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**Chronic ethanol feeding in mice decreases expression of genes for major structural bone proteins in
a Nox4-independent manner.**

Kim B. Pedersen¹, Michelle L. Osborn², Alex C. Robertson¹, Ashlee E. Williams¹, James Watt¹,
Alexandra Denys¹, Katrin Schröder³, Martin J. Ronis^{1*}

Affiliations: ¹Department of Pharmacology & Experimental Therapeutics, Louisiana State Health
Sciences Center (LSUHSC) -New Orleans, LA; ²Comparative Biomedical Sciences, LSU School of
Veterinary Medicine, Baton Rouge, LA; ³Institute of Physiology I, Goethe-University, Frankfurt,
Germany

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Running Title

Independent effects of Nox4 and ethanol on bone biology

Corresponding author

Martin Ronis PhD, Department of Pharmacology & Experimental Therapeutics, Louisiana State Health Sciences Center-New Orleans, 1901 Perdido St., New Orleans, LA 70112. Phone 504-568-4514, Fax 504-568-2361, Email: mronis@lsuhsc.edu.

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Abbreviations

ALT, alanine transaminase; ANOVA, analysis of variance; C_T, cycle threshold; CTX, carboxy-terminal collagen crosslinks; EtOH, ethanol; μ CT, micro-computed tomography; Nox, NADPH oxidase; qRT-PCR, quantitative reverse transcription polymerase chain reaction; RNA-Seq, RNA sequencing; ROS, reactive oxygen species

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Abstract

Bone loss in response to alcohol intake has previously been hypothesized to be mediated by excessive production of reactive oxygen species (ROS) via NADPH oxidase (Nox) enzymes. Nox4 is one of several Nox enzymes expressed in bone. We investigated the role of Nox4 in the chondro-osteoblastic lineage of the long bones in mice during normal chow feeding and during chronic ethanol feeding for 90 days. We generated mice with a genotype (*PrxCre* +/- *Nox4 fl/fl*) allowing conditional knockout of Nox4 in the limb bud mesenchyme. Adult mice had 95% knockdown of Nox4 expression in the femoral shafts. For mice on regular chow, only whole-body Nox4 knockout mice had clearly increased cortical thickness and bone mineral density in the tibiae. When chronically fed a liquid diet with and without ethanol, conditional Nox4 knockout mice had slightly reduced dimensions of the cortical and trabecular regions of the tibiae ($P < 0.1$). The ethanol diet caused a significant reduction in cortical bone area and cortical thickness relative to a control diet without ethanol ($P < 0.05$). The ethanol diet further reduced gene expression of *Frzb*, *Myh3* and several genes encoding collagen and other major structural bone proteins ($P < 0.05$), while the Nox4 genotype had no effects on these genes. In conclusion, Nox4 expression from both mesenchymal and non-mesenchymal cell lineages appears to exert subtle effects on bone. However, chronic ethanol feeding reduces cortical bone mass and cortical gene expression of major structural bone proteins in a Nox4-independent manner.

Significance statement

Excessive alcohol intake contributes to osteopenia and osteoporosis, with oxidative stress caused by the activity of NADPH oxidases hypothesized to be a mediator. We tested the role of NADPH oxidase 4 (Nox4) in osteoblast precursors in the long bones of mice with a conditional Nox4 knockout model. We found that Nox4 exerted effects independently of alcohol intake and ethanol effects on bone were Nox4 independent.

Introduction

Chronic alcohol consumption affects bone turnover and predisposes to the development of osteopenia and osteoporosis in humans (Luo et al., 2017; Watt et al., 2019). In rodent models, chronic intake of ethanol also leads to reduction of bone mass (Chen et al., 2011; Mercer et al., 2014; Watt et al., 2018). In osteoblasts, alcohol exposure is associated with increased parameters of oxidative stress, i.e. an excessive, deleterious production of reactive oxygen species (ROS) (Chen et al., 2008). Dietary antioxidants prevent ethanol-mediated loss of bone mass in vivo (Chen et al., 2011; Alund et al., 2017). We therefore hypothesized that bone loss caused by alcohol intake is mediated by oxidative stress.

NADPH oxidases are enzymes that generate ROS in the form of superoxide and/or hydrogen peroxide. NADPH oxidase 2 (Nox2) and NADPH oxidase 4 (Nox4) are both expressed in murine bone. The role of Nox2 and Nox4 in ethanol-mediated bone loss has previously been investigated using whole-body knockout mice. Knockout of p47^{phox}, which is an essential cofactor for functional Nox2 activity, partly protected against bone loss but only blocked ethanol-induced increases in osteoclastogenesis and bone resorption (Mercer et al., 2014). Whole-body knockout of Nox4 in male mice actually increased trabecular bone loss during ethanol feeding, possibly by impairing normal osteogenic processes such as mesenchymal stem cell proliferation (Watt et al., 2018). On the other hand, female mice of a different Nox4 knockout genotype maintained on a regular rodent diet have been reported to have increased bone mass and decreased osteoclastogenesis relative to wild type animals (Goettsch et al., 2013). Whether these apparently disparate effects of knocking out Nox4 are due to different Nox4 knockout models, different dietary regimens, or sex and age differences are currently unknown. To get a better understanding of the role of Nox4 on bone homeostasis, we generated a genetic model (*PrxCre +/- Nox4 fl/fl*) in which knockdown of Nox4 occurs in cells of the chondroblastic/osteoblastic lineage in the long bones of mice. Both male and female mice were investigated after being maintained on regular mouse chow and after being exposed to 90 days of chronic ethanol feeding using Lieber-DeCarli liquid diets. The tibial bone microstructure and the femoral shaft transcriptome were analyzed.

Materials and methods

Animals

PrxCre mice with CRE recombinase under control of a rat *Prx1*-derived promoter/enhancer (B6.Cg-Tg(Prrx1-cre)1Cjt/J, Cat. No. 005584,) were purchased from The Jackson Laboratory, Bar Harbor, ME. The genotype of hemizygous animals is termed *PrxCre* +/- . The Nox4 knockout model, in which exons 1 and 2 (relative to NM_0157605) are flanked by LoxP sites (Fig. 1A), was obtained under a Material Transfer Agreement with Drs. Ralf Brandes and Katrin Schröder, University of Frankfurt, Germany and has been previously described (Goettsch et al., 2013). The genotype of homozygous animals is termed *Nox4 fl/fl*. Experimental conditional knockout and control animals were the progeny of crossing *PrxCre* +/- *Nox4 fl/fl* males with *Nox4 fl/fl* females. Since the *Prrx1* gene promoter/enhancer is expressed in the female germline (Logan et al., 2002), whole-body Nox4 knockout alleles, termed *Nox4 0*, were generated in breedings with PrxCre expressed in the female parent. Experimental whole-body knockout animals were the progeny of crossing *Nox4 0/0* males with *Nox4 0/0* females. Whole-body Nox4 knockout mice lacking exon 4 of the *Nox4* gene and wildtype C57Bl/6J mice of genotypes *Nox4 -/-* and *Nox4 +/+* were purchased from The Jackson Laboratory (Cat. Nos. 022996 and 000664, respectively). Genotyping primers used for distinguishing the *Nox4 fl/fl* allele and the *Nox4 +* wild-type allele were ttctaagtagccttggtggtgc and tgcctccaatcatgaaagtg. The *Nox4 0* allele was verified with genotyping primers tgggtgcacaaccattctagg and tgcctccaatcatgaaagtg. The presence of the CRE transgene was tested with genotyping primers acctgaagatgttcgcgattatct and accgtcagtagtgagatatctt. The Institutional Animal Care and Use Committee (IACUC) of LSU Health Sciences Center approved all animal experiments in accordance with the Guide for the Care and Use of Laboratory Animals (U.S. National Institutes of Health).

