Pannexin 3 deletion reduces fat accumulation and inflammation in a sex-specific manner

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Abstract

Pannexin 3 (PANX3), a channel-forming glycoprotein, facilitates nutrient-induced inflammation in vitro, and genetic linkage data suggest that it regulates body mass index. In this study, we characterized global *Panx3* knockout (KO) mice in the context of forced exercise (FEX) and high fat diet (HFD). Wildtype (WT) mice placed on either a western diet or a HFD showed increased expression of Panx3 mRNA and protein in adipose tissue, while FEX reduced its expression. We did not observe any overt differences between WT and *Panx3* KO female mice; however, sedentary (SED) *Panx3* KO male mice fed a chow diet had lower body weight and lower fat mass from 12 weeks that persisted until 30 weeks of age compared to SED WT. Remarkably, the body weight and fat mass reduction in *Panx3* KO mice was similar in magnitude to the effect of 6 weeks of FEX in WT mice. Additionally, *Panx3* KO mice demonstrated lower inflammatory status in white adipose tissue compared to SED and FEX WT mice. When challenged with a HFD, male *Panx3* KO mice exhibited less weight gain and lower inflammatory status in quadriceps muscle and white adipose tissue. These data suggest that *Panx3* regulates fat accumulation and inflammation in white adipose and skeletal muscle tissue of male mice and may act as a molecular switch that is controlled by nutrients and exercise.

Introduction

Lack of physical activity and excessive caloric consumption are major underlying causes of obesity (1). While exercise training and caloric deficit are effective treatments for obesity, many find these interventions difficult to sustain (2). Genetic factors increase susceptibility to weight gain (3), and understanding which genetic factors underlie obesity will assist clinicians in determining the best pharmacotherapeutic options for a given patient.

Pannexins (PANX1, 2, 3) are glycoproteins that form membrane channels to allow the passage of ions and metabolites for autocrine and paracrine signaling in a variety of cells (4). Previous reports have shown that PANX1 is expressed in adipocytes and has a functional role in immune cell recruitment (5), adipocyte hypertrophy and fat accumulation (6), glucose metabolism (7), and thermoregulation in brown fat (8). While PANX3 has been shown to form channels at cell and endoplasmic reticulum membranes of osteoblasts (9; 10), skeletal muscle (11), and chondrocytes (10; 12; 13), recent evidence suggests it may also play a role in adipogenesis and inflammation (16; 19). Halliwill and colleagues found that the *Panx3* gene is linked to body mass index in male mice, using a systems approach involving qualitative trait loci (QTL) mapping, gene expression, and gene expression correlation network analysis (16). This group also identified *Panx3* as a component of an important adipocyte signaling and differentiation gene network through *homeodomain-interacting protein kinase 2* (Hipk2) (19). Interestingly, the ablation of the Hipk2 reduced adipogenesis in white adipose tissue and was protective against weight gain under high fat diet (HFD) conditions (19). These studies provide indirect evidence that *Panx3* may be involved in the molecular mechanism of fat accumulation.

A consequence of excessive fat accumulation is inflammation of adipose tissue (20-23). This inflammation is thought to contribute to many comorbidities including insulin resistance (22; 24-27). Our group previously reported that the saturated-fatty acid palmitate activated cell-intrinsic pro-inflammatory programs in isolated muscle cells and concomitantly increased *Panx3* expression (28). Additionally, we demonstrated that PANX3 channels allowed passage for adenosine triphosphate (ATP) release, which in turn attracted monocytes towards the muscle cells (28). This *ex-vivo* paradigm would suggest that PANX3 may be a contributor to nutrient-induced skeletal muscle inflammation by acting as a conduit for ‘find me’ signals to immune cells. While

Panx3 was expressed at low levels in skeletal muscle tissue of mice fed either a chow or a HFD diet, HFD induced the expression of *Panx3* in adipose tissue (28). This was the first published finding of *Panx3* expression in mouse adipose tissue. However, its role in diet-induced obesity, fat accumulation, inflammation and insulin resistance *in vivo* has not been investigated.

Considering the evidence above, we sought out to determine the physiological effects of a global deletion of *Panx3* in mice exposed to forced exercise (FEX) and dietary excess. While there were minimal differences between WT and *Panx3* KO female mice, in males, *Panx3* KO mice had lower body weight, fat mass and inflammation in fat tissue compared to WT mice to the same extent as 6 weeks of forced treadmill running, and this potentially beneficial phenotype was still evident when challenged with caloric excess. This would suggest that the deletion of *Panx3* may have metabolic health benefits to male mice which we observe to be associated with an attenuation of fat accumulation and inflammation. In addition, our studies suggest sex-specific roles of *Panx3* in metabolism.

Results

Male, but not female, *Panx3* KO mice weigh less and have less fat mass at 12 to 24 weeks of age compared to WT mice.

To determine the effect of deleting *Panx3* on body weight, composition, and blood glucose regulation, female and male congenic WT and *Panx3* KO (KO) mice were fed *ad libitum* on rodent chow diet and weighed bi-weekly until 24 weeks of age. In female mice, there were no significant differences in body weight (Fig. 1A), fat mass (Fig. 1B), lean mass (Fig. 1C), or blood glucose tolerance (D & E) between genotypes. In contrast, male *Panx3* KO mice weighed significantly less than controls from 20 – 24 weeks of age (Fig. 1F). Fat mass was lower in *Panx3* KO compared to

WT mice at 12 weeks, and this difference was more prominent at 24 weeks of age (Fig. 1G), while there were no differences in lean mass at these time points (Fig. 1H). No significant differences in glucose tolerance were observed in male mice (Fig. 1I & J). A subset of male mice was assessed using a metabolic cage to determine if there are differences in sleep, activity, food consumption, and respiration (Supp. 1). There were no differences in any of these measures between genotypes. These results suggest that under standard diet conditions the deletion of *Panx3* reduces whole body fat accumulation in male, but not female mice, with no effects on lean mass up to 24 weeks of age or on glucose tolerance.

Panx3 expression in adipose tissue is induced by high fat diet and inhibited by forced exercise.

We next determined if diet and activity can influence PANX3 protein level in adipose tissue. A cohort of male mice were fed a normal chow diet or a high fat diet (HFD) from 12 to 28 weeks of age. Interestingly, PANX3 protein levels were higher in the HFD animals compared to the chow fed animals (Fig. 2A & B), which was similar to the levels detected in the *Panx3* KO mice on either diet. When measuring body weight, *Panx3* KO mice had significantly lower body weight/initial body weight compared to WT mice (Fig. 2C). These results support previous findings of ours that Panx3 expression is induced by dietary factors in a mouse model.

