to the Doctor Meshail Okla for her input on glucose metabolism and insulin resistance and to Lone Christiansen for excellent technical assistance. Some analyses have been performed by OPEN Lab, Odense University Hospital (OUH), financially supported by the Research Council at OUH.

Disclosure of Potential Conflicts of Interest

The authors declare that there is no conflict of interest.

Data Availability Statement

RNA sequencing and processed data have been deposited in the Gene Expression Omnibus (GEO) database, accession code number is GSE194075. Metabolomics raw data are available on Mendeley database at DOI: 10.17632/3jyb26mz62.1. Primary BMSCs (RNAseq) and femurs (Metabolomics) were obtained from female C57BL/6J mice at 5 months of age fed with normal or high fat diet and submitted or not to ovariectomy. All experimental procedures were approved by the Danish Animal Ethical committee (2017-15-0201-01210).

Author's contributions:

DA and FF contributed equally to this work.

DA and FF: involved in design, conception, data generation, data acquisition, data interpretation, and manuscript writing. AC: involved in bioinformatic data analysis and interpretation. ND: involved in animal experiments. CS, GK, JH, NF: involved in data acquisition. AR: involved in bioinformatic data analysis and interpretation and manuscript revision. MT: involved in design, conception, and manuscript revision. MK involved in conception, supervision of the work, data interpretation, manuscript writing and revision.

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Figure legends

Figure 1: Effect of HFD-induced obesity on bone parameters. (A) Study design. Bone parameters as evaluated by μ CT, 8 weeks after the surgery, (B) Representative images for μ CT 3D reconstruction of

trabecular compartment (C) trabecular bone parameters were evaluated as bone volume per total volume (BV/TV), trabecular number (Tb.N), trabecular thickness (Tb.Th), and trabecular separation (Tb.Sep). (D) Representative images for μ CT 3D reconstruction of cortical compartment (E) Cortical diaphysis bone measurements as cortical thickness (Co.Th) and porosity %. (F) Markers for bone turnover, Ratio P1NP/BS (Bone surface- mm^2) ($n = 10$ per group) for bone formation (left panel) and Ratio CTX-1/BS ($n = 9$ per group) for bone resorption (right panel) were measured in serum. Data are presented as mean $\pm$ SEM ($n = 7$ -10 mice/group). One way ANOVA, * $p < 0.05$, ** $p < 0.005$; *** $p < 0.0005$.

Figure 2: Effect of HFD on body composition and glucose metabolism. Evaluation of the body composition at the endpoint of the study (8 mice/group). (A) Body weight (B) Fat mass percentage and Lean mass (absolute value). Glucose metabolism as evaluated by (C) Fasting blood glucose (mg/dL), (D) Glucose tolerance test (GTT) and (E) area of the curve (AOC). (F) Representative photomicrographs of Insulin staining of pancreas of ND-SHAM, ND-OVX, HFD-SHAM & HFD-OVX mice (left panel). Scale bar: 500 μm . Quantified β -cell area expressed as percentage of total area (right panel). (G) HOMA-IR as calculated $[(\text{Fasting Blood Glucose (mg/dL)} * \text{Fasting Insulin (mIU/L)})/405]$. (H) Relative HOMA $\beta = 20 \times [\text{FI (mIU/L)} / (\text{FBG (mmol/L)} - 3.5) (\%)]$. Data are presented as mean $\pm$ SEM ($n = 17$ for A to E and $n = 5$ to 8 for F to H), * $p < 0.05$, ** $p < 0.005$; *** $p < 0.005$; 1-way ANOVA.

Figure 3: Effect of HFD on bone marrow and peripheral fat depot. (A) Left panel, representative images for μ CT-3D reconstruction of proximal tibia bone marrow adiposity from the side of the primary spongiosa, scale bar = 250 μm . Right panel, quantification of bone marrow adiposity in proximal tibia (%). (B) Quantification of mRNA expression levels of Insulin signaling-related genes (*Irs1*, *Irs2*, *Insr*), adipogenic genes (*Adipoq*, *Cebpa*, *Ppar γ 2*) and inflammatory genes (*Tnfa*, *Il1 β*) in SAT (left panel) and VAT (right panel) (ND-SHAM ($n = 6$), ND-OVX ($n = 8$), HFD-SHAM ($n = 8$) and HFD-OVX ($n = 8$)) (C)

Western blot of AKT, pS473-AKT and β -actin and densitometry evaluation of p-S473-AKT vs. AKT in undifferentiated BMSCs with and without 15 minutes of insulin stimulation (100 nM). Data are presented as mean $\pm$ SEM from three independent experiments (** $p < 0.0005$).

Figure 4: Effect of HFD-induced obesity and estrogen deficiency on osteogenic molecular signature in BMSCs. (A) Heat map showing a Jaccard index based on the overlap of up-and downregulated genes ($p < 0.05$) in BMSCs from ND-OVX, HFD-SHAM and HFD-OVX mice compared to control mice ND-SHAM. (B) Venn diagram showing the overlap among genes that are up-or downregulated ($p < 0.05$) in BMSCs from ND-OVX, HFD-SHAM and HFD-OVX mice compared to control mice ND-SHAM. (C) Heat map showing the enrichment of gene ontology terms related to bone development, bone morphogenesis, ossification, osteoblast differentiation and skeletal system morphogenesis among the up and down-regulated genes in BMSCs from ND-OVX, HFD-SHAM and HFD-OVX mice compared to control mice ND-SHAM (D) Heat map showing the log fold change of genes from the indicated gene ontology pathways.

Figure 5: HFD-induced obesity up-regulated senescence in bone, bone marrow and BMSCs. Quantification of mRNA expression levels using qPCR ($n = 10$) of (A & C) senescence associated markers and (B & D) senescence associated secretory phenotype markers (SASP) in bone (A & B) and bone marrow (C & D) of ND-SHAM, ND-OVX, HFD-SHAM and HFD-OVX mice at the end of the experiment. (E) Heat map showing enrichment analysis of genes, up and down regulated, in BMSCs of ND-OVX, HFD-SHAM and HFD-OVX mice that are under control of the indicated pathways (SPEED pathway analysis). (F) Box plot showing the log fold change in expression levels of genes linked to senescence in BMSCs from ND-OVX, HFD-SHAM and HFD-OVX mice compared to control mice ND-SHAM. Mann-Whitney test with ** $p < 0.01$. (G) Senescent cells percentage (cells not immunostained for LMNB1). (H)

Immunostaining for Lamin B1 (LMNB1) (a negative marker of cellular senescence) performed on tibial sections. Lack of staining is indicating senescent cells (black arrow). Scale bar: 50 μ m. Data are presented as mean $\pm$ SEM, * $p < 0.05$, ** $p < 0.005$; *** $p < 0.0005$. two-tailed unpaired Student's t test and 1-way ANOVA.

Figure 6: Full bone metabolic profiling. Composition in metabolites of full bones from ND-SHAM, ND-OVX, HFD-SHAM and HFD-OVX groups. Global metabolomic analyses of metabolites from full femurs using liquid chromatography-mass spectrometry (LC-MS). **(A)** PLS-DA score plot of the ND-SHAM, ND-OVX, HFD-SHAM and HFD-OVX groups in metabolomic analysis. **(B)** Variable importance in projection (VIP) from PLS-DA analysis, colored boxes on the right indicate the relative concentrations of the corresponding metabolites in each group under study (black arrows are indicating those with gradual variation following the modelization pattern suggested by the PLS-DA). **(C)** Heat map of the top 25 metabolites that are differentially expressed in the full bones of ND-SHAM, ND-OVX, HFD-SHAM and HFD-OVX mice. **(D)** Metabolic pathway analysis. Matched pathways are displayed as circles and color and size of each circle are based on p value and pathway impact value, respectively. The pathways with the highest statistical significance scores are indicated as follows: (i) Purine metabolism, (ii) Pyrimidine metabolism, (iii) Alanine, Aspartate and Glutamate metabolism, (iv) Arginine biosynthesis, (v) Taurine and Hypotaurine metabolism and (vi) TCA cycle.