Ethanol feeding

Ethanol (EtOH) was provided as a variant of the liquid high-fat Lieber-DeCarli diet. The control diet contained 176.2 g/L DYET # 710260 Lieber-DeCarli Ethanol Diet and 38.1 g/L DYET # 402850 Maltose Dextrin for a protein:carbohydrate:fat caloric ratio of 24:30:47 and a concentration of 1000 kcal/L. The ethanol diets were made by replacing total food calories with ethanol while maintaining the 24:30:47 protein:carbohydrate:fat ratio and 1000 kcal/L. Mice were housed two to a cage. Ethanol diets were provided *ad libitum*, while volumes of the control diet were adjusted weekly to pair-fed mice to ensure the same caloric intake as in the ethanol-fed mice. Feeding was initiated with 13 – 15 week old mice. The ethanol concentration was increased from 0% to 25% of dietary calories over 15 days, maintained at 25% for 2 months, and increased to 28% in the last 14 days before euthanasia. The mice were 27 – 28 weeks old when euthanized. The number of mice fed the ethanol diet were 10 male *Nox4 fl/fl*, 10 male *PrxCre +/- Nox4 fl/fl*, 9 female *Nox4 fl/fl* and 9 female *PrxCre +/- Nox4 fl/fl*. The number of mice fed the control diet were 9 male *Nox4 fl/fl*, 9 male *PrxCre +/- Nox4 fl/fl*, 10 female *Nox4 fl/fl* and 6 female *PrxCre +/- Nox4 fl/fl*.

Mouse dissection

Mice were euthanized by CO₂. Body weights and liver weights were recorded. Blood was collected from the right ventricle of the heart. The blood was clotted for 20 minutes at room temperature followed by centrifugation at 2000 rcf for 30 minutes at 4°C. Serum was collected and stored at -80°C. The tibiae were fixed in 10% formalin. For RNA isolation, the femoral shafts and femoral bone marrow were isolated and stored at -80°C for later isolation of high-quality RNA as previously described (Pedersen et al., 2019). Liver aliquots were frozen and stored at -80°C. Additional liver aliquots and a whole kidney were incubated overnight in RNAlater with subsequent storage at -80°C for later isolation of RNA. RNA from the femoral marrow, as well as the kidney and liver, were isolated by the TRI Reagent method. RNA from the femoral shaft was isolated by the TRI Reagent – RNeasy hybrid protocol previously described (Pedersen et al., 2019). For later protein isolation, whole femurs were frozen at -80°C. Liver triglyceride

content was determined with a colorimetric triglyceride quantification assay kit ab65336 from Abcam (Cambridge, MA).

Serum markers

The serum contents of alanine transaminase (ALT), osteocalcin, and carboxy-terminal collagen crosslinks (CTX) were determined with commercial kits (Cat. No. 700260 from Cayman Chemical, Ann Arbor MI, Cat. No. 60-1305 from Quidel, San Diego, CA, and Cat. No. LS-F21349 from LSBio, Seattle, WA).

Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR).

qRT-PCR was conducted as previously described (Pedersen et al., 2019) with RNA samples diluted to a concentration of 5 ng/ μ L. Primers are listed in Table 1.

Micro-computed tomography (μ CT)

Trabecular and cortical bone morphology of formalin-fixed tibiae were scanned and their morphology assessed using a μ CT 40 system from SCANCO Medical AG (Switzerland). All specimens were scanned using a voltage of 55 kVp, a current of 145 μ A, an integration time of 200 ms, with a voxel resolution of 6 μ m for trabecular bone and 12 μ m for cortical bone. Two trabecular regions interior to the cortex and primary spongiosa were analyzed: one segment located 0.4-0.7 mm distal to the proximal growth plate and a second segment located 0.3-1.2 mm distal to the end of the primary spongiosa. A single cortical region was analyzed and was located 4.8 – 4.2 mm proximal to the tibia-fibula junction. Thresholds to differentiate bone from the surrounding soft tissue were set to 486 and 703 mg HA/ccm for the trabecular and cortical regions, respectively.

RNA Sequencing (RNA-Seq)

RNA-Seq service was purchased from Genewiz (South Plainfield, NJ). Samples were of male femoral shaft RNA with each sample being a pool of RNA from 2 mice. The samples were from mice of

genotypes *Nox4 fl/fl* or *PrxCre +/- Nox4 fl/fl* fed the ethanol or control diets with 3 samples of each condition. Gene ontology analysis was done using the Gene Ontology Resource with PANTHER Overrepresentation Test (The Gene Ontology Consortium, 2019).

Western blotting

Femur shafts proteins were isolated by a Minute Total Protein Extraction Kit for Bone Tissue (#SA-02-BT from Invent Biotechnologies, Inc., Plymouth, MN) using the denaturing buffer from the kit supplemented with protease inhibitors. Protein-bound nitrotyrosine was detected with a rabbit anti-nitrotyrosine antibody (# 06-284 from MilliporeSigma, St. Louis, MO) at a 1:1000 dilution followed by a horseradish peroxidase-conjugated anti rabbit IgG (NA934VS from GE Healthcare) at a 1:20,000 dilution. Nitrotyrosine Bovine Serum Albumin (#89542 from Cayman Chemical Company, Ann Arbor, MI) was a positive control. Blots were developed with SuperSignal West Pico PLUS Chemiluminescent Substrate (# 34577 from Thermo Scientific, Rockford, IL). Proteins on the blotting membrane were visualized by staining with Naphthol Blue Black (N3393 from MilliporeSigma).

Statistical analysis

2-way and 3-way ANOVAs were carried out. For qRT-PCR data, the ANOVAs were conducted on the cycle threshold (C_T) values. For RNA-Seq, a 2-way ANOVA of \log_2 -transformed normalized counts of each gene was conducted for assessment of main effects of genotype and diet, as well as the interaction between genotype and diet. The test probabilities were not adjusted for the multiplicity of genes. Figures illustrate means and standard errors of the parameters.

Results

The genetic model ensures conditional Nox4 knockdown.

Nox4 fl/fl mice have exons 1 and 2 of the Nox4 gene flanked by LoxP sites (Fig. 1A). *PrxCre* is expressed in limb bud mesenchyme as well as part of the craniofacial mesenchyme (Logan et al., 2002), so *PrxCre* +/- *Nox4 fl/fl* mice should have knockdown of Nox4 in these tissues. With a primer set amplifying an amplicon stretching from exon 1 to exon 3 of Nox4 mRNA by qRT-PCR, Nox4 expression from the knockout region in the femoral shaft is indeed reduced by more than 95% and not detected in whole-body knockout mice of genotype *Nox4 0/0* (Fig. 1B). There are described human Nox4 mRNA isoforms without exons 1 and 2, such as transcript variant 6 (NCBI Reference Sequence NM_001291929.1), where translation occurs from alternate translation initiation sites. We therefore also designed a qRT-PCR assay for total Nox4 mRNA with an amplicon stretching from exon 3 to exon 5. This assay confirmed that total Nox4 mRNA in the femoral shaft is knocked down by more than 95% in *PrxCre* +/- *Nox4 fl/fl* mice (Fig. 1C). The expression of Nox4 in the femoral bone marrow was too low to quantify accurately. Nox2 mRNA expression in bone was not affected by the Nox4 genotype (Fig. 1D). As expected, mice of the *PrxCre* +/- *Nox4 fl/fl* genotype do not show reduced Nox4 expression in liver and kidney (Figs. 1E and 1F). The kidney is the major organ expressing Nox4, at least in humans (Shiose et al., 2001). While there is no detection of Nox4 mRNA from the knockout region in the kidney of *Nox4 0/0* mice, total kidney Nox4 mRNA was only reduced by 36% (Fig. 1F), suggesting that the majority of kidney Nox4 mRNA lacks exons 1 and 2.