A subset of male mice were fed a western diet containing 45% kcals from fat or a normal chow diet and allocated to a SED or FEX group. On normal chow, very low to undetectable levels of *Panx3* were observed in visceral adipose tissue of SED and FEX mice (Supp. 2A & B). However, SED Western diet animals had significantly higher levels of *Panx3* expression compared to both chow fed groups (Supp. 2A & B). Interestingly, FEX animals fed the Western diet had low to

undetectable levels of *Panx3* in their visceral adipose tissue (Supp. 2A & B). These data suggests that diet and exercise each, yet inversely, can regulate Panx3 expression in adipose tissue.

Male *Panx3* KO mice fed a HFD have less inflammation in epidydimal adipose and skeletal muscle tissue.

Considering HFD regulated PANX3 expression in adipose tissue, we then set out to determine if *Panx3* KO mice are protected from diet-induced weight gain and inflammation. *Panx3* KO and WT mice were placed on a HFD (60% kcal from fat) from 12 to 28 weeks of age. At sacrifice, liver, quadriceps muscle and epidydimal white adipose tissues (eWAT) were collected for analysis of inflammatory markers. Macrophage marker *Emr1* and *Itgax* (CD11c), pro-inflammatory genes *Tnfa*, *Nos2*, *Il12a*, *Ccl2* and *Il6* and anti-inflammatory markers *Arg1*, *Mrc1*, *Il10* and *Chi3l3* were included in analyses. A pro-inflammatory score was calculated as the ratio of pro-inflammatory over anti-inflammatory markers and reflects the inflammatory status of the tissue. *Panx3* KO mice had significantly lower overall inflammatory index in eWAT ($p = 0.0438$) (Fig. 3A & B), quadriceps ($p = 0.0007$) (Fig.3 C & D), but no significant difference in liver ($p = 0.3838$, Fig. 3 E & F) compared to WT mice. These results suggest that *Panx3* KO male mice have less skeletal muscle and fat tissue inflammation compared to WT mice while on a HFD.

***Panx3* deletion has a similar effect on body weight and body composition as 6 weeks of forced exercise in male mice.**

Considering that exercise regulates *Panx3* expression in visceral adipose tissue, we sought to determine if there would be differences in the response to FEX between WT and *Panx3* KO mice. Female and male WT and *Panx3* KO mice were randomly allocated to either a sedentary (SED) or a FEX protocol from 24 to 30 weeks of age. In females, *Panx3* KO mice weighed significantly

less than WT mice regardless of activity at 30 weeks of age (genotype: $p = 0.0159$) (Fig. 4A). There were no differences in either fat mass and fat mass corrected for body weight (Fig. 4B & C) or lean mass and lean mass corrected for body weight (Fig. 4D & E) mass between genotypes, however, both WT and *Panx3* KO mice fat mass (Fig. 4B), and fat mass corrected for body weight (Fig. 4C), was reduced in response to FEX.

In males, SED *Panx3* KO mice weighed significantly less than SED WT mice, while there were no differences between genotypes for the FEX groups (Fig. 4F). SED *Panx3* KO mice had significantly lower fat mass and fat mass corrected for body weight compared to SED WT mice (Fig. 4 G & H). Interestingly, FEX significantly decreased fat mass in WT mice, while FEX had no additional effect on fat mass in *Panx3* KO mice, as SED *Panx3* KO mice had similar fat mass to FEX WT mice (Fig. 4G & H). This suggests that deletion of *Panx3* alone has a profound effect on fat mass that was not further enhanced by exercise. While there were no significant differences in raw lean mass among the groups (Fig. 4I), when lean mass was corrected for body weight, SED *Panx3* KO had significantly higher lean mass compared to SED WT mice (Fig. 4J). These results suggest that the deletion of *Panx3* reduces body weight, fat mass, and increasing lean mass to the same extent as 6 weeks of FEX in male WT mice.

Female *Panx3* KO mice have higher inflammation in quadriceps and liver tissues compared to WT mice

Changes in adiposity and lean mass could be due to inflammatory activation of adipose, liver, and skeletal muscle tissue. Therefore, we next determined if *Panx3* deletion influences inflammatory gene expression in these metabolic tissues. Female WT and *Panx3* KO mice from both SED and FEX groups were sacrificed, and inflammatory genes were measured as described above. While

there were no differences in inflammatory index of the visceral fat (Fig. 5B), female *Panx3* KO mice had significantly higher inflammatory indexes for both quadriceps (Fig. 5A) and liver (Fig. 5C) compared to WT mice. This would suggest that in female mice the deletion of *Panx3* leads to higher inflammation in the quadriceps and liver tissues.

Male *Panx3* KO mice have lower inflammation in quadriceps and visceral adipose tissue compared to WT mice.

Male WT and *Panx3* KO mice from both SED and FEX groups were sacrificed, and liver, quadriceps muscle and visceral white adipose tissues were collected for analysis of inflammatory markers as described above, and an inflammatory index was calculated. SED WT mice had a significantly higher inflammatory index compared to the FEX animals regardless of genotype, however no group was different than SED *Panx3* KO in the quadriceps (genotype x activity, $p = 0.0156$) (Fig. 6A). In visceral adipose tissue, SED *Panx3* KO mice had significantly lower inflammation compared the SED WT and FEX mice (genotype x activity, $p = 0.0138$) (Figure 6B), however there were no differences among the groups for liver inflammatory index (Fig. 6C). These results suggest that deletion of *Panx3* results in lower inflammation in skeletal muscle, to the same extent as 6 weeks of FEX, and to a greater degree in white adipose tissue.

Discussion

A number of studies have shown that *Panx3* has diverse roles in the development and pathophysiology of skin (14-18), bone (9; 14; 29), and cartilage (10; 12; 13). While there have been indirect reports for a role of *Panx3* in controlling body mass index (16) and adipogenesis (19), there is no information directly linking *Panx3* to fat accumulation and body weight. Here we show that diet and exercise are regulators of Panx3 expression in adipose tissue, and that the

deletion of *Panx3* in C57BL/6 mice results in reduced body weight in adulthood. Furthermore, in male mice, where this phenotype seems to be more pronounced, it is accompanied by less fat accumulation, with a similar effect as 6 weeks of FEX. Additionally, the lower fat mass in male *Panx3* KO mice was accompanied by a reduced inflammatory index in both the quadriceps muscle and visceral white adipose tissue, with the inflammation in visceral white adipose tissue being even lower than that in FEX WT mice. This suggests that *Panx3* expression may have profound implications for regulating visceral white adipose tissue inflammation. In a second experiment exploring the effect of *Panx3* deletion in a HFD context, we observed that *Panx3* KO mice are modestly protected against diet induced weight gain. Interestingly, even when challenged with HFD, *Panx3* KO mice had a lower inflammatory index in skeletal muscle and white adipose tissue. This suggests that the protective anti-inflammatory effects of *Panx3* deletion is maintained even when the body is challenged with an extreme HFD.