Supplementary Figure 1: Effect of HFD-induced obesity on bone parameters. μ CT analysis of proximal tibia after 12 weeks of ND, HFD and surgery. **(A)** Representative images for μ CT 3D reconstruction of trabecular compartment **(B)** trabecular bone parameters were evaluated as bone volume per total volume (BV/TV), trabecular number (Tb.N), trabecular thickness (Tb.Th), and trabecular separation (Tb.Sep). **(C)** Representative images for μ CT 3D reconstruction of cortical compartment **(D)**

Analysis of cortical diaphysis bone measurements was evaluated as cortical thickness (Co.Th) and Porosity %. **(E)** Osteoclasts parameters as evaluated by TRAcP staining on bone sections OcS/BS and OcN/BPm. **(F)** Bone turnover markers: P1NP ($n = 10$ per group) for bone formation (left panel) and CTX-1 ($n = 9$ per group) for bone resorption (right panel) were measured in serum. Data are presented as mean $\pm$ SEM ($n = 8$ mice/group), * $p < 0.05$, ** $p < 0.05$; *** $p < 0.0005$. One-way ANOVA.

Supplementary Figure 2: Molecular Phenotype in bone and bone marrow microenvironment:

Quantification of mRNA expression levels of osteoblast and osteoclast related genes **(A & C)** and adipocyte related genes **(B & D)** in bone **(A & B)** and bone marrow **(C & D)** using qPCR ($n = 8$) in ND-SHAM, ND-OVX, HFD-SHAM and HFD-OVX mice. Data are presented as mean $\pm$ SEM, * $p < 0.05$, ** $p < 0.005$; *** $p < 0.0005$. two-tailed unpaired Student's t test.

Supplementary Figure 3: Gene expression changes:

MA plot showing gene expression changes in cultured BMSCs in **(A)** ND-OVX, **(B)** HFD-SHAM and **(C)** HFD-OVX mice compared to control mice ND-SHAM. Differentially expressed genes ($p < 0.05$) are highlighted. Up- and downregulated genes were subjected to gene ontology analysis. Heat map showing the p -value for the top 20 biological terms.

Supplementary Figure 4: RNAseq

(A&B) Box plot showing the log fold changes for genes with distinct patterns when comparing RNA-seq levels of cultured BMSCs from ND-OVX, HFD-SHAM and HFD-OV to ND-SHAM (**upper panels- upregulated and lower panels- downregulated**). **(C)** Heat map showing the enrichment of genes clusters (1 to 9 clusters of genes grouped based on their pattern of expression as represented from **(A)** for target

genes of the indicated pathways using SPEED analysis). **(D)** Heat map showing the enrichment of gene clusters in **(A)** for gene ontology terms.

Supplementary Figure 5: Bone metabolic profiling. **(A)** PCA score plot of the experimental groups (ND-SHAM, ND-OVX, HFD-SHAM and HFD-OVX) in metabolomic analysis. **(B)** The reliability of the established PLS-DA model was evaluated by the explained variation R^2 and the predicted total variation Q^2 , which is calculated by cross-validation. The expected R^2 and Q^2 , highly dependent on their application model, should be more than 0.5 and 0.4, respectively, for a significant biological model.

AGING CELL AUTHOR CHECKLIST. *Authors should submit this checklist together with their manuscript. Please ensure that you have read the Author Guidelines in detail before submission.*

Title	High Fat Diet (HFD)-Induced Obesity Augments the Deleterious Effects of Estrogen Deficiency on Bone: Evidence from ovariectomized Mice.						
Authors	Dalia Ali, Florence Figeac, Atenisa Caci, Nicholas Ditzel, Clarissa Schmal, Greet Kerckhofs, Jesper Havelund, Nils Færgeman, Alexander Rauch, Michaela Tencerova, and Moustapha Kassem						
Manuscript Type	Research Article						
Total Character Count (including spaces)¹	65133						
Word count of Summary²	222						
Number of papers cited in the References³	87						
Listing of all Tables (Table1, Table 2 etc)⁴	Supplementary Table 1: differentially expressed metabolites in ND-SHAM, ND-OVX, HFD-SHAM and HFD-OVX. ANOVA						
	Supplementary Table 2: Normal diet and high fat diet composition						
Figure specifications (please complete one row per figure)⁵	Colour	Greyscale	Black and white	Single column (80mm)	Double column (180mm)	Size of figure at full scale (mm x mm)	Smallest font size used in the figure at full scale (minimum 6pt)
Figure no.	(yes/no)	(yes/no)	(yes/no)	(yes/no)	(yes/no)	(insert details)	(insert details)
1	yes	no	no	no	yes	279.654mm x 395.732mm	9
2	yes	no	no	no	yes	279.654mm x 395.732mm	9
3	yes	no	no	no	yes	279.654mm x 395.732mm	9
4	yes	no	no	no	yes	279.654mm x 395.732mm	9
5	yes	no	no	no	yes	279.654mm x 395.732mm	9
6	yes	no	no	no	yes	279.654mm x 395.732mm	9

¹ The maximum character count allowed is 50,000 (incl. spaces) for Primary Research Papers and Reviews, 10,000 for Short Takes.

² Summary should not exceed 250 words.

³ Primary Research Papers can contain a maximum of two tables. If more are needed they should replace some of the Figures or can be placed in the Supporting Information.

⁴ A maximum of 45 references is allowed for Primary Research Papers and 20 references for Short Takes.

⁵ A Primary Research Paper may contain up to 6 figures and a Short Take up to 2 figures. Authors are encouraged to provide figures in the size they are to appear in the journal and at the specifications given.