Whole-body knockout of Nox4 increases thickness and density of cortical bone

We assessed the effects of conditional and whole-body knockout of Nox4 on the bone microstructure of the tibiae for adult mice fed regular rodent chow (Figs. 2-3). We compared mice in early adulthood at 13 weeks of age and in later adulthood at 32 weeks of age. The mice continued gaining weight during this period from an average of 20.8 g to 27.6 g for females and from 27.6 g to 37.5 g for males. At 32 weeks of age, *PrxCre* +/- *Nox4 fl/fl* mice had an average lower body weight than *Nox4 fl/fl* mice in females (26.0

g vs. 30.9 g; $P = 0.07$) and males (33.8 g vs. 40.0 g; $P = 0.05$). All trabecular bone parameters were affected by sex (Figs. 2A – H) with less trabecular bone present in females than males. There was a loss of trabecular bone as the animals aged from 13 to 32 weeks, but an increased trabecular bone mineral density during that same period (Figs. 2A – 2H). There were no main effects of the *Nox4* genotype on the trabecular bone parameters. Cortical bone parameters (Fig. 3) showed significant interactions between age and sex reflecting that females have a more pronounced increase in cortical bone parameters with age than males (Fig. 3B – 3E). The *Nox4* genotype had small, but highly significant ($P < 0.001$) main effects on cortical bone with *Nox4* 0/0 mice showing higher levels of cortical bone area/total area, cortical thickness, and cortical bone mineral density than *Nox4* fl/fl mice. For 32-week-old female mice, the highest mean levels of cortical bone area/total area and cortical thickness occurred for the *PrxCre* +/- *Nox4* fl/fl genotype, suggesting that local conditional *Nox4* knockdown may be enough to affect cortical bone. Otherwise, the *Nox* 0/0 mice exhibited the highest means, indicating stronger effects for whole-body than for conditional knockdown of *Nox4* expression.

Chronic alcohol consumption affects body weight, liver weight and bone turnover

We wished to determine effects of conditional *Nox4* knockdown on bone characteristics after chronic alcohol consumption for 3 months. In the past, our laboratory has typically provided alcohol to mice as liquid Lieber-DeCarli-based diets in which carbohydrates are exchanged for alcohol (Mercer et al., 2014; Watt et al., 2018). Unfortunately, the high content of fat and simple carbohydrates in control diets without alcohol tend to cause as much hepatic damage as the alcohol-containing diets, and it becomes difficult to separate alcohol from carbohydrate effects (discussed previously (Shearn et al., 2018)). For the current study, we therefore decided to replace a fraction of the whole control diet with alcohol and thereby maintain the same protein:carbohydrate:fat ratio in alcohol and control diets. We achieved nearly identical average caloric intakes in alcohol-fed and control-fed animals (14.2 kcal/mouse/day in both alcohol-fed and control-fed males, 12.6 kcal/mouse/day for ethanol-fed females and 12.7 kcal/mouse/day for control-fed females).

Animals gained weight throughout the feeding period (Fig. 4A). The average weights of ethanol- and control-fed animals started to diverge after 2 months resulting in significantly lower body weight for the ethanol diet when the mice were euthanized (Fig. 4B). The ethanol diet also led to a highly significant elevation of the liver weight relative to body weight (Fig. 4C). The content of hepatic triglycerides and serum ALT, which is a marker of hepatic injury, was increased in ethanol-fed females, but not males (Figs. 4D, 4E).

The concentration of serum osteocalcin, a marker of bone formation, was slightly lower in alcohol-fed animals (Fig. 4F). The bone resorption marker CTX was elevated in alcohol-fed females, but not males (Fig. 4G). The effects observed on these parameters are all changes typically observed with chronic alcohol consumption. While the sex also had clear effect on the parameters, the genotype did not affect any of them significantly.

Chronic alcohol consumption and Nox4 depletion have independent effects on tibial bone structure

The bone microstructure of tibiae from the mice in the ethanol feeding experiment was analyzed by μ CT. Trabecular microstructure below the primary spongiosa showed the same significant sex differences for trabecular total volume, trabecular bone volume, bone volume/total volume, trabecular volume and trabecular separation as observed in mice maintained on the regular chow diet. However, the diet or genotype had no statistically significant main effects (data not shown). Since the density of trabecular bone turned out to be relatively low in this region, we also analyzed a region closer to the proximal growth plate (Fig. 5A – 5H). The ethanol diet still showed no significant effect. However, trabecular total volume was slightly, but significantly ($P < 0.001$), reduced in the pair-fed *PrxCre +/- Nox4 fl/fl* mice on the control diet. There was also a trend ($P = 0.09$) for reduced trabecular bone volume in the pair-fed *PrxCre +/- Nox4 fl/fl* mice. Finally, there was a significant interaction between genotype and sex ($P = 0.027$) for trabecular thickness reflecting a slight reduction in thickness in pair-fed *PrxCre +/- Nox4 fl/fl* females, but not males.

Cortical bone of the tibiae showed the same sex differences as those observed in 32-week-old mice on the regular chow diet, i.e. a smaller total cortical area, but thicker and denser cortex in females (Figs. 6A, 6D, 6E). The ethanol diet significantly reduced bone area, bone area/total area, and cortical thickness (Figs. 6B, 6C, 6D). The *PrxCre* +/- *Nox4* *fl/fl* genotype resulted in slightly but significantly reduced cortical bone area (Fig. 6B). There was also a trend ($P = 0.07$) for reduced total cortical area (Fig. 6A).

We conclude that the chronic ethanol consumption decreased cortical bone mass while the conditional knockdown of *Nox4* tended to decrease the dimensions of cortical bone and the trabecular region close to the growth plate.

Chronic alcohol consumption reduces gene expression of major structural bone proteins.

We assessed the effects of chronic alcohol consumption and the conditional *Nox4* knockdown on the femoral shaft transcriptome by RNA-Seq. Due to cost considerations, we conducted RNA-Seq for only mice of one sex and chose males since they gave the higher RNA yield. The samples for RNA-Seq were of good quality with an average RNA Integrity Number from 7.9 to 8.8. The expression of 18044 genes were analyzed for animals of the genotypes *PrxCre* +/- *Nox4* *fl/fl* and *Nox4* *fl/fl* fed either the ethanol diet or the control diet (Supplemental File 1). The two most highly expressed genes in *Nox4* *fl/fl* males fed the control diet were the major bone collagen genes *Col1a1* and *Col1a2*. Surprisingly, the fourth most highly expressed gene was *Hbb-bs* encoding the s form of the beta polypeptide of adult hemoglobin. This expression must come from bone marrow erythrocyte precursors. Thus, while the RNA isolated from the femoral shafts is highly enriched in bone RNA, there is also a non-negligible contribution of RNA from bone marrow cells adhering to the femur shaft.

From 2-way ANOVAs conducted for each gene, 2117 and 635 genes showed significance ($P < 0.05$) for the main effects of the diet and the genotype, respectively, while 945 genes had a significant interaction between diet and genotype. Expression of the housekeeping gene *Gapdh* was not significantly affected by the treatments as measured by RNA-Seq or qRT-PCR (Fig. 8H). Since 902 genes can be expected to show significance at $P < 0.05$ by chance when 18044 genes are analyzed, the ethanol diet affects gene

expression of more genes than could be expected by chance. For most of these genes, ethanol exerts a relatively modest, less than 2-fold, change in gene expression. Only 35 genes are regulated significantly ($P < 0.05$) and at least 2-fold (Fig 7A). Six of these are immunoglobulin genes, suggesting ethanol effects on bone marrow cells. The strongest downregulation by ethanol (5.2-fold, $P = 0.002$) was for the gene for Myosin heavy chain 3 (*Myh3*), of which certain mutations can cause skeletal abnormalities (Carapito et al., 2016). Another gene known to have direct bone effects is *Frzb* encoding Frizzled Related Protein (Enomoto-Iwamoto et al., 2002). It was down-regulated 2.1-fold ($P = 0.0006$) by the chronic ethanol feeding. Ethanol regulation of *Myh3* and *Frzb* was confirmed by qRT-PCR (Fig. 8B and 8X).