We have previously shown, in cultured myotubes, that *Panx3* propitiates the cell-intrinsic pro-inflammatory effects of dietary fatty acid palmitate (Pillon et al., 2014). Blocking of PANX3 channels reduced the capacity of cultured skeletal muscle cells to recruit monocytes. While in the present study we did not quantify the immune cell recruitment to adipose or skeletal muscle tissue, the reduced expression of pro-inflammatory genes suggests that deletion of *Panx3* may interrupt high fat diet-induced inflammation. Additionally, or alternatively, *Panx3* deletion results in an inability to accumulate lipids in adipocytes, thus, inhibiting adipocyte hypertrophy and stress (Haczeyni et al., 2018). While we measured overall fat accumulation in this study, we did not assess the morphology of adipose tissue between genotypes, so we are unable to conclude if this diminished adipose tissue inflammation is associated with altered adipocyte size or number. Interestingly, total adiponectin, a measure of metabolic health, was significantly lower in male

SED *Panx3* KO mice compared to SED WT mice (Supp. 5F). While this outcome would suggest that the SED WT mice are metabolically healthier than the *Panx3* KO mice, the HMW adiponectin, which is a better measure than total adiponectin, was not significantly different (Supp. 5G & H). Similarly, there were no significant differences in blood glucose tolerance among the male groups (Supp. 4B). The difference in total adiponectin levels between SED WT and Panx3 KO mice may be the result of reduced number of adipocytes in the *Panx3* KO mice, and thus fewer cells that secrete adiponectin (Meyer et al., 2013). Additionally, we observed no overt differences in circulating metabolic measures such as triglycerides, cholesterol, and insulin (Supp. 4). These results suggest that the deletion of *Panx3* in male mice results in tissue specific inflammatory changes, that however do not influence overall metabolic health.

While consistent daily exercise is a pillar of health, much of the literature suggests that exercise alone cannot reduce adiposity in people with obesity, and dietary interventions are necessary (30; 31). In mouse models, the extent to which exercise can influence body weight is dependent on the diet and the nature of the exercise intervention (voluntary versus forced) (32; 33). In this study, we found that FEX attenuated weight gain in WT mice (Supp. 3F), while in *Panx3* KO mice FEX had no further effect on reducing body weight (Supp. 3F). This suggests *Panx3* is necessary for weight gain in male adult C57BL/6 mice. Additionally, we observed a significant decrease in fat mass of male WT mice from 24 to 30 weeks of age as a result of FEX (Supp. 3G & H) while there were no changes in *Panx3* KO mice. Considering that there were significant differences in fat mass between male WT and *Panx3* KO mice, it appears that the attenuation of body weight gain in the *Panx3* KO mice is predominantly the result of a reduced fat depot. The magnitude of difference in fat mass between SED WT and Panx3 KO mice was similar to the effect of 6 weeks of FEX, would suggest that manipulating *Panx3* expression may have

profound effects on fat accumulation in mice. This reduction in fat mass may be what reduced tissue inflammation in *Panx3* KO mice.

Exercise can have clinically significant effects on chronic systemic inflammation (34-36). Studies in mouse models show that exercise can attenuate visceral white adipose tissue inflammation (37), specifically, the recruitment of M1-like macrophages and CD8+ T cells upon exposure to HFD (38). In this study we found that male FEX WT mice had a lower inflammatory index in visceral white adipose tissue compared to SED mice, suggesting that the exercise intervention had an anti-inflammatory effect in this tissue. Interestingly, male SED *Panx3* KO mice had similar measures of fat accumulation, inflammation, and plasma metabolic parameters as FEX WT mice, with no additional benefit from FEX in *Panx3* KO mice. This would suggest that the deletion of *Panx3* has a predominant effect on these measures, and that the addition of FEX does not induce any further adaptations.

While *Panx3* levels are undetectable in the chow fed WT animals, they were significantly elevated in mice fed a western diet or HFD, suggesting that *Panx3* expression is sensitive to dietary factors, as gleaned from previous work in cell culture models (28). Moreover, FEX was able to counter diet-induced *Panx3* upregulation (Supp. 2A & B). This would suggest that exercise is able to inhibit the signalling responsible for *Panx3* expression caused by dietary factors. However, future studies will be needed to determine what signaling pathways are responsible for the induction and suppression of *Panx3* expression by diet and exercise. Our previous data along with those reported by others indicate that the toll-like receptor 4 (TLR4)/nuclear factor -kB (NF-kB) pathway is activated by the saturated fatty acid palmitate (42), and we previously showed that this pathway mediated the expression of *Panx3 (28)*. Conversely, moderate aerobic exercise is known

to down regulate TLR4 receptors, which would down regulate the proinflammatory NF-kB pathway and may thus inhibit *Panx3* expression (43).

The comparison of sexes is an understudied area, especially in the field of pannexin biology. In this study we included both male and female littermates, and thus were able to determine interactions between *Panx3* genotype and sex in response to HFD and FEX. Interestingly, while there was some effect of deleting *Panx3* on body weight gain in females later in life, we found that the effects of deleting *Panx3* were more evident in males for weight gain, fat accumulation, and tissue inflammation. Considering male C57BL/6 mice are much more susceptible to weight gain and fat expansion under a variety of dietary conditions compared to female mice (44), this may explain why we observed a greater effect in males compared to females. Furthermore, quantitative trait loci data linking *Panx3* to body mass index were specific to male mice (16). However, previous reports have demonstrated sexual dimorphisms in response to diet and exercise in terms of body weight gain and metabolism, which are dominated by a strong male effect (44). In contrast, female mice have been shown to have an enhanced anti-inflammatory response in white adipose tissue under HFD conditions, which may have protective effects against additional body weight gain (45). The estrogen receptor also appears to play a protective role in metabolic disease and obesity (46). We did not measure sex hormones in this study, and there are no published reports of estrogen and testosterone levels in *Panx3* KO mice. However, *Panx3* is expressed in Leydig cells (47) and therefore may influence testosterone production, which has been shown to be an important hormone in the pathogenesis of obesity (48-51).

Conclusion

We have shown that the deletion of *Panx3* can attenuate body weight gain due to a reduction in adipose tissue accumulation, and lower inflammatory status in metabolic tissues such as skeletal muscle and white adipose tissue in male mice. This genotype effect was equivalent to that observed after 6 weeks of daily FEX, suggesting that *Panx3* may play a significant role in regulating weight gain, fat accumulation and inflammation outcomes. Future research is needed to determine which cell types are responsible for these effects of *Panx3*, as this study used a global KO model. Findings such as those reported in this study could lead to novel strategies to target PANX3 channel function at selected tissues and time points, as an adjunct strategy in the comprehensive management of obesity and inflammatory diseases.