Obesity
Estrogen
Deficiency

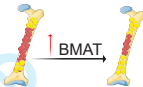


Bone Quality



Aging Page 34 of 47

Bone Phenotype

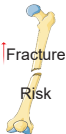


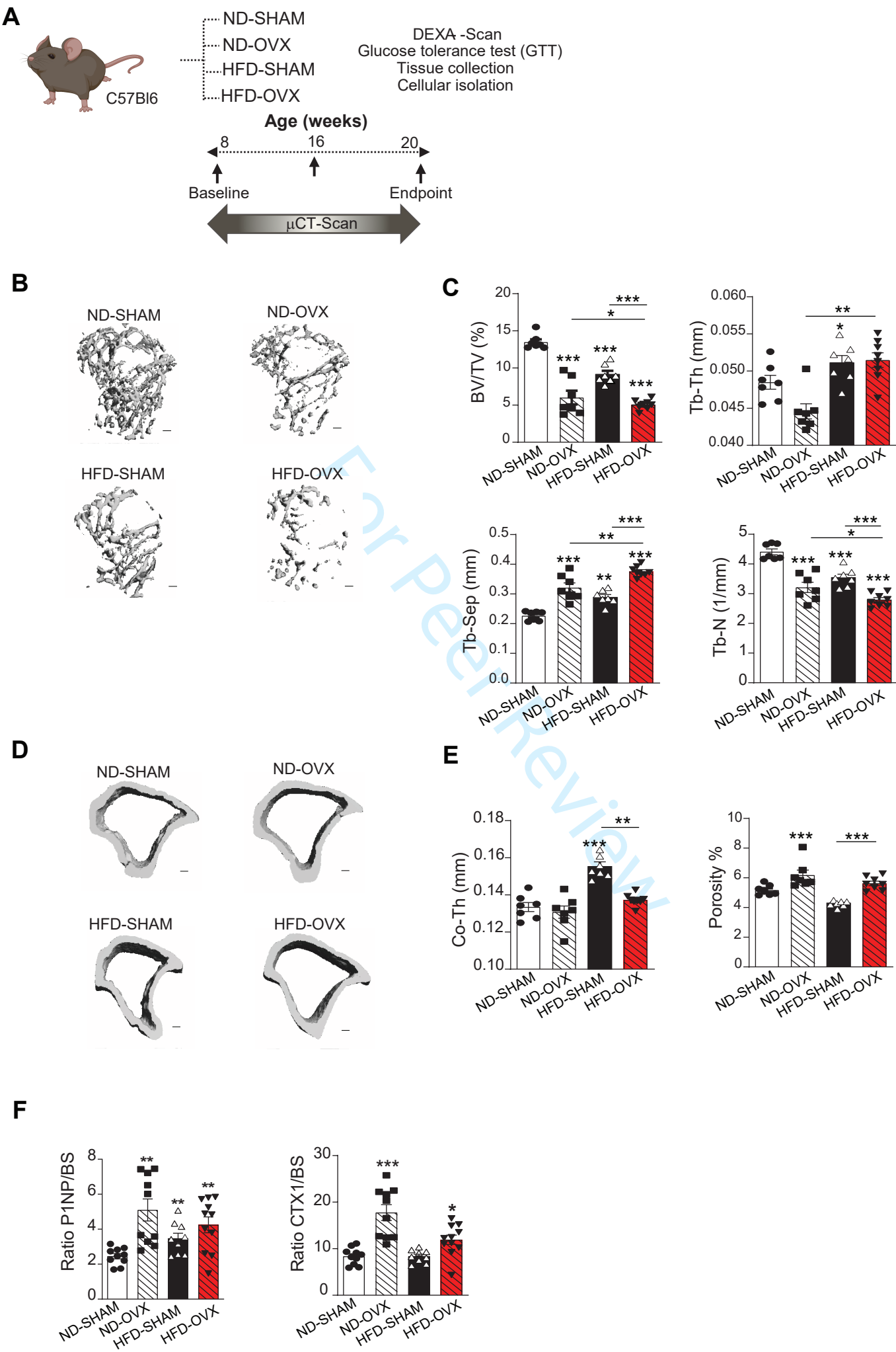
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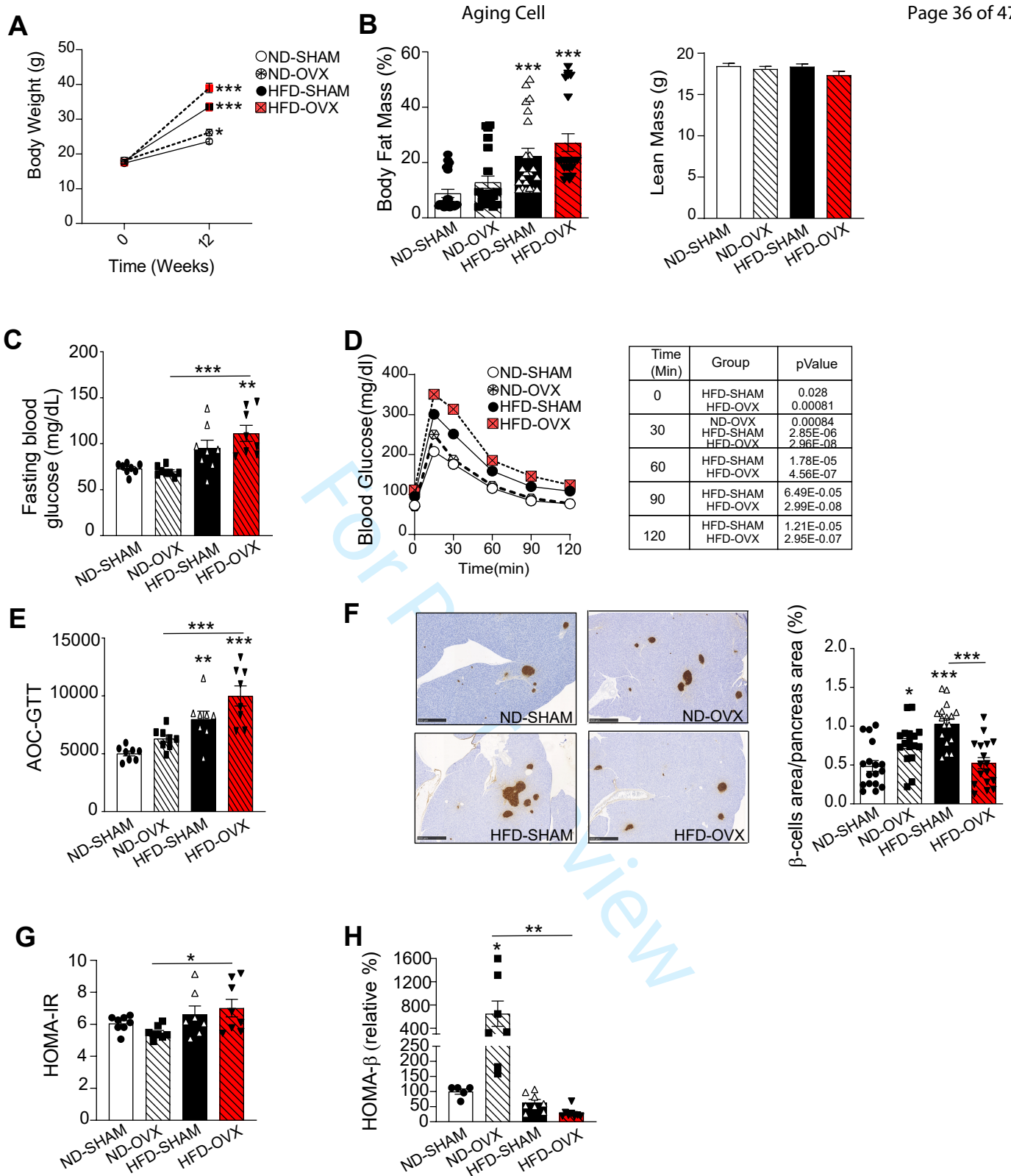
↑ SASP