Nox4 itself is the most downregulated gene for the *PrxCre* +/- *Nox4* fl/fl genotype (Fig. 7B). Two genes, *Cyp2s1* and *Gm45226*, show strong upregulation for the *PrxCre* +/- *Nox4* fl/fl genotype. The regulation of Nox4 and *Cyp2s1* was confirmed by qRT-PCR (Fig. 8A and 8I). The *Cyp2s1* and *Gm45336* genes are located on chromosome 7, as is *Nox4*. We speculated that the upregulation could be due to excision of cis-regulatory inhibitors for *Cyp2s1* and *Gm45336* expression located between the LoxP sites of the *Nox4* gene rather than a decrease in Nox4 activity. This was tested by comparing *Cyp2s1* induction in *Nox4* 0/0 mice to induction in the Nox4 knockout (*Nox4* -/-) animals previously used (Watt et al., 2018) where exon 4 required for enzymatic activities was deleted, rather than exons 1 and 2. However, none of the whole-body knockout models showed *Cyp2s1* induction (Supplemental Figure 1A). We next speculated that the *PrxCre* +/- genotype itself could lead to *Cyp2s1* induction. We determined *Cyp2s1* expression in the femur shaft in a subset of 13 week old male mice kept on normal chow. There was only *Cyp2s1* induction in the conditional knockout genotype *PrxCre* +/- *Nox4* fl/fl, but not in the whole-body knockout *Nox4* 0/0 genotype (Supplemental Figure 1B). This demonstrates that it is the presence of the *PrxCre* transgene construct rather than decreased Nox4 activity that increases *Cyp2s1* expression. Apart from these genes mentioned above, the *Nox4* genotype had little overall effect on gene expression. There were 84 genes showing significant ($P < 0.05$) interaction between genotype and diet with at least a 2-fold difference in the diet-mediated change for the *Nox4* fl/fl and the *PrxCre* +/- *Nox4* fl/fl genotype. No gene ontology classes were found to be significantly overrepresented. Presumably from bone marrow cells

adhering to the shaft, there were five *Igkv* immunoglobulin genes with different variable regions appeared to have a statistically significant interaction effect (Fig. 7C). However, this number is not substantially different from the 3.8 ($= 76 * 0.05$) genes of the 76 *Igkv* genes quantified in the RNA-Seq data set that by chance can be expected to show statistical significance at $P < 0.05$.

Previous research from our group has indicated that expression of osteocalcin is downregulated while expression of Nox2 and RANKL is upregulated by ethanol feeding in growing female rodents fed liquid diets from ages 6 to 14 weeks (Chen et al., 2011; Mercer et al., 2014; Watt et al., 2018). The RNA-Seq data set, as well as qRT-PCR, showed no significant main diet effect for these 3 genes in the current experimental design in male mice when EtOH exposure was begun during adulthood at age 13 weeks. However, sex did have a significant effect on these genes (Figs. 8E, 8F, 8G).

As most significant effects mediated by the ethanol diet are less than a 2-fold change, we speculated that such modest changes in highly expressed genes could be biologically relevant. Looking at the 100 most highly expressed genes for the *Nox4 fl/f* mice fed the control diet, there were 16 genes with a significant main diet effect (Fig. 7D) as compared to 5 genes expected by chance. Compared to the mouse reference genome, the list shows a 26X and >100X overrepresentation of genes involved in ossification and collagen fibril organization, respectively. It included all 6 collagen genes among the top 100 genes, as well as 3 other genes encoding structural bone matrix proteins (*Sparc*, *Ibsp* and *Dcn*). All these bone matrix genes are downregulated 19-44% by ethanol. We have previously observed downregulation of *Colla1* expression by ethanol in wild-type mice, and in this study, we further confirmed the ethanol-mediated downregulation of *Col2a1* expression by qRT-PCR (Fig. 8D). We conclude that ethanol downregulates the expression of *Myh3*, *Frzb* and several genes encoding major structural bone proteins in the femoral shaft.

Discussion

The role of Nox4 in bone morphology and bone turnover has previously been addressed by using whole-body Nox4 knockout mice. Female Nox4 knockout mice with the same genotype as the *Nox4* 0/0 animals showed increased trabecular width, trabecular thickness and trabecular density in the distal femur (Goettsch et al., 2013). In the context of ethanol feeding, our laboratory found decreased bone mass in both trabecular and cortical bone of the tibiae in males of a different Nox4 knockout strain (Watt et al., 2018). Our μ CT data of the tibiae in the current study suggest that these seemingly contrasting effects of Nox4 deletion on bone morphology may actually both occur. Thus, *Nox4* 0/0 mice kept on regular chow had increased cortical thickness and bone mineral density compared to *Nox4* fl/fl mice. On the other hand, *PrxCre* +/- *Nox4* fl/fl mice fed the liquid Lieber-DeCarli diets tended to have a reduced bone mass for both the trabecular area near the growth plate and for the cortical region as compared to those of the *Nox4* fl/fl mice. The latter may represent a liquid diet/genotype interaction. For mice on the chow diet, the *Nox4* 0/0 seemed to have larger effects on cortical bone than the *PrxCre* +/- *Nox4* fl/fl genotype (Fig. 2). This suggests that these morphological changes are primarily due to knockout of Nox4 in cells that are not of the mesenchymal lineage, such as osteoclasts, as previously suggested (Goettsch et al., 2013). Females in general had smaller dimensions of tibial bone but higher bone mineral density. Aging from 13 to 32 weeks of age led to a decrease in trabecular bone and an increase in bone mineral density (Fig. 2).

We used an ethanol feeding model in which the ratios between protein, fat, and carbohydrate were the same in ethanol and control-fed mice. The ethanol intake had clear effects on body weight and relative liver weight (Fig. 3). There were also small, but highly significant effects on cortical bone (Fig. 5). Surprisingly, there were none of the ethanol effects on the trabecular bone morphology that we found in our previous studies (Mercer et al., 2014; Watt et al., 2018). This discrepancy may be due to a higher ethanol concentration in the previous studies (30% vs. 25-28%), or due to the exchange of only carbohydrate calories with ethanol in previous research. As in our previous study with whole-body Nox4 knockout animals (Watt et al., 2018), both ethanol intake and Nox4 depletion led to reduced cortical bone

mass. Females responded more to the ethanol diet in terms of liver triglycerides, serum ALT and serum CTX. Yet, for bone morphology, there were no pronounced sex differences in the response to ethanol.

Interpretation of gene expression data from the femoral shafts needs to take into account that part of the RNA that originates from bone marrow cells. Only if a gene shows clearly higher expression in RNA isolated from the femoral shaft than in RNA isolated from the marrow, can the gene unequivocally be concluded to be expressed in the shaft. We have observed approximately 50-fold higher *Frzb* expression in the femoral shaft than in the marrow and essentially no *Myh3* expression in the marrow (data not shown). Thus, the regulation of *Frzb* and *Myh3* depicted in Fig. 7B is a shaft phenomenon.