Materials & Methods

Animals and ethics

Experiments performed on animals were approved by the Animal Care Committee of the University Council on Animal Care at the University of Western Ontario, London ON, Canada (UWO # 2019-069), and in accordance with relevant guidelines and regulations. *Panx3* KO mice were generated as described previously (12). These mice were backcrossed with the WT mice: C57BL/6 N strain mice from Charles River Canada (Saint-Constant, PQ) until a congenic line was obtained (minimum of 10 backcrossed generations). Mice were weaned at 3 weeks of age and fed either a chow diet (6.2% kcals from fat), Western (45% kcals from fat) or a HFD (60% kcals from fat, Test Diet 58Y1) as described in the respective sections. At sacrifice, mice were sacrificed using carbon dioxide. Immediately after, blood was collected via cardiac puncture, visceral fat, skeletal muscle, and liver were collected and immediately snap frozen and stored at -80.

Forced Exercise (FEX) Protocol

At 24 weeks of age, mice were randomized to either SED or FEX groups. The FEX groups were forced to run on a treadmill (Columbus Instruments) for 1 hour a day, 5 days a week, at a speed of 11 m/s, and a 10º incline, for 6 weeks. The mice were encouraged to run using a bottle brush bristle and a shock grid at the end of the treadmill as per the animal ethics protocol. Mice were acclimatized for 10 mins before each session, which consisted of being in the treadmill with no belt movement.

Body Mass Composition

Fat and lean mass composition were measured at 24 and 30 weeks of age using quantitative magnetic resonance (echo-MRI) mobile unit (Avian Facility of Advanced Research, University of Western Ontario, London, ON, Canada) as described by (6). Measurements were taken in triplicate to verify the results.

Blood Glucose Tolerance and Plasma Analysis

Blood glucose tolerance testing was conducted at 24 (pre-exercise) and 30 (post-exercise) weeks of age. Mice were fasted 4 hours prior to testing. Fasted blood glucose was measured via a glucometer (OneTouch Ultra). Glucose tolerance testing was conducted by administration of 1 g/kg of glucose by intraperitoneal injection, and blood glucose was monitored at 0, 15, 30, 60, and 120 minutes via tail vain. At sacrifice, blood was collected, and plasma was isolated. ELISAs (ALPCO, NH, USA) were performed following the manufacturer's protocol for insulin, and cholesterol was assessed by CHOD-PAP kit (Roche Diagnostics, Indianapolis, IN) and triglyceride

analysis was conducted by Triglycerol/Glycerol kit (Roche Diagnostics, Indianapolis, IN) following manufacturer's protocols.

Metabolic Analysis

Metabolic analysis was assessed using the Comprehensive Lab Animal Monitoring System (CLAMS) with the Oxymax software (Columbus Instruments, Columbus, OH, USA) at the Robarts Research Institute. Mice were individually caged and acclimated for 24 hours prior to measurement of food consumption, water consumption, energy expenditure, volume of oxygen (VO2) and carbon dioxide (VCO2), respiratory exchange ratio (RER), total activity, total ambulatory activity, and sleep duration, as described previously (6).

Tissue Gene Expression Analysis

RNA Extraction

Tissue RNA extraction were performed using total RNA isolation (TRIzol) reagent (Life Technologies) and phenol-chloroform phase separation. Samples were homogenized in TRIzol, mixed with chloroform and centrifuged at 12, 000 x g for 15 minutes at 4°C. The aqueous phase containing the RNA was isolated. Isopropanol was added and samples incubated at room temperature for 30 minutes to precipitate the RNA. The extracted RNA was pelleted by centrifugation at 12, 000 x g for 10 minutes at 4°C. The pellets were washed with 70% ethanol, centrifuged at 7,500 x g for 5 minutes, and then again washed with 100% Ethanol and centrifuged lastly at 7,500 x g for 5 minutes. The samples were stored in -80°C.

cDNA Synthesis

NanoDrop 2000c spectrophotometer (NanoDrop) was used to quantify the extracted RNA concentration and its purity. SuperScript variable input linear output (VILO) kit for complementary deoxyribonucleic acid (cDNA) synthesis (Life Technologies) was used to synthesize cDNA. cDNA synthesis reaction was performed in 10 µL volume to which up to 125 ng/µL RNA was added and a final concentration of 1X VILO Reaction mix and 1X SuperScript Enzyme mix were loaded. the 96-well plate containing the samples were incubated at 42°C for 60 min, then 85°C for 5 min on a C1000 thermal cycler (Bio-Rad), then stored at -20°C.

qPCR Reaction

20 ng of cDNA was used for each reverse transcriptase quantitative polymerase chain reaction (RT-qPCR) reaction along with 1X TaqMan Fast Advanced Master Mix, and predesigned TaqMan probes (Life Technologies) for the following target genes: Arg1 (Mm00475988_m1), Mrc1 (Mm00485148), IL-10 (Mm00439614), Chi3l3 (Mm00657889), Emr1 (Mm00802529_m1), IL-12a (Mm00434165), CCL2 (Mm00441242), Itgax (Mm00498701_m1), Nos2 (Mm00440502_m1), and Tnf (Mm00443258_m1) on a StepOne Plus Real-Time PCR System (Life Technologies). Samples were held at 95°C for 20 seconds, then cycled from 95°C for 1second to 60°C for 20 s for 40 cycles. Gene expression of target genes were normalized to average of the housekeeping genes *Abt1* (Mm00803824_m1), *Hprt* (Mm03024075_m1), and/or *Eef2* (Mm01171435_gH) using the ΔΔCt method.

Protein Analysis

Lysates were extracted with: 1% Triton X-100, 150mM NaCl, 10mM Tris, 1mM EDTA, 1mM EGTA, 0.5% NP-40 or a RIPA buffer (50mM Tris-HCl pH 8.0, 150mM NaCl, 1% NP-40 (Igepal), 0.5% sodium deoxycholate). Each buffer contained 1 mM sodium fluoride, 1 mM sodium

orthovanadate, and half of a tablet of complete-mini EDTA-free protease inhibitor (Roche, Mannheim, Germany). Protein was quantified by bicinchoninic acid (BCA) assay (Thermo Scientific). Protein lysates (40μg) were separated by 10% SDS-PAGE and transferred onto a nitrocellulose membrane using an iBlotTM System (Invitrogen, USA). Membranes were blocked with 3% bovine serum albumin (BSA) with 0.05% Tween-20 in 1X phosphate buffer saline (PBS), and incubated with anti-human PANX3 antibody (1:1000; PANX3 CT), and anti-GAPDH antibody (1:1000; Millipore Cat# MAB374). For detection, IRDye® -800CW and -680RD (Life TechnologiesTM, USA) were used as secondary antibodies at 1:10,000 dilutions and imaged using a Li-Cor Odyssey infrared imaging system (Li-Cor, USA). Western blot quantification and analysis was conducted using Image Studio™ Lite (LI-COR Biosciences).