↑ Senescent Cells

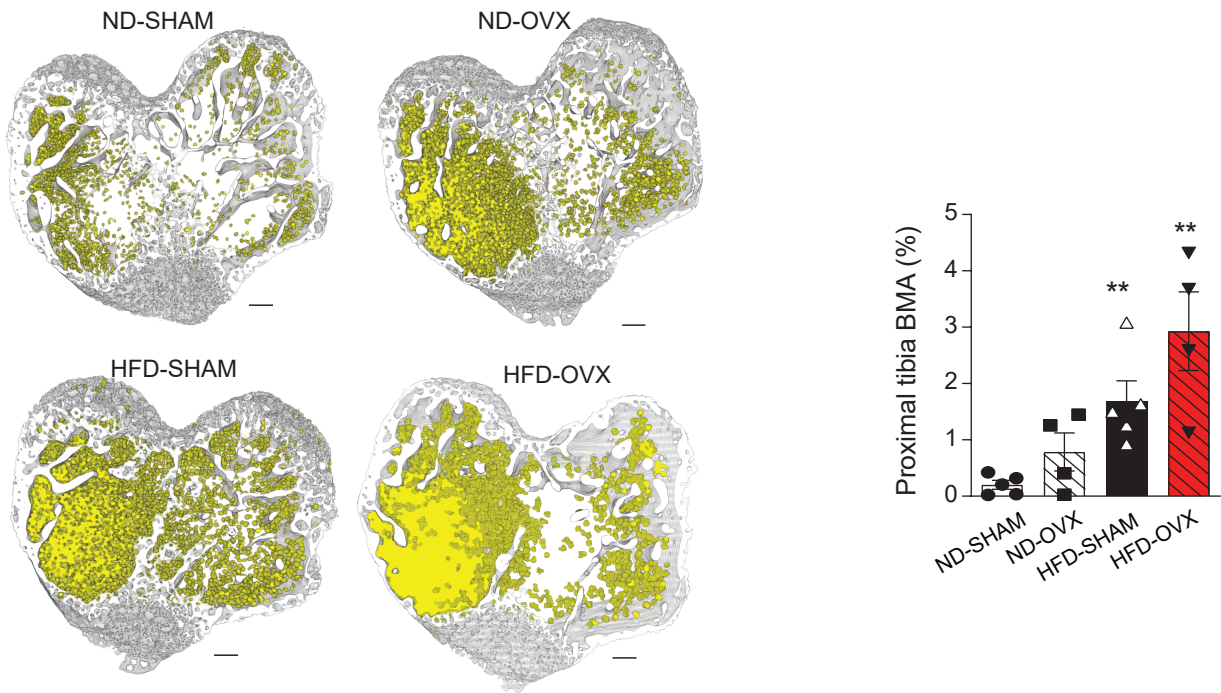
↓ Anabolic Bone Formation Pathways



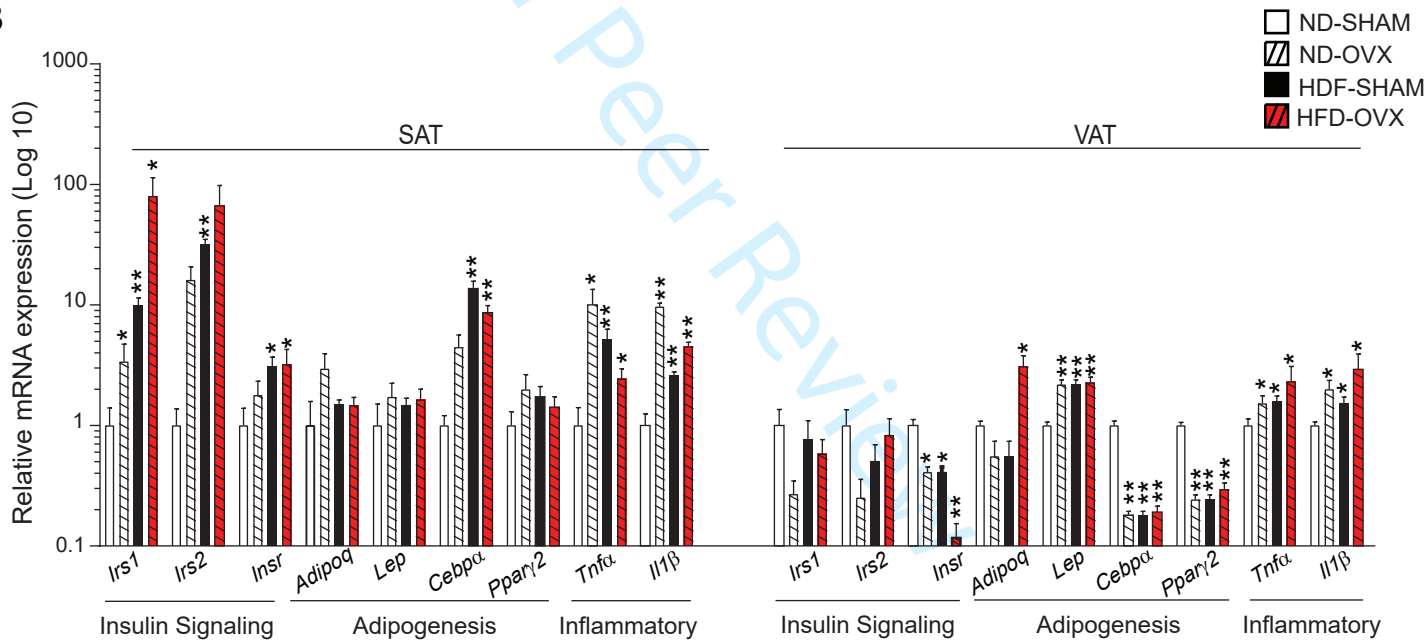




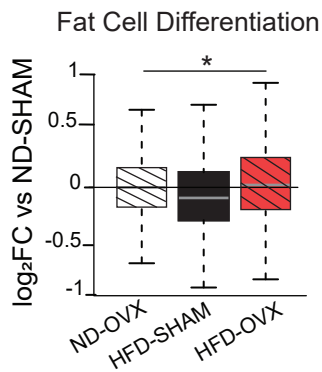
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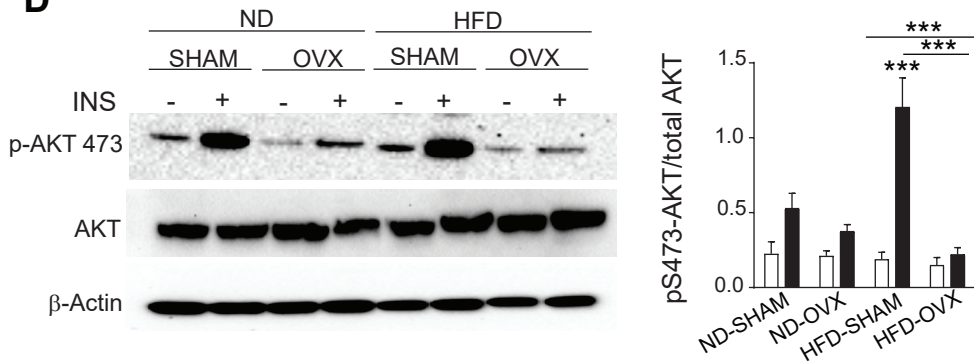