Apart from downregulation of Nox4 expression and the strong upregulation of Cyp2s1 and Gm45336 in *PrxCre +/- Nox4 fl/fl* animals that seems regulated by the PrxCre transgene construct itself, there were hardly any effects of the conditional Nox4 knockdown on gene expression in the femoral shaft. However, the RNA-Seq data set also revealed that the most highly expressed NADPH oxidase gene was Nox2 (*Cybb*), with approximately 650 times higher expression than Nox4. Nox4 deletion may thus only lead to a marginal decrease in NADPH oxidation. The lack of strong effects of Nox4 depletion on the shaft gene expression suggests that the observed bone morphology effects in the conditional Nox4 knockout mice are mediated by mesenchyme-derived cells acting earlier in development and/or residing outside of the bone matrix itself.

In contrast to previous studies, we did not observe ethanol-mediated effects on Nox2 or RANKL mRNA expression in the femoral shaft. RANKL expression in the femoral bone marrow was likewise unaffected by ethanol (data not shown). A recently implemented technical improvement in our laboratory is the ability to consistently isolate RNA of high quality from mouse bones (Pedersen et al., 2019), reducing the risk of finding spurious changes due to variations in the RNA integrity. In combination with the higher number of animals in the ethanol feeding experiment compared to previous studies, the lack of induction of these two genes by the current ethanol diet is a robust finding. It is possible that RANKL, an inducer of bone resorption, is upregulated in other bones leading to the increased serum CTX observed in females. Lack of ethanol regulation of Nox2 and RANKL in the femur shaft may also reflect a different

diet composition with a lower blood ethanol concentration resulting from a final dietary dose of 28 % of calories compared to 30% of calories in other studies. In a 1-week ethanol binge-drinking model of mice, we recently did observe a robust induction of both femoral shaft and femoral one marrow RANKL mRNA (data not shown).

Since Nox4 had surprisingly little effect on gene expression in the femoral shaft, we also tested whether Nox4 and ethanol feeding affected ROS in the shaft. As an indicator of ROS, nitrotyrosine determined by immunohistochemistry was previously observed to become increased with chronic alcohol feeding of female mice, particularly in the bone marrow under the tibial growth plate (Alund et al., 2017). We quantified nitrotyrosine in femoral shaft proteins from male mice from the chronic ethanol feeding study using highly sensitive Western blots allowing detection of < 2 ng of nitrotyrosine BSA. The levels of nitrotyrosine were very low and not clearly affected by either ethanol feeding or conditional knockdown of Nox4 (Supplemental Figure 2). It is possible that the lower concentration of dietary alcohol in the current study compared to previous studies results in lower blood ethanol concentrations and thus less generation of ROS. On the other hand, the data suggests that the observed ethanol effects on bone structure and femoral gene expression are independent of local ROS in the bone matrix, at least ROS leading to protein nitrotyrosinylation.

In the current study, we did observe the previously noticed downregulation of *Col1a1*, as well as of 5 other highly expressed collagen genes. Downregulation of collagen genes and other genes for structural bone proteins might be one mechanism whereby ethanol decreases bone quality. Whether the downregulation of *Frzb* and *Myh3* expression has consequences for the bone morphology will have to be determined in future research. The Nox4 genotype had no significant effect on any of these genes.

We did not address the influence of sex on global gene expression in the femoral shaft. However, there was a significant main effect of sex for 6 of the 8 genes we analyzed by qRT-PCR, suggesting that sex has a strong influence on the femoral gene expression.

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In summary, we conclude that chronic ethanol feeding in mice decreases cortical bone mass and reduces expression of *Frzb*, *Myh3* and genes for major structural bone proteins independently of *Nox4* gene expression within the osteoblast lineage.

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Authorship Contributions

Participated in research design: Pedersen, Watt, Ronis

Conducted experiments: Pedersen, Osborn, Robinson, Williams, Watt, Denys

Contributed mouse genotype: Schröder

Performed data analysis: Pedersen, Robinson, Ronis

Wrote or contributed to the writing of the manuscript: All authors

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Footnotes

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The authors declare that they have no conflicts of interest.

Legends for figures

Figure 1. Conditional knockdown of Nox4 expression in long bones. (A) Model for PrxCre-mediated excision of exons 1 and 2 of the Nox4 gene. Indicated are genotyping amplicons for distinguishing the various Nox4 alleles. (B-D) The concentrations of Nox4 mRNA from the knockout (K/O) region, total Nox4 mRNA, and Nox2 relative to β -actin mRNA were determined in the femoral shaft and femoral bone marrow from 13 weeks old mice by qRT-PCR. There were 5 – 11 mice per group. (E-F) The concentration of Nox4 mRNA was determined in liver and kidney aliquots from 32 weeks old female mice. There were 4 mice per group. *, ***: $P < 0.05$, $P < 0.001$ vs. the *Nox4 fl/fl* genotype.

Figure 2. Sex and age affect trabecular bone of mice maintained on standard chow. Males and females of genotypes *Nox4 fl/fl*, *PrxCre Nox4 fl/fl*, and *Nox4 0/0* at 13 and 32 weeks of age were analyzed. There were 5 – 12 mice per group. 3-way ANOVAs were conducted with sex, age and genotype as the three factors. The panels indicate significant main effects and important interaction effects. (A - H) Trabecular bone parameters for a region of the tibiae 0.3 – 1.2 mm below the primary spongiosa. (J - N) Cortical bone parameters for the tibiae.

Figure 3. Age, sex and the *Nox4* genotype affects cortical bone of mice maintained on standard chow. Males and females of genotypes *Nox4 fl/fl*, *PrxCre Nox4 fl/fl*, and *Nox4 0/0* at 13 and 32 weeks of age were analyzed. There were 5 – 12 mice per group. 3-way ANOVAs were conducted with sex, age and genotype as the three factors. The panels indicate significant main effects and important interaction effects. (A - E) Cortical bone parameters for the tibiae.

Figure 4. Chronic ethanol feeding for 3 months affects body composition and bone turnover. (A) The diagram illustrates the time course of the body weights for males and females fed either the ethanol diet or control diet for 3 months. (B-D) Body weight, relative liver weight, and the concentration of liver triglycerides were determined at the end of the chronic ethanol feeding experiment. (E-G) Serum concentrations of ALT, Osteocalcin and CTX were determined. 3-way ANOVAs were conducted with sex, diet and genotype as the three factors. The panels indicate significant main effects. For ALT and CTX, a separate 2-way ANOVA for just the data for females were calculated.

Figure 5. Trabecular data for mice from the chronic ethanol feeding experiment. (A-H) Trabecular bone parameters for a region of the tibiae 0.4 - 0.7 mm distal to the proximal growth plate. 3-way ANOVAs were conducted with sex, diet and genotype as the three factors. The panels indicate significant main effects.

Figure 6. Cortical data for mice from the chronic ethanol feeding experiment. (A-E) Cortical bone parameters for the tibiae. 3-way ANOVAs were conducted with sex, diet and genotype as the three factors. The panels indicate significant main effects.

Figure 7. RNA-Seq analysis of femoral shafts of mice from the chronic ethanol feeding experiment. RNA-Seq was conducted on femoral shaft RNA isolated from male mice. A 2-way ANOVA was conducted for each gene with diet and genotype as the two factors. The test probabilities were not

adjusted for the multiplicity of genes. Volcano plots depicting the test probability for each gene against the fold change in gene expression are shown for the main effect of diet (**A**), the main effect of genotype (**B**) and the interaction effect between diet and genotype (**C**). Points above the red horizontal dashed line represents genes with test probabilities $P < 0.05$. Red points indicate genes with at least a 2-fold change in gene expression and a test probability $P < 0.05$. (**D**) Among the 100 genes with the highest expression levels in *Nox4* *fl/fl* mice on the control diet, the change in gene expression caused by ethanol is depicted for the 16 genes that show a significant ($P < 0.05$) main diet effect.