Statistical Analysis

Statistical analyses were performed using Graph Pad Prism (GraphPad, San Diego, CA). Outliers were removed from data sets using the outlier test from GraphPad Prism Ver 6.07. Body weight progression was analysed using a repeated measures ANOVA. Body weight, fat mass, and lean mass measures were analysed using an un-paired t-test at 12 and 24 weeks of age. For 30 week and *ex vivo* analysis for body weight, fat mass, lean mass, insulin, cholesterol, triglycerides, and AUC a two-way ANOVA with genotype and activity as factors was used. For blood glucose tolerance curves a three-way factorial ANOVA was used with genotype x activity x time as factors. To determine the relationship between age, genotype, and activity, a three-way repeated measures factorial ANOVA was used to assess body weight, body composition, and blood glucose AUC in supplemental figures. Data are presented as mean ± SEM. N (animals).

Acknowledgements and conflict of interest statement

The authors declare no conflict of interest of any kind with the current manuscript. We thank the funding agencies that supported this work including: Petro-Canada Young Innovator Award – Western University to SP, Ontario Graduate Scholarship to CBW, CIHR Foundation Grant (FRN:FDN-143203) to AK.

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Figure Legends

Figure 1: Male *Panx3* KO mice weigh less and have less fat mass at 12 to 24 weeks of age compared to WT mice.

Male and female WT and Panx3 KO mice fed a normal chow diet and body weights were recorded bi-weekly until 24 weeks of age (A & B). There were no effects from genotype on body weight for female mice (A), however in males, there was an interaction between genotype and age ($p < 0.0001$) resulting in Panx3 KO mice weighing less from 20 – 24 weeks of age (B). At 12 and 24 weeks of age there were no differences in fat mass (C) or lean mass (E) in female mice, however, while there were no differences in lean mass (F) at either time points for males, Panx3 KO mice had lower fat mass (D) at both time points, as determined by ECHO-qMRI measurements. At 24 weeks of age, glucose tolerance testing was performed and no difference in area under the curve (AUC) between WT and Panx3 KOs was measured for either female (G) or male (H) mice. ns: not significant, *$p < 0.05$, ****$p < 0.0001$, mean ± SEM.

Figure 2: High fat diets cause PANX3 protein expression in fat tissue and *Panx3* KO mice show modest protect of weight gain on a HFD.

Animals fed a high fat diet (60% kcal from fat) were fed from 12 to 28 weeks of age and visceral fat was analysed for Panx3 protein expression (A & B). Panx3 was significantly higher in WT mice that were fed a HFD compared to all other groups. ns: not significant, N = 3, n – 3. Different letters indicate significantly different ($p < 0.05$). Male WT and *Panx3* KO mice were fed on an HFD (60% kcal from fat) from 12-28 weeks of age. When analysing weight gain from initial weight (C), there was a genotypic effect ($p = 0.0382$) in which the *Panx3* KO mice gained weight slower than the WT mice (n = 13 – 16).

Figure 3: Male *Panx3* KO mice are protected from HFD induced inflammation compared to WT mice.

WT and *Panx3* knockout (KO) mice were allocated to either a sedentary (SED) or forced exercise (FEX) group from 24 to 30 weeks of age. Epididymal white fat (eWAT) (A & B), quadriceps (C & D) and liver (E & F) tissue was collected, and mRNA expression was analyzed by RT-qPCR for macrophage marker Emr1 and Itgax (CD11c), pro-inflammatory genes Tnfa, Nos2, IL-12a,

Ccl2 and IL-6 and anti-inflammatory markers Arg1, Mrc1, IL-10 and Chi3l3. An inflammatory index score was calculated as the ratio of pro-inflammatory over anti-inflammatory markers and reflects the inflammatory status of the tissue. Ko mice had significantly lower inflammatory indexes in both eWAT (B) and quadriceps (D) compared to WT mice, however no differences in liver tissue (F). N = 5, * = $p < 0.05$, ** = $p < 0.01$.

Figure 4: *Panx3* deletion has a similar effect on body weight and body composition as 6 weeks of forced exercise in male mice.

At 24 weeks of age both male and female WT and Panx3 knockout (KO) mice were randomly allocated to either sedentary (SED) or forced exercise (FEX) groups until 30 weeks of age. In females, KO mice weighed significantly less regardless of activity (genotype: $p = 0.0159$) (A), however there were no differences in fat mass (B & C) or lean mass (D & E) between genotypes. In males, SED KO mice weighed significantly less than SED WT mice ($p = 0.0017$) (F), however not significantly different that FEX WT or KO mice. SED KO mice had significantly less fat mass compared to SED WT ($p = 0.0041$) (G &H), however there was no significant difference between FEX KO or WT mice (G & H). There were no significant difference for lean mass (I) among the groups, however when corrected for body mass (J), SED KO mice had significantly more lean mass compared to SED WT. n = 3-4, *$p = 0.05$, **$p = 0.01$, ***$p = 0.001$, ****$p < 0.0001$.

Figure 5: Female *Panx3* KO mice have higher inflammatory index in quadriceps and liver tissues compared to WT mice.

WT and Panx3 knockout (KO) mice were allocated to either a sedentary (SED) or forced exercise (FEX) group from 24 to 30 weeks of age. Quadriceps (A & D), visceral fat (B & E), and liver (C & F) tissue was collected and mRNA expression was analyzed by RT-qPCR for macrophage marker Emr1 and Itgax (CD11c), pro-inflammatory genes Tnfa, Nos2, IL-12a, Ccl2 and IL-6 and anti-inflammatory markers Arg1, Mrc1, IL-10 and Chi3l3. An inflammatory index score was calculated as the ratio of pro-inflammatory over anti-inflammatory markers and reflects the inflammatory status of the tissue (A – C). KO mice had significantly higher inflammatory indices in both quadriceps (A) and liver (C) tissues regardless of activity, however there was no difference in visceral fat tissue (B). n = 3-5, *$p<0.05$. ns: not significant, *$p < 0.05$, **$p < 0.01$, mean ± SEM. Different letters indicate significantly different ($p < 0.05$).

Figure 6: Male *Panx3* KO mice have lower inflammatory index in quadriceps and visceral fat tissues compared to WT mice.