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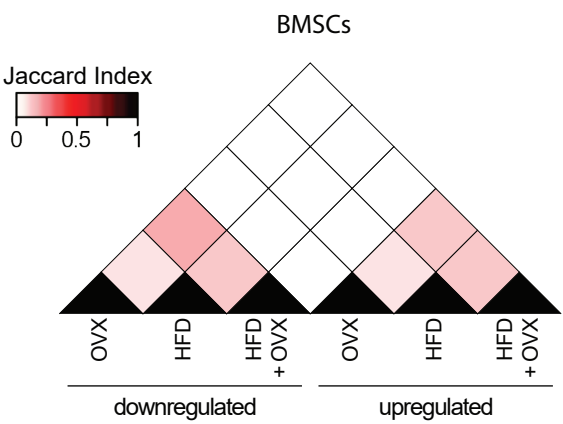
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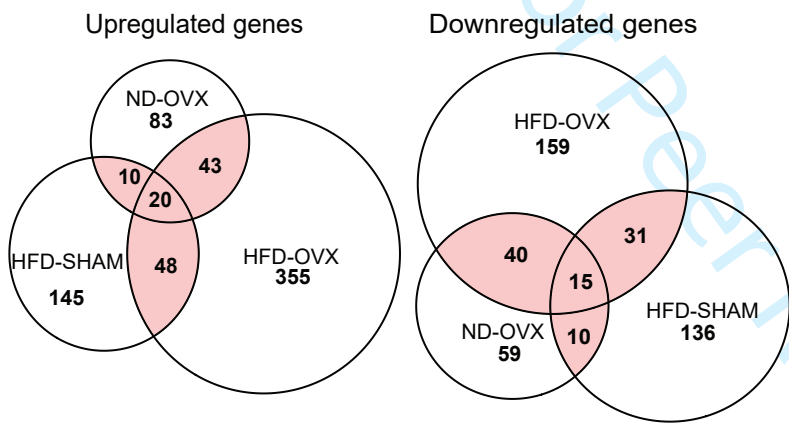
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