Figure 8. qRT-PCR gene expression analysis replicates findings from RNA-Seq. Gene expression was determined for RNA isolated from the femur shafts of mice from the chronic ethanol feeding experiments by qRT-PCR. The expression levels were normalized to the concentration of total RNA. The expression levels were determined for total Nox4 mRNA (A), Frzb (B), Myh3 (C), Col2a1 (D), osteocalcin (E), RANKL (F), Nox2 (G), GAPDH (H) and Cyp2s1 (I). 3-way ANOVAs were conducted with sex, diet and genotype as the three factors. The panels indicate significant main effects.

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Table 1. qRT-PCR primers

Gene	Gene symbol	Forward primer	Reverse primer
Nox4 knockout region	<i>Nox4</i>	acc tct gcc tgc tca ttt gg	cct agg ccc aac att tgg tga
Total Nox4	<i>Nox4</i>	gga tca cag aag gtc cct agc	gtt gag ggc att cac caa gtg
Nox2	<i>Cybb</i>	ggg atg aat ctc agg cca at	gcc gtc cat aca gag tct tc
β-Actin	<i>Actb</i>	aga tga ccc aga tca tgt ttg aga	cca gag gca tac agg gac agc
GAPDH	<i>Gapdh</i>	cat ctt cca gga gcg aga cc	cct tca agt ggg ccc cg
RANKL	<i>Tnfsf11</i>	cag cat cgc tct gtt cct gta	ctg cgt ttt cat gga gtc tca
Cyp2S1	<i>Cyp2s1</i>	ttg gca tcc gtt tgc cct at	gag aac atc tcg tag gcc tgg
Osteocalcin	<i>Bglap</i>	agc ctt cat gtc caa gca gga g	gac tga ggc tcc aag gta gcg
Col2a1	<i>Col2a1</i>	ggc cag gat gcc cga aaa tta	cgc acc ctt ttc tcc ctt gt
Myh3	<i>Myh3</i>	ggc caa act gat cac tcg ga	atg gac tcc ctc ctc tgc at
Frzb	<i>Frzb</i>	aca act atg tca tcc ggg ct	tga cgg tgt ccc ttg gaa tg