WT and Panx3 knockout (KO) mice were allocated to either a sedentary (SED) or forced exercise (FEX) group from 24 to 30 weeks of age. Quadriceps (A & D), visceral fat (B & E), and liver (C & F) tissue was collected, and mRNA expression was analyzed by RT-qPCR for macrophage marker Emr1 and Itgax (CD11c), pro-inflammatory genes Tnfa, Nos2, IL-12a, Ccl2 and IL-6 and anti-inflammatory markers Arg1, Mrc1, IL-10 and Chi3l3. An inflammatory index score was calculated as the ratio of pro-inflammatory over anti-inflammatory markers and reflects the inflammatory status of the tissue (A – C). In quadriceps tissue, SED KO mice were not significantly different than FEX WT or KO groups, however SED WT mice was significantly higher (A). In Visceral fat, SED KO mice had significantly lower inflammatory indexes compared to both SED and FEX WT mice (B). No differences between groups was observed in the liver (C). n = 3-5, *$p<0.05$. ns: not significant, *$p < 0.05$, **$p < 0.01$, mean ± SEM. Different letters indicate significantly different ($p < 0.05$).

Supplemental 1: Male WT and *Panx3* KO mice have similar metabolic activity, sleep and food consumption in metabolic cages

Male WT and Panx3 KO mice were placed in a metabolic cage to determine if there are any differences in metabolic activity, food consumption, activity, and sleep. There were no significant differences between WT and Panx3 KO mice for any of the measures. N=4.

Supplemental 2: Western diet induces the expression of Panx3 in visceral white adipose tissue, while FEX attenuates its expression in male WT mice.

Male WT mice were fed either a chow or a Western diet from 12 to 30 weeks of age. At 24 weeks of age, half the mice were subjected to a forced exercise protocol as described in the methods. At 30 weeks of age, visceral fat was excised at sacrifice and mRNA was isolated to determine the levels of Panx3. In chow fed animals, there was low to non-detectable levels of Panx3, while the SED Western diet animals had Panx3 expression detectable. However, Panx3 was significantly lower in the Western diet fed animals that were performed the FEX protocol. $N = 3$, $P < 0.05$. Different letters indicate significantly different.

Supplemental 3: Male *Panx3* KO mice do not gain weight between 24 and 30 weeks of age regardless of activity.

Male and female WT and *Panx3* KO mice were randomly allocated to either a sedentary (SED) or a forced exercise (FEX) group between 24 and 30 weeks of age. Body weight was measured bi-weekly. There were no significant differences in body weight between female WT and *Panx3* KO mice at any time point (A), However, there was a main effect for genotype for body weight/initial body weight. Suggesting, *Panx3* KO mice gain slower over time compared to WT mice (C). For male mice, there was a main effect for genotype for body weight (B) and body weight/initial body weight (D), suggesting *Panx3* KO mice weigh less and gain weight slower than WT mice. When analyzing body weights at 24 and 30 weeks of age to determine the effect of activity, *Panx3* KO female mice weighed significantly less than WT mice, however there was no effect from the FEX for either genotype (E). For male mice, SED *Panx3* KO mice weighed significantly less than SED WT mice and did not gain weight over time (F).

Supplemental 4: *Panx3* deletion has no effect on glucose tolerance, insulin, cholesterol, or triglyceride plasma levels.

At 24 weeks of age, female and male WT and *Panx3* KO mice were randomly allocated to either a sedentary (SED) or forced exercise (FEX) protocol until 30 weeks of age. After which, blood glucose tolerance testing was completed (A & B). Area under the curve (AUC) analysis (A) revealed that while there was no significant difference between SED WT ($n = 3$) and SED or FEX *Panx3* KO ($n = 3$) mice ($p = 0.0618$), the FEX WT mice ($n = 3$) had significantly lower AUC compared to SED WT ($p < 0.05$). At sacrifice, blood was collected and analyzed for circulating insulin (C), cholesterol (E), and triglycerides (G) ($N = 20$, $n = 5$), with no significant effects for genotype for any of these measures. In male mice, there were no significant differences for genotype observed with and out FEX for AUC (B). After testing, mice were randomly allocated to either a sedentary (SED) or forced exercise (FEX) protocol for 6 weeks. After which, blood

glucose tolerance testing was completed again (B). There were no significant effects for circulating insulin (D), cholesterol (F), and triglycerides (H) (N = 20, n = 5). *p<0.05.

Supplemental 5: Male *Panx3* KO mice have lower total adiponectin compared to WT mice

Female and male WT and Panx3 KO mice were allocated to either a sedentary (SED) or forced exercise (FEX) group from 24 to 30 weeks of age. Blood was collected at sacrifice and analyzed for markers of inflammation. In female mice, there were no significant differences among any of the groups for either total adiponectin (A), HMW Adiponectin (B), HMW/Total Adiponectin (C), Serum Amyloid A (SAA) (D), or IL6 (E). For male mice, SED WT mice had significantly higher Total Adiponectin (F) compared to SED KO mice, however there were no significant differences in among the groups for HMW Adiponectin (G), HMW Adiponectin/Total Adiponectin (H) or SAA (I). For IL6, there was a main effect for genotype (p = 0.0078) suggesting that *Panx3* KO mice have lower levels of circulating IL6 compared to WT mice, regardless of activity (J).n = 4-5, *p < 0.05. no significance (ns).