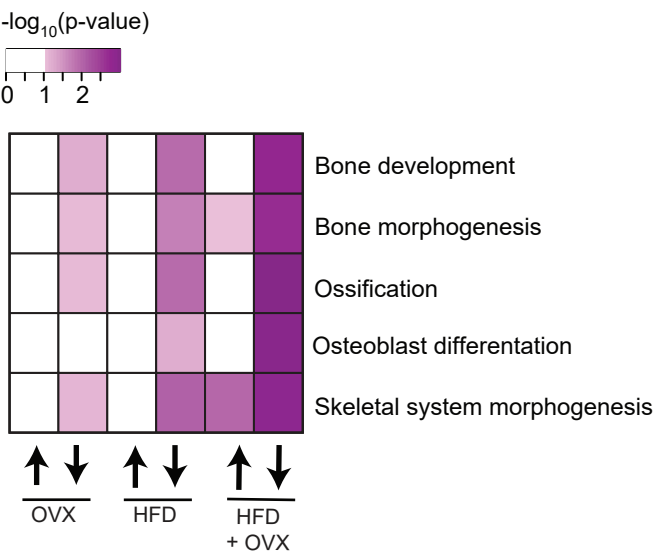
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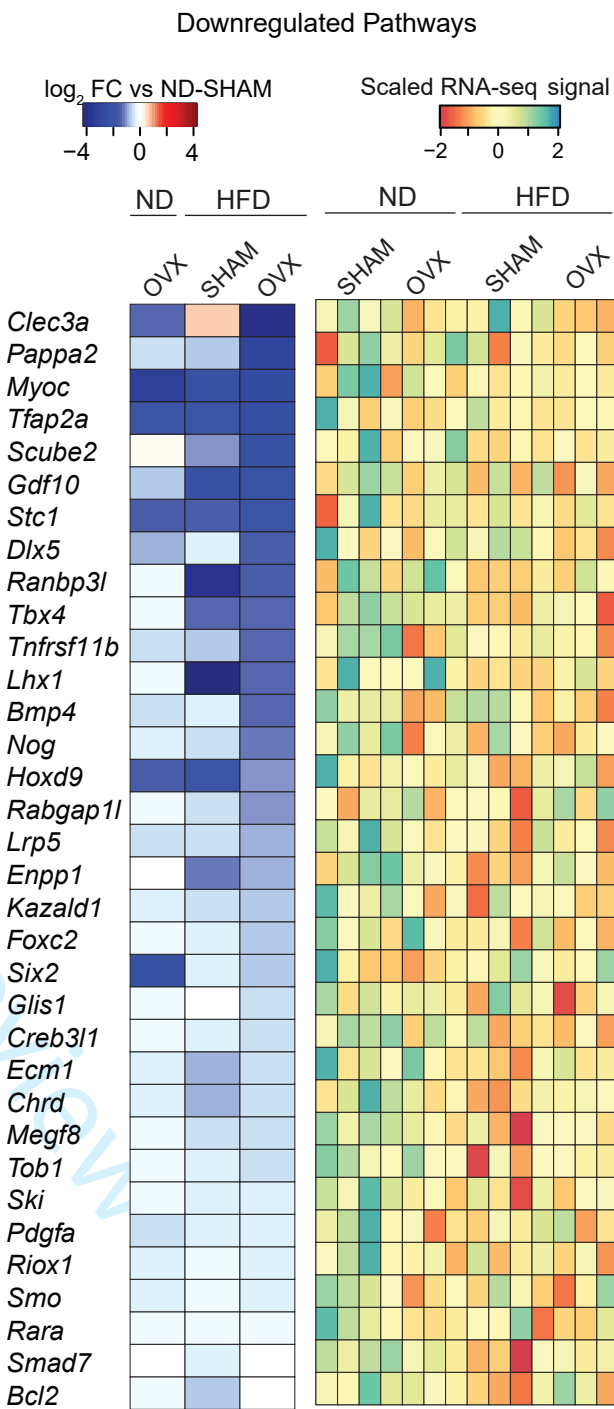
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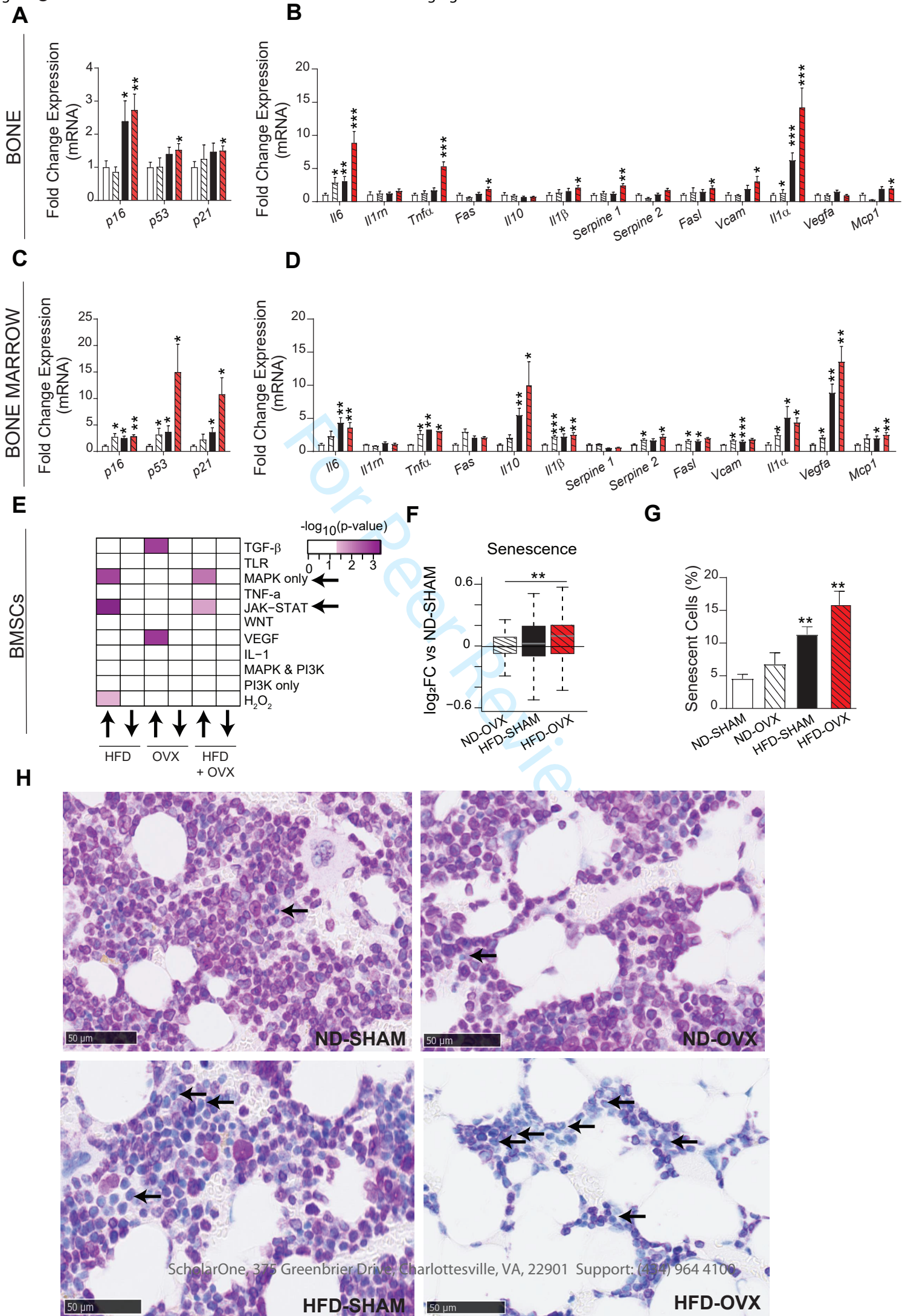
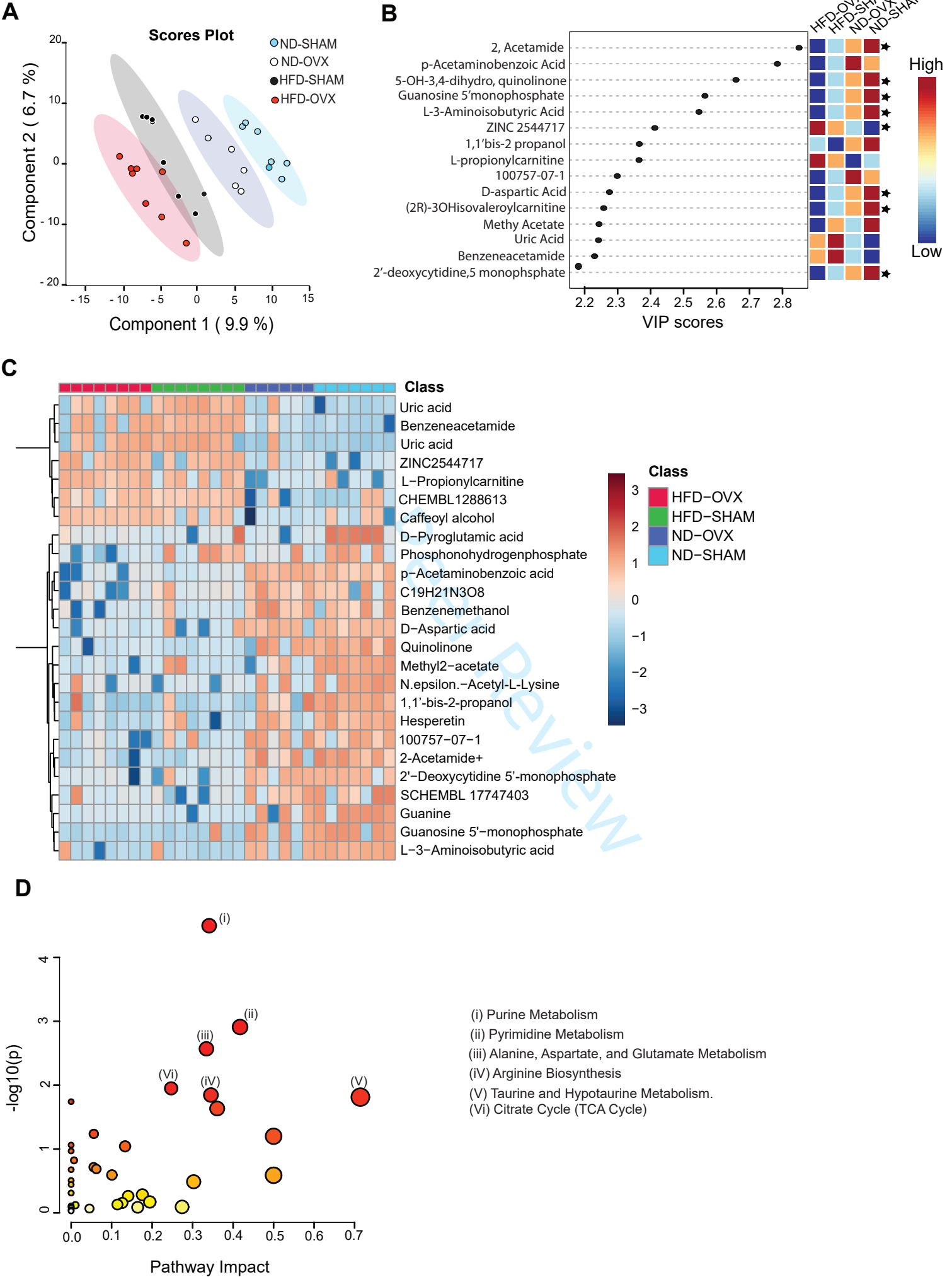