Figure 1

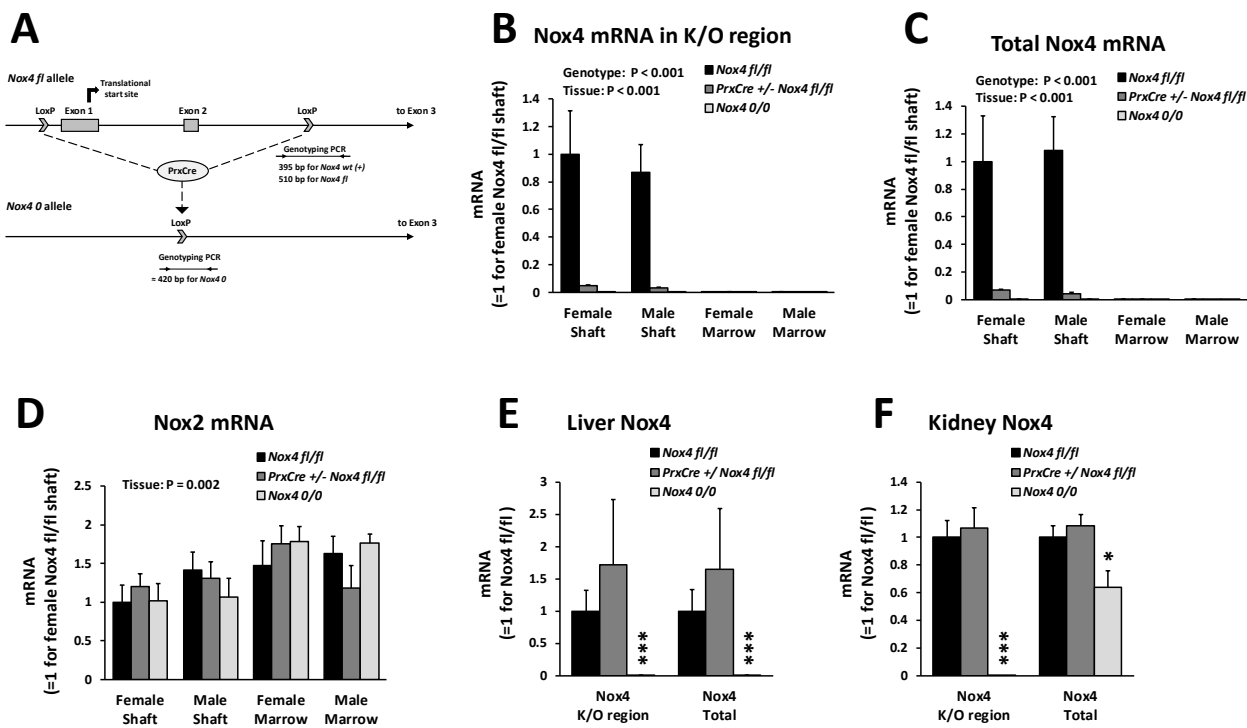


Figure 2

Trabecular parameters

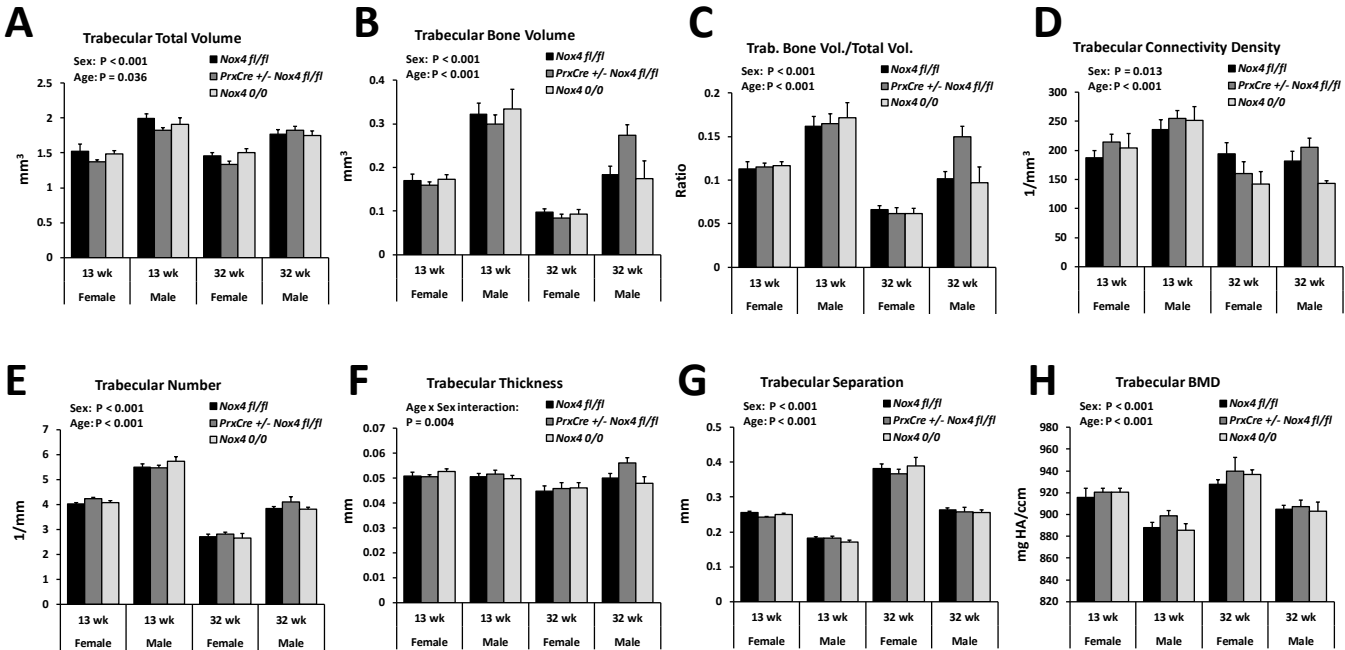


Figure 3

Cortical parameters

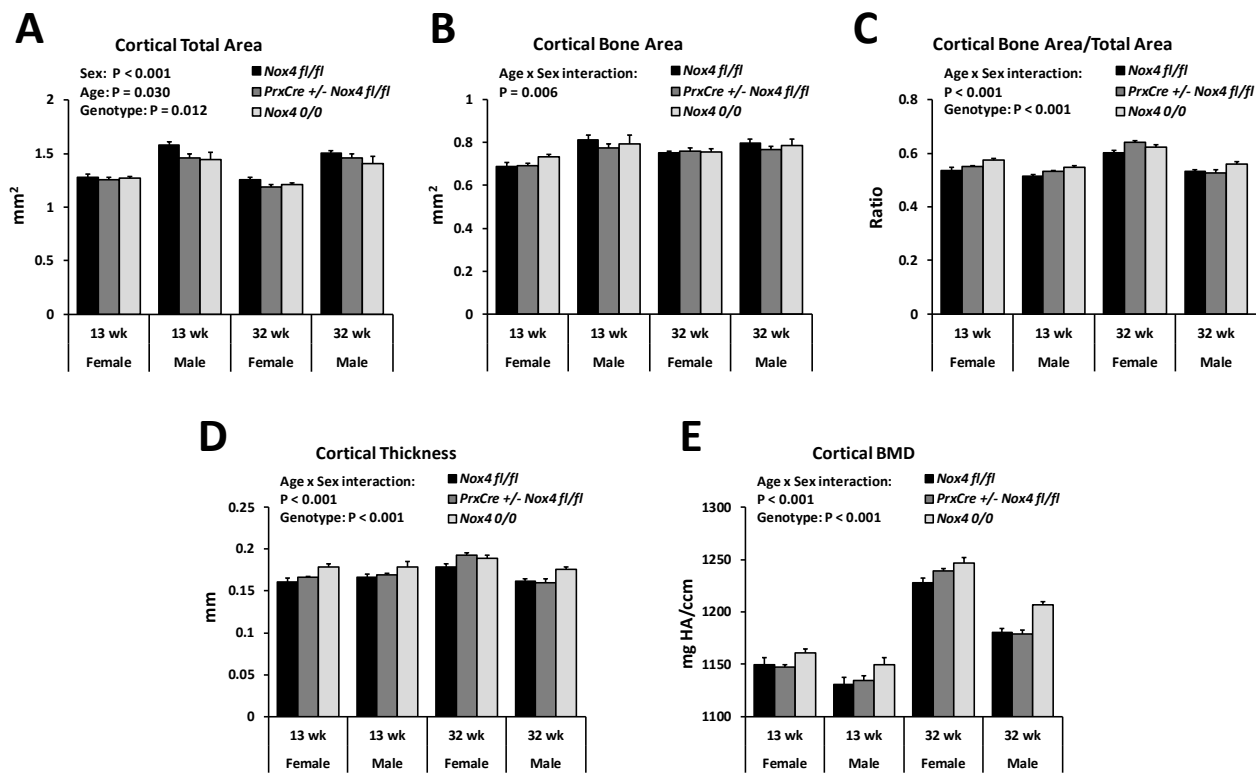


Figure 4