Figure 1

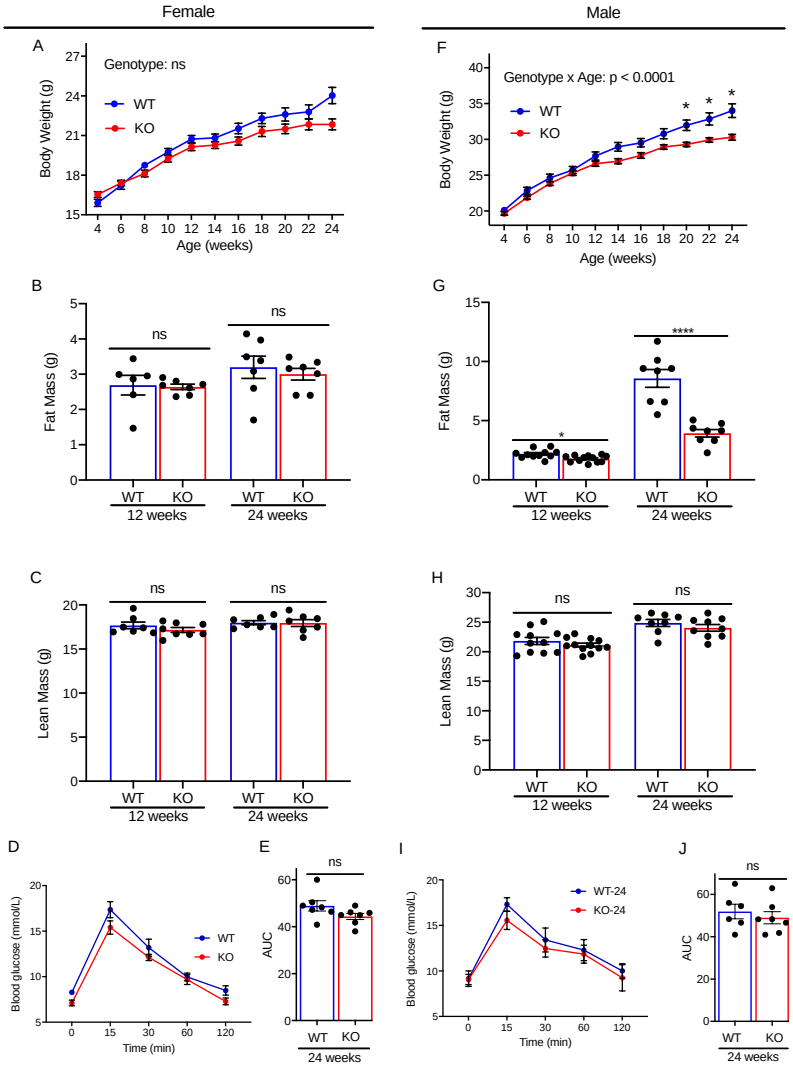

Female
Male
A
Genotype: ns
WT
KO
Body Weight (g)
Age (weeks)
F
Genotype x Age: p < 0.0001
B
ns
Fat Mass (g)
12 weeks
24 weeks
G
****
C
Lean Mass (g)
H
D
Blood glucose (mmol/L)
Time (min)
E
AUC
I
WT-24
KO-24
J

Figure 2

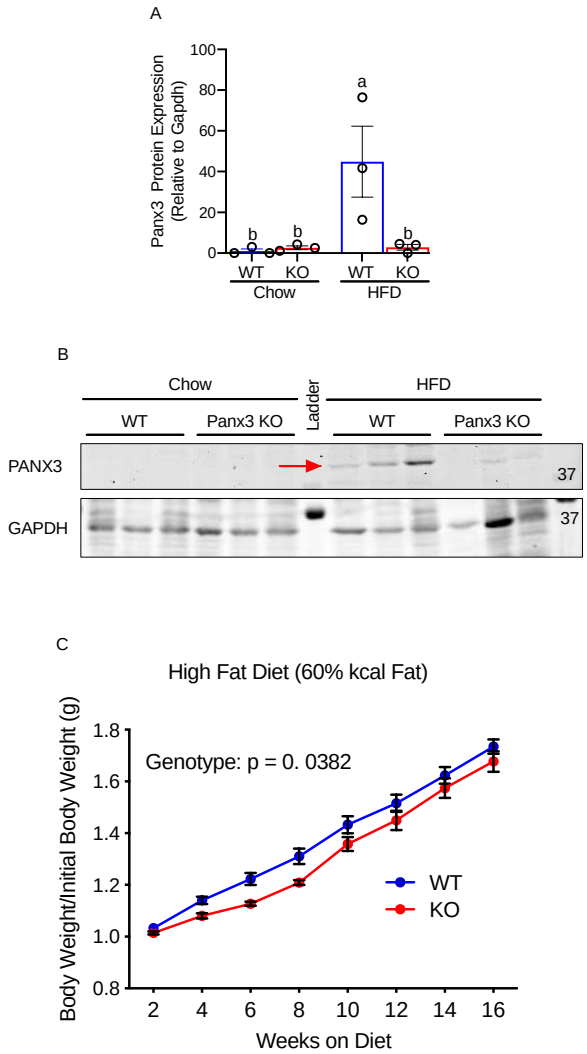
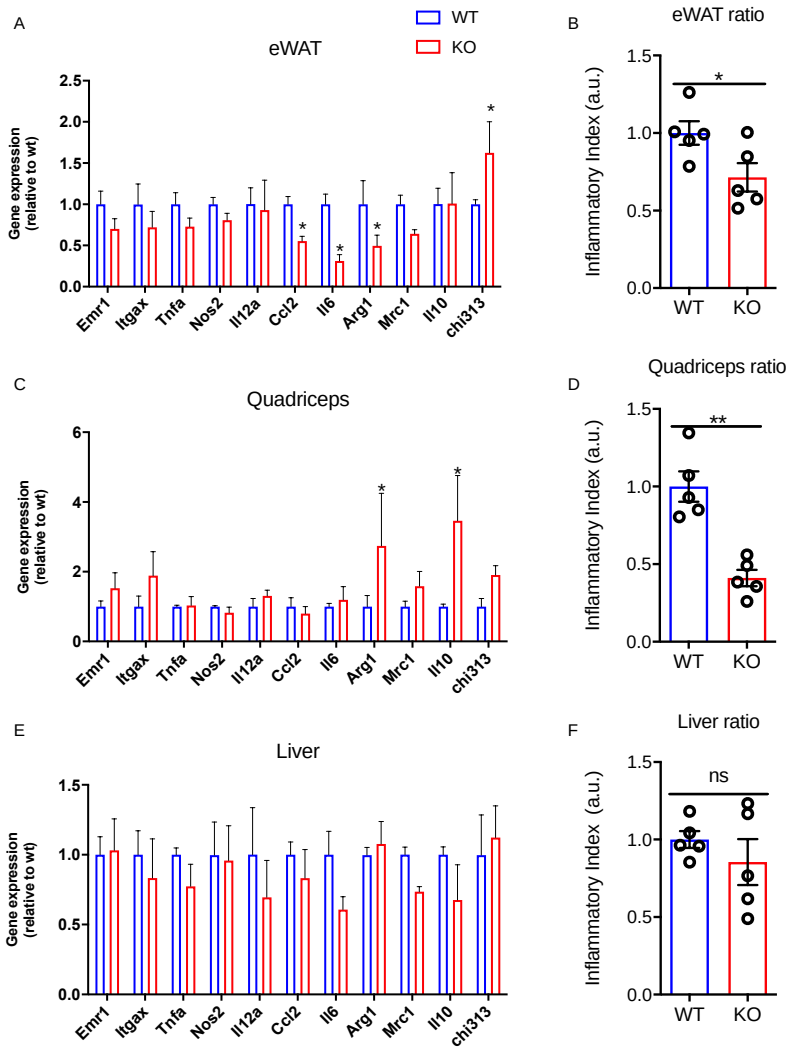