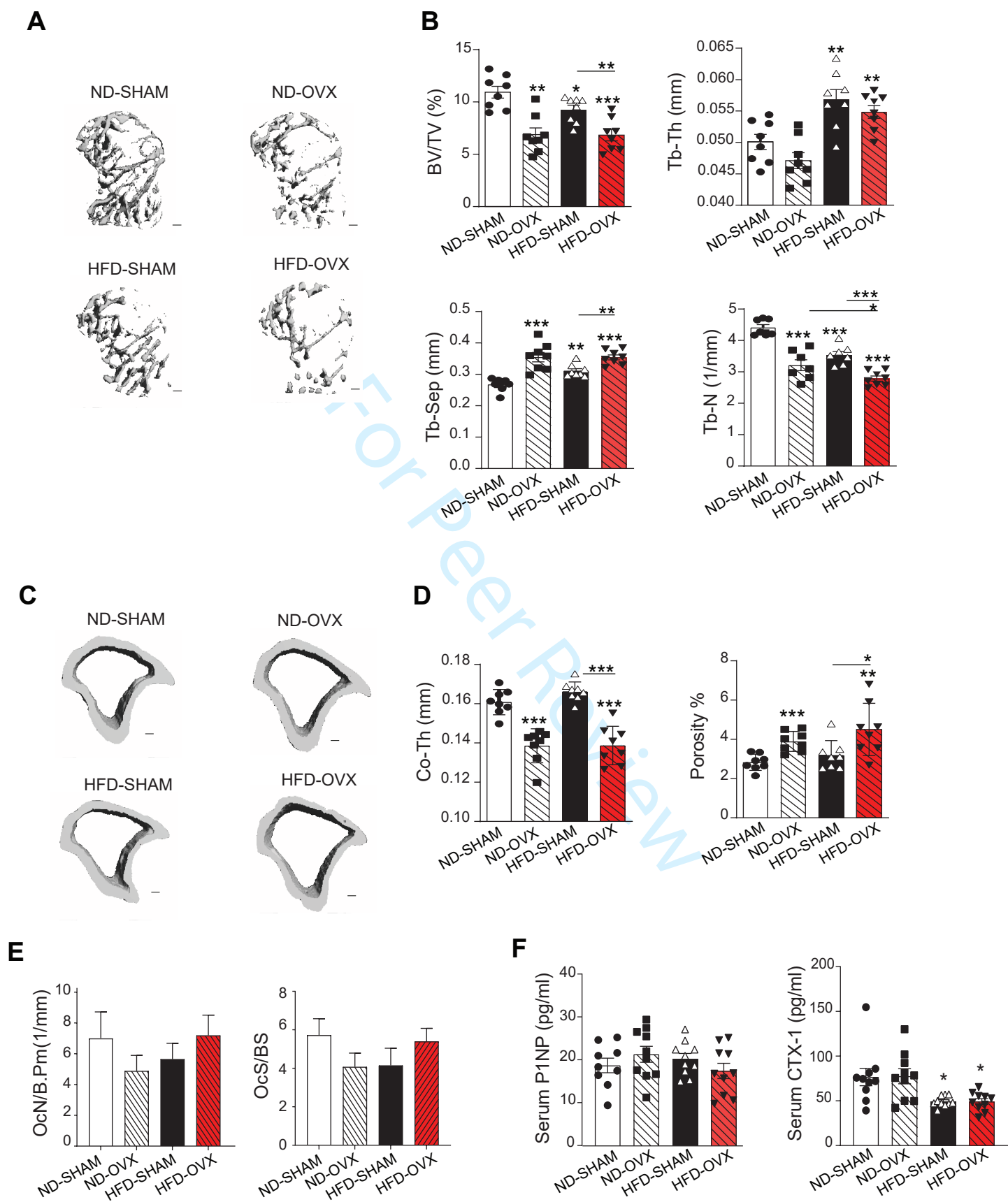
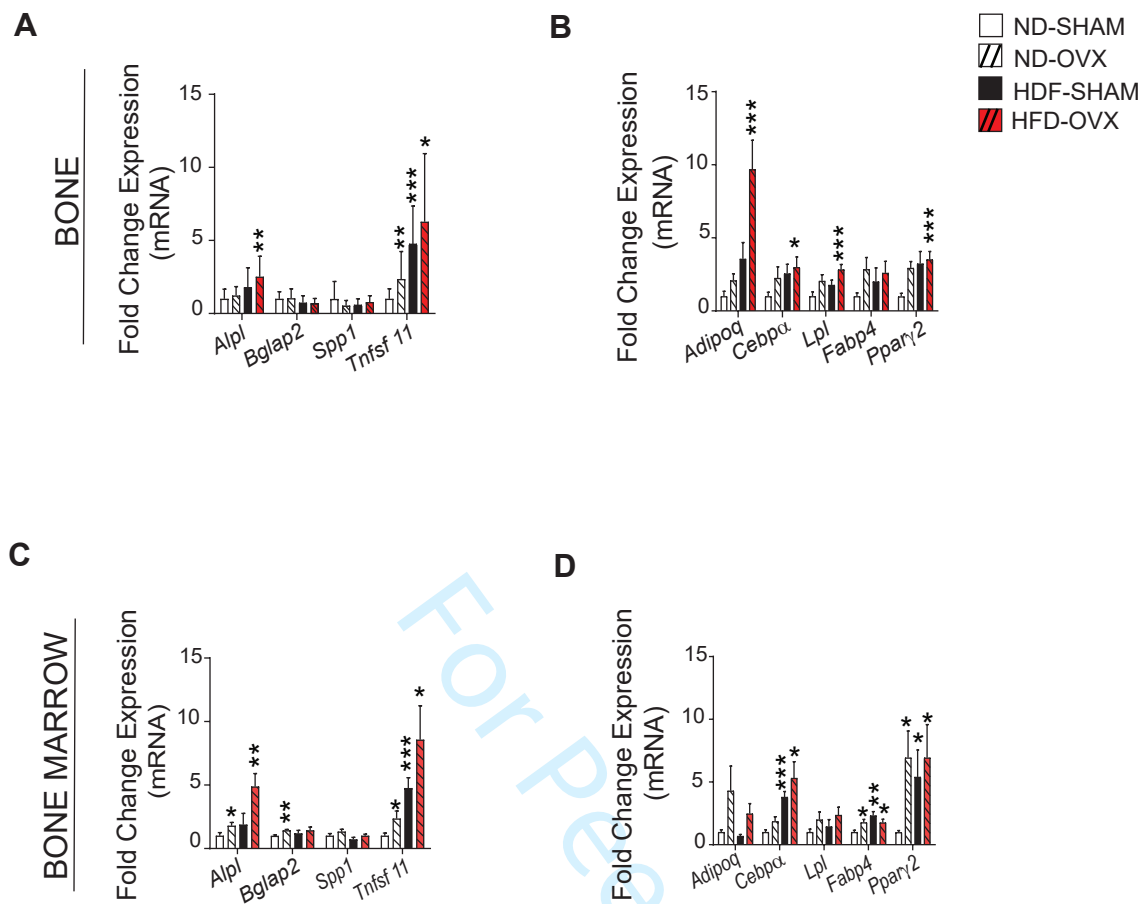
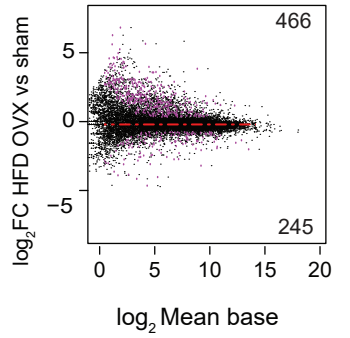
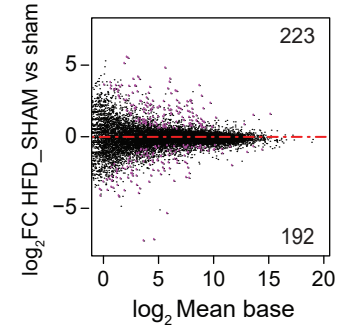
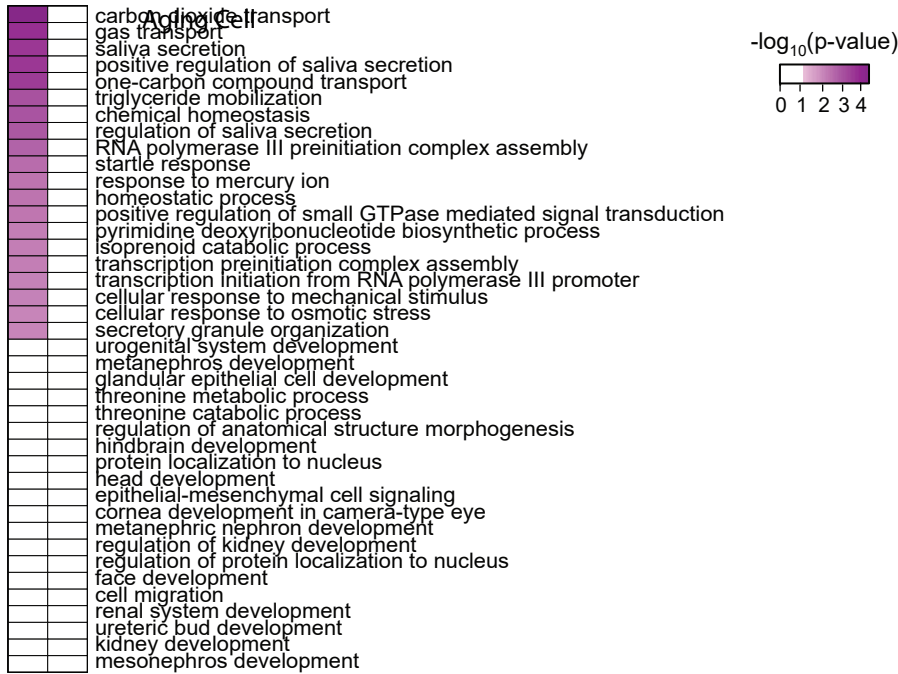
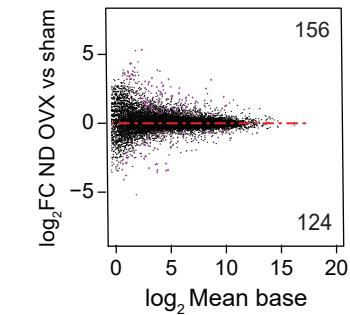