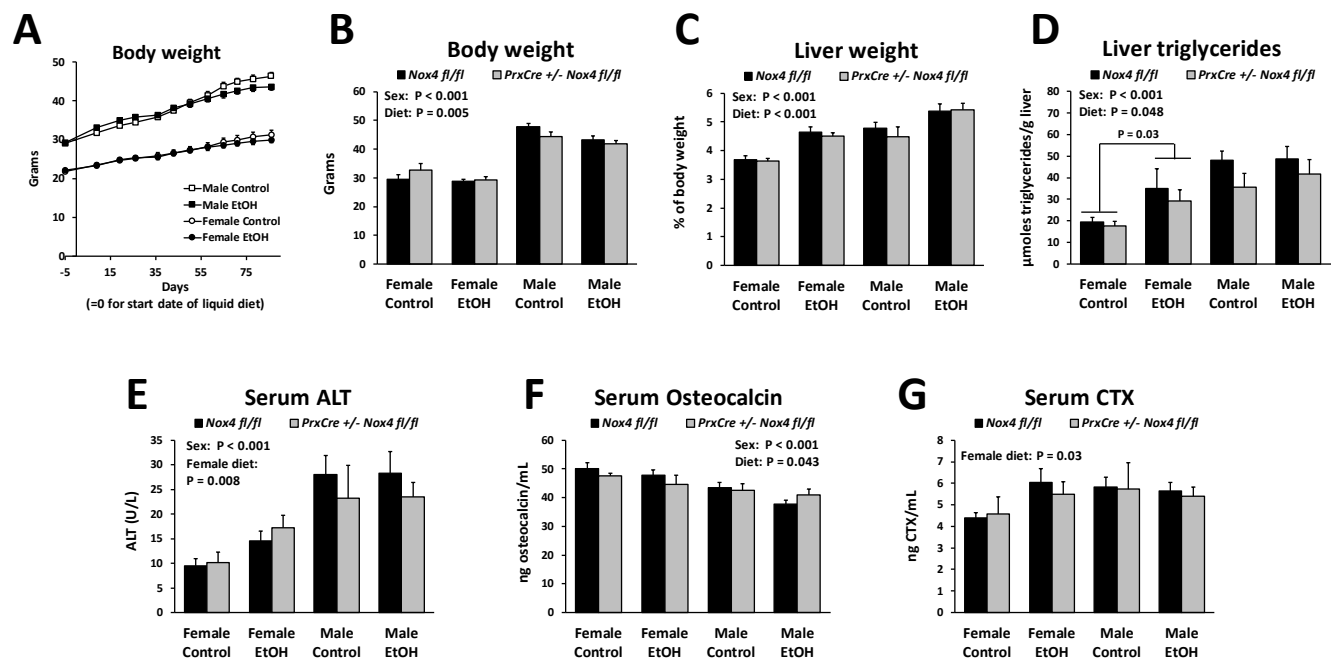


Figure 5

Trabecular parameters

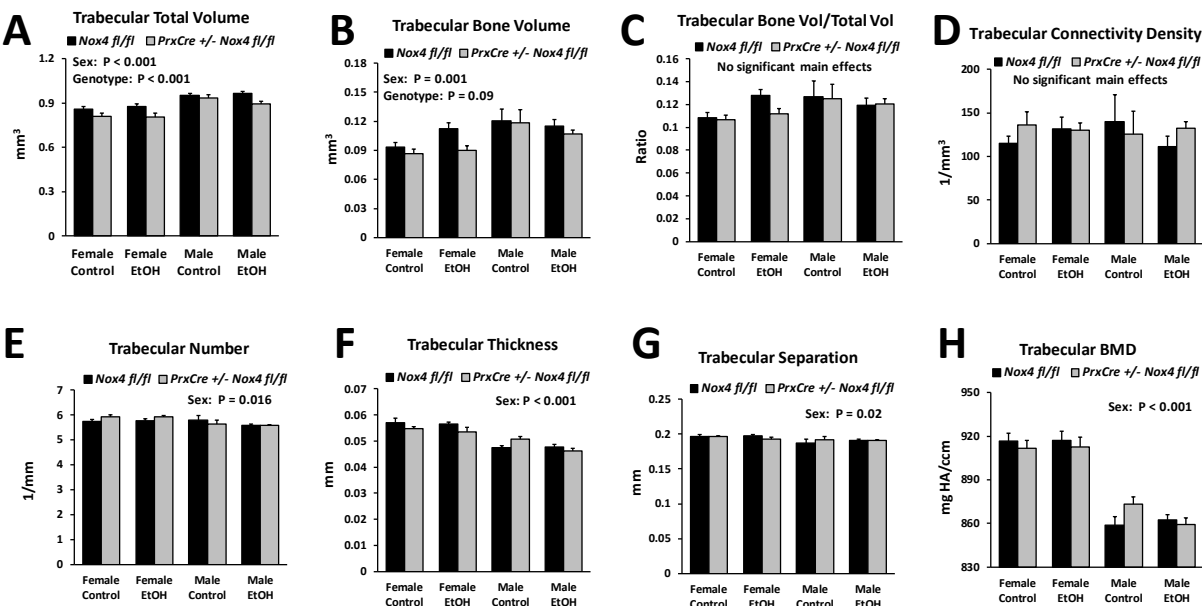


Figure 6

Cortical parameters

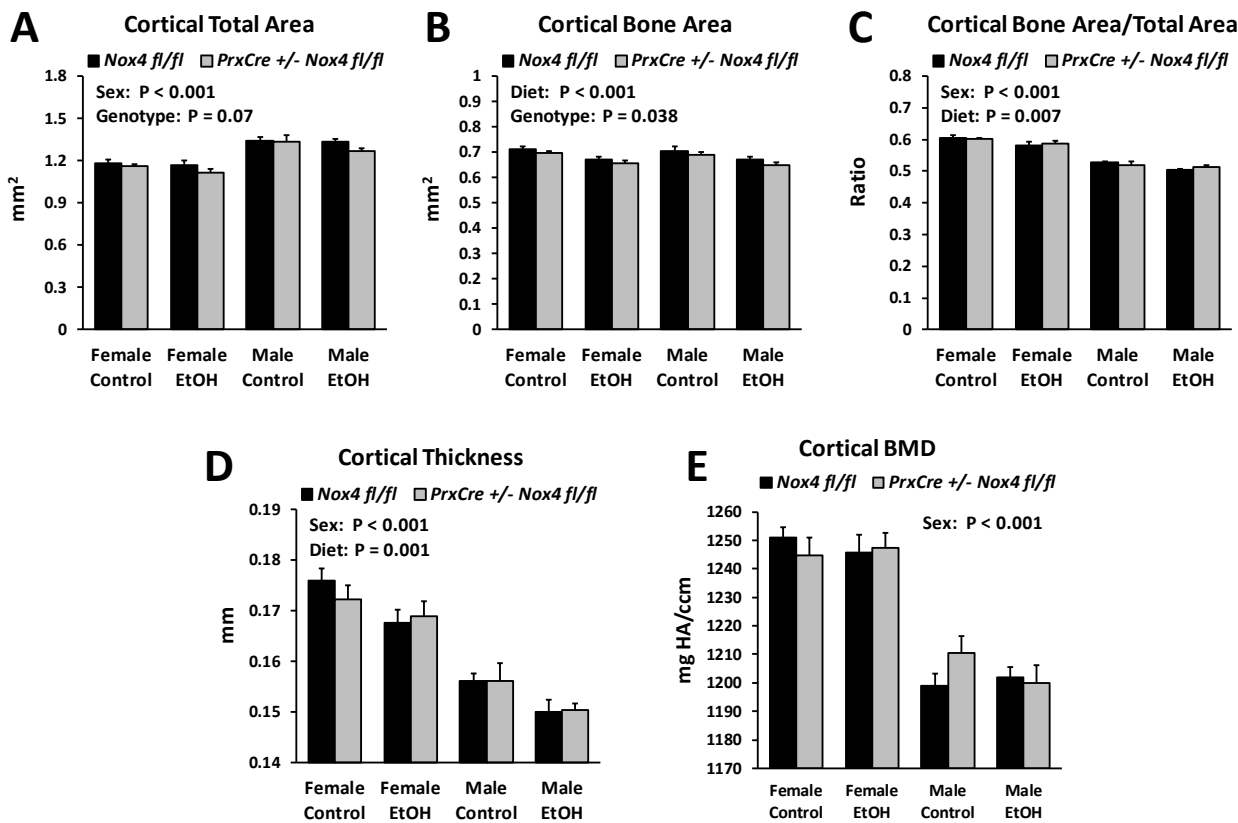


Figure 7

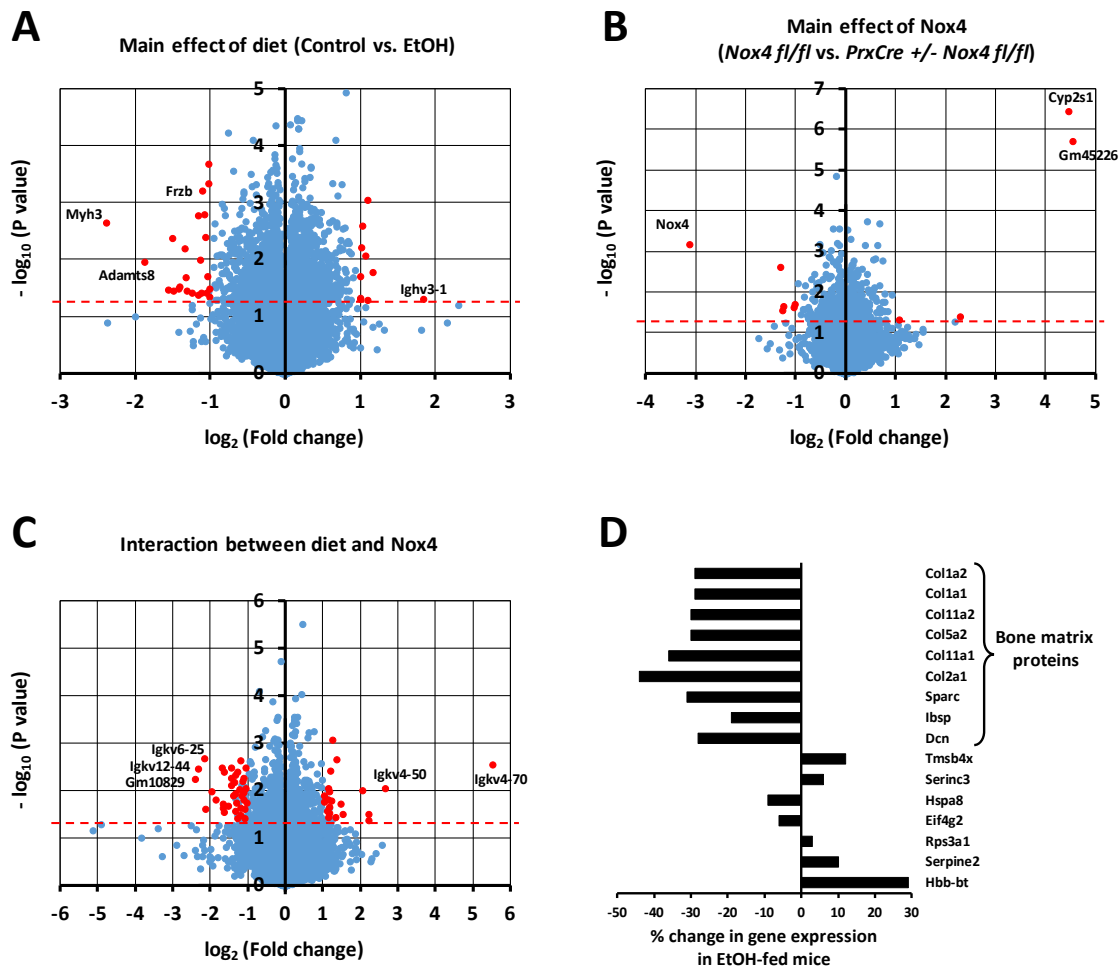


Figure 8