A
Panx3 Protein Expression
(Relative to Gapdh)
100
80
60
40
20
0
a
b
b
b
WT
KO
WT
KO
Chow
HFD
B
Chow
HFD
Ladder
WT
Panx3 KO
WT
Panx3 KO
PANX3
GAPDH
37
37
C
High Fat Diet (60% kcal Fat)
Genotype: p = 0. 0382
Body Weight/Initial Body Weight (g)
1.8
1.6
1.4
1.2
1.0
0.8
2
4
6
8
10
12
14
16
Weeks on Diet
WT
KO

Figure 3


A
eWAT
WT
KO
Gene expression (relative to wt)
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2.0
1.5
1.0
0.5
0.0
Emr1
Itgax
Tnfa
Nos2
Il12a
Ccl2
Il6
Arg1
Mrc1
Il10
chi313
B
eWAT ratio
Inflammatory Index (a.u.)
1.5
1.0
0.5
0.0
*
WT
KO
C
Quadriceps
Gene expression (relative to wt)
6
4
2
0
D
Quadriceps ratio
Inflammatory Index (a.u.)
**
E
Liver
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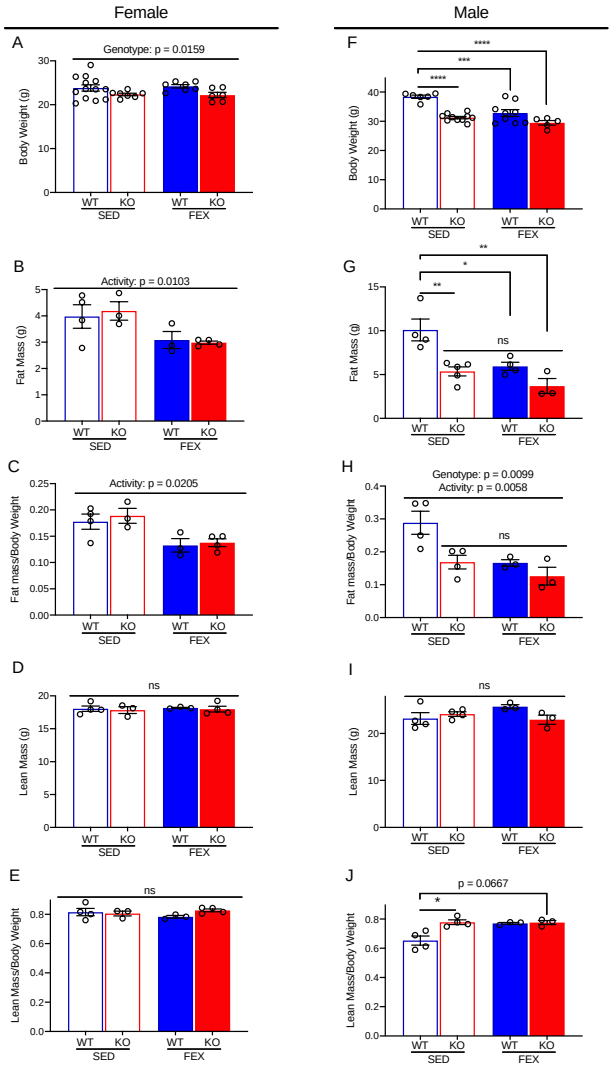
F
Liver ratio
Inflammatory Index (a.u.)
ns

Figure 4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B
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Fat Mass (g)
C
Activity: p = 0.0205
Fat mass/Body Weight
D
ns
Lean Mass (g)
E
ns
Lean Mass/Body Weight
F
****
***
****
Body Weight (g)
G
**
*
**
ns
Fat Mass (g)
H
Genotype: p = 0.0099
Activity: p = 0.0058
ns
Fat mass/Body Weight
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Lean Mass (g)
J
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*
Lean Mass/Body Weight
WT
KO
SED
FEX

Figure 5

Females

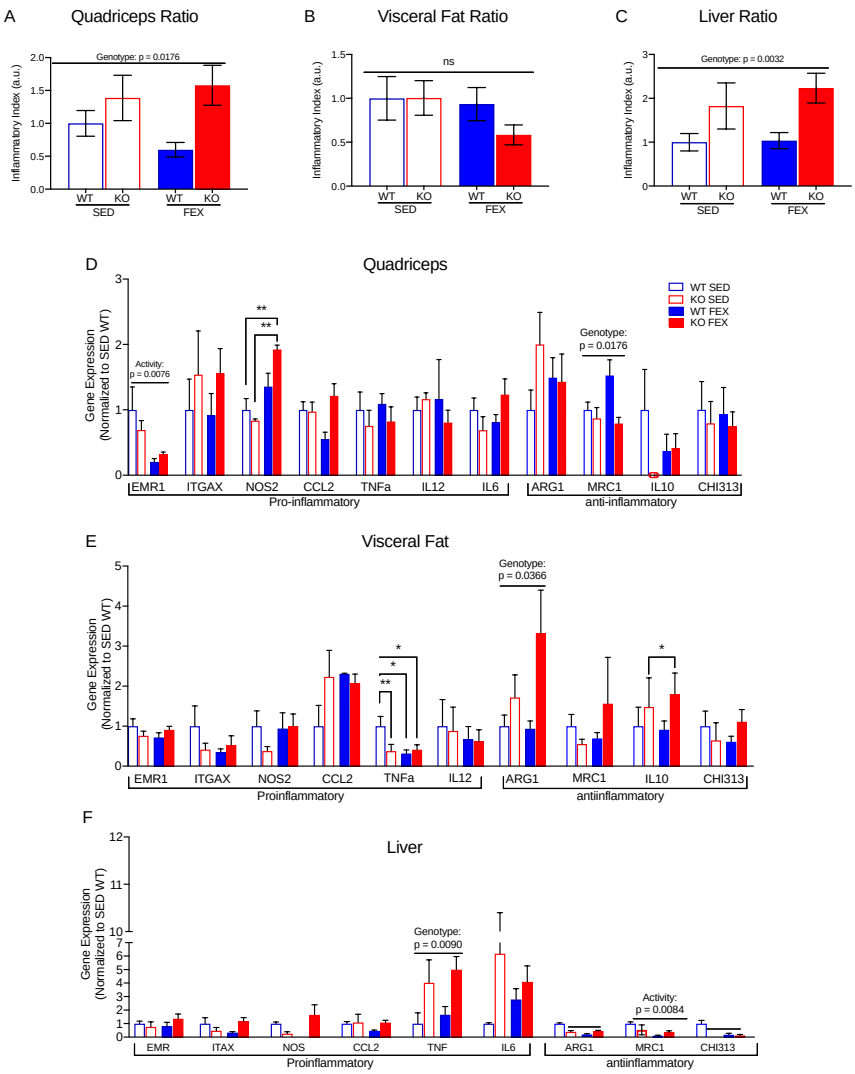