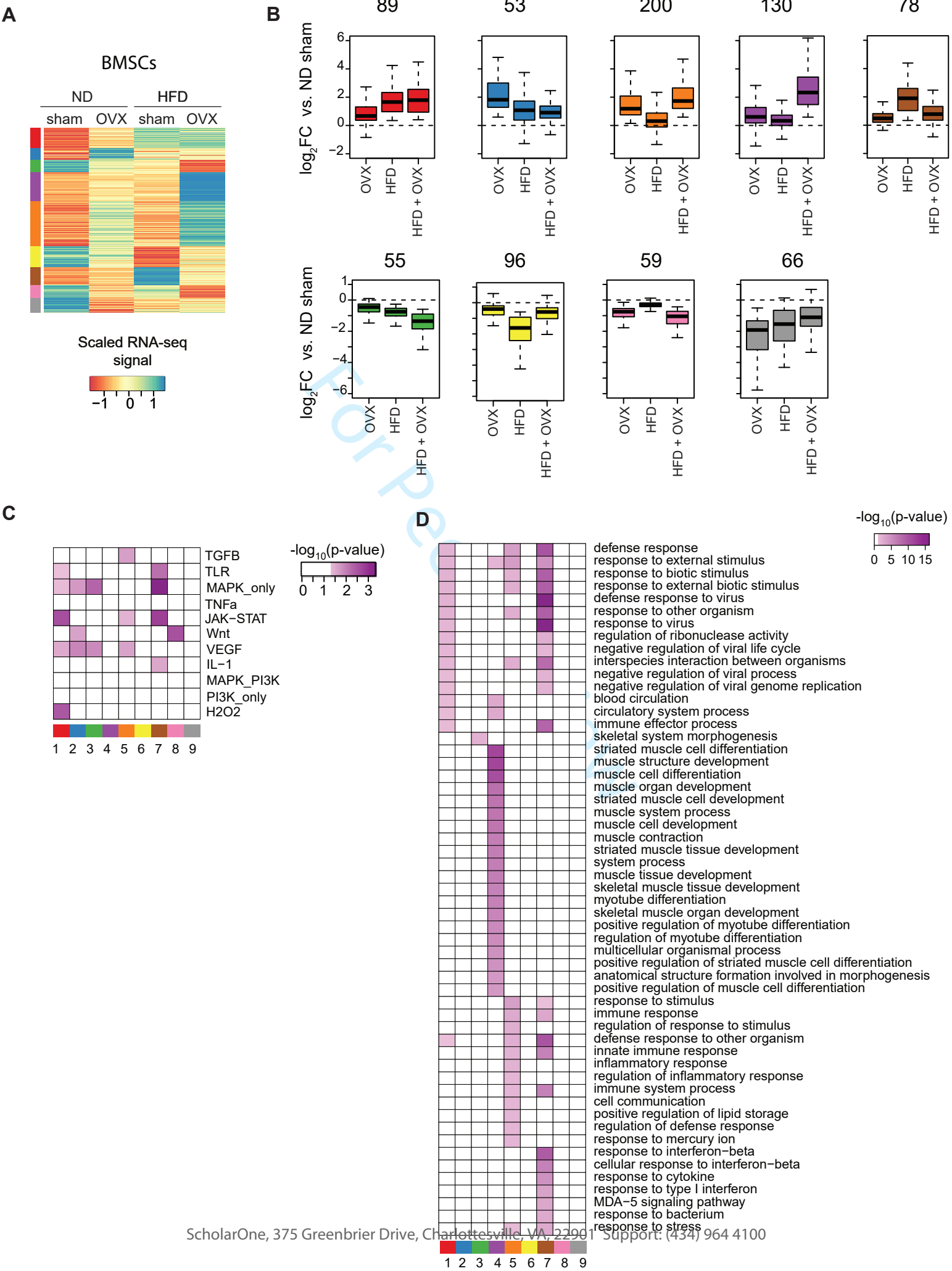
Figure 6



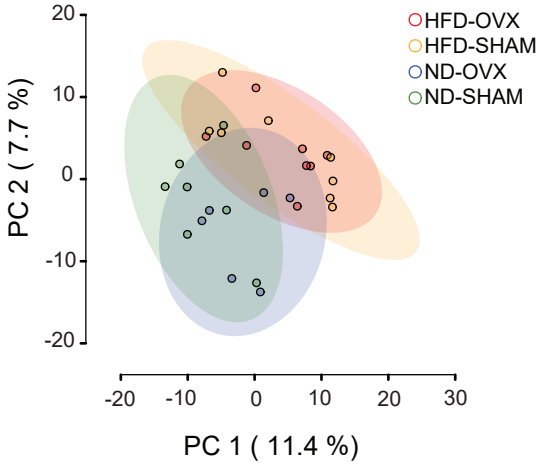




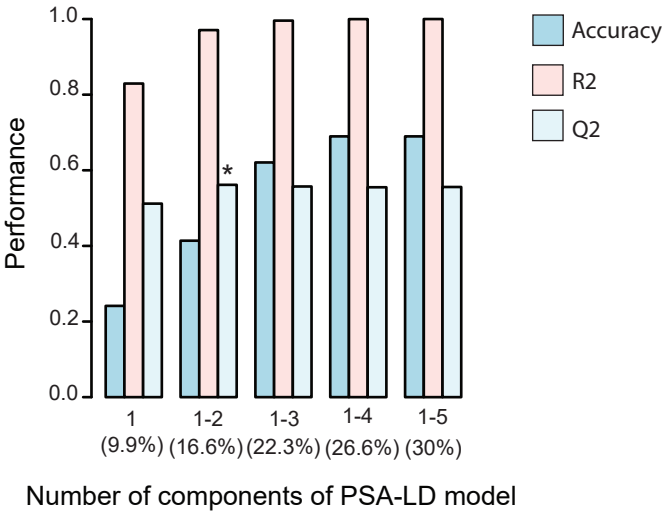




A



B



For Peer Review

No	Name	Formula	m/z	Adduct	p.value	FDR	Pathway
1	4-Acetamidobenzoic acid	C ₉ H ₉ NO ₃	178.0508	[M-H]-	3E-06	0.0015	Folate biosynthesis (linked with purine metabolism) https://www.genome.jp/pathway/map00790+C00568
2	2-(4-Acetamido-N-[2-(benzotriazol-1-yl)acetyl]anilino)-N-(2-methylbutan-2-yl)-2-(1-methylpyrrol-2-yl)acetamide	C ₂₈ H ₃₃ N ₇ O ₃	516.2728	[M + H] ⁺	2E-05	0.005	Tryptophan metabolism https://www.genome.jp/pathway/map00380+C02693 Gradual impact
3	Benzeneacetamide, N-(2-((4-chloro-2-(2-chlorobenzoyl)phenyl)methylamino)-2-oxoethyl)-	C ₂₄ H ₂₀ Cl ₂ N ₂ O ₃	455.0902	[M + H] ⁺	5E-05	0.0081	NA
4	Uric acid	C ₅ H ₄ N ₄ O ₃	335.0491	[2M-H]-	9E-05	0.0123	Purine metabolism https://www.genome.jp/pathway/map00230
5	5-Hydroxy-3,4-dihydro-2(1H)-quinolinone	C ₉ H ₉ NO ₂	162.0559	[M-H]-	0.0001	0.016	Folate biosynthesis (linked with purine metabolism) https://www.genome.jp/pathway/map00790+C00568 Gradual impact
6	Guanosine 5'-monophosphate	C ₁₀ H ₁₄ N ₅ O ₈ P	364.0652	[M+H] ⁺	0.0003	0.0251	Purine metabolism Gradual impact
7	AZD8848	C ₂₉ H ₄₃ N ₇ O ₅	570.3407	[M + H] ⁺	0.0004	0.0291	Oligoribonucleotide
8	L-3-Aminoisobutyric acid	C ₄ H ₉ NO ₂	102.0559	[M-H]-	0.0004	0.0291	Pyrimidine metabolism Gradual impact
9	L-Propionylcarnitine	C ₁₀ H ₁₉ NO ₄	218.1387	[M+H] ⁺	0.0005	0.0291	Lipid metabolism
10	Uric acid	C ₅ H ₄ N ₄ O ₃	167.0209	[M-H]-	0.0006	0.0335	Purine metabolism https://www.genome.jp/pathway/map00230
11	4-Amino-3,5-dichloro-α-[[[6-[2-(2-pyridinyl)ethoxy]hexyl]amino]methyl]benzenemethanol	C ₂₁ H ₂₉ Cl ₂ N ₃ O ₂	426.1696	[M + H] ⁺	0.0009	0.0402	NA
12	1,1'-((2,7-Di-4-morpholinyl-6-phenyl-4-pteridinyloxy)bis-2-propanol	C ₂₆ H ₃₅ N ₇ O ₄	510.2834	[M + H] ⁺	0.0009	0.0402	Morpholines??

Supplementary Table 1 : differentially expressed metabolites in ND-SHAM, ND-OVX, HFD-SHAM and HFD-OVX. ANOVA.

Supplementary Table 2

Normal Diet - R70

Ingredients:

Oatmeal, barley, wheat bran, wheat flour, beet fiber, potato protein, minerals, lysine, vitamins and trace elements.

Nutritional value %:

Crude protein 14.5
 Crude fat 4.5
 of which linoleic acid 1.0
 Carbohydrate 60.1
 Fiber 4.9
 Ash 5.0
 Water <11
Average available energy kJ/100g 1254
(calculated content)

Minerals % :

Calcium 0,7
 Phosphorus 0,5
 sodium chloride 0,4
 Magnesium 0,25
 Potassium 0,8

Trace elements mg/kg:

Copper 12
 Iron 145
 Manganese 68
 Zinc 80
 Iodide 1,3
 Cobalt 0,5
 Selenium 0,1

Vitamins (added amount)

A 9 000 IU/kg
 D 1 000 IU/kg
 E 50 mg/kg
 K3 15 mg/kg
 B1 6 mg/kg
 B2 5 mg/kg
 B6 5 mg/kg
 B12 0,07 mg/kg
 Biotin 0,4 mg/kg
 Folic acid 1,0 mg/kg
 Calcium pantothenate 15 mg/kg
 Niacin 20 mg/kg

Aminoacids% (calculated contents)

Lysine 0,8 (here of 0,3% added)
 Methionine 0,3
 Cysteine 0,2
 Threonine 0,5
 Arginine 0,7
 Histidine 0,5
 Valine 0,6
 Isoleucine 0,6
 Leucine 0,9
 Tyrosine 0,4
 aspartic acid 0,9
 Serine 0,7
 Glutamic acid 3,1
 Proline 1,1
 Glycine 0,7
 Alanine 0,7

Supplementary Table 3

Hight Fat Diet (12492- Rodent diet with 60% fat).

Class Description	Ingredients	
Protien	Casein, Lactic, 30 Mesh	200 g
Protein	Cystine, L	3,0 g
Carbohydrates	Lodex 10	12 g
Cabohydrates	Sucrose, Fine Granulated	72,80 g
Fiber	Solka Floc, FCC200	50 g
Fat	Lard	245 g
Fat	Soybean oil, USP	25 g
Minerals		50 g
	Potassium Citrate Monohydrate	
	Calcium Phosphate, Dibasic	
	Calcium Carbonate light, USP	
	Sodium Chloride	
	Magnesium Sulfate, Heptahydrate	
	Magnesium Oxide, Heavy, DC, USP	
	Ferric Citrate	
	Manganese Carbonate Hydrate	
	Zinc CarbonateChromium Potassium Sulfate	
	Chromium Potassium Sulfate	
	Copper Carbonate	
	Ammonium Molybdate Tetrahydrate	
	Sodium Flouride	
	Sodium Selenite	
	Potassium Iodate	
Vitamin		1 g
	Vitamin E Acetate, 50%	
	Niacin (B3)	
	Biotin, 1%	
	Pantothenic acid, d, Calcium B5	
	D3 100,000 IU/gm	
	B12, 0.1% Mannitol	
	A Acetate, 500,000IU/gm	
	Pyridoxine Hcl, B6	
	Riboflavin, B2	
	Thiamine Hcl, B1	
	Folic acid	
	Menadione Sodium Bisulfite	
Dye	Dye, Blue FD&C #1, Alum. Lake 35-42%	
Total :		773,85 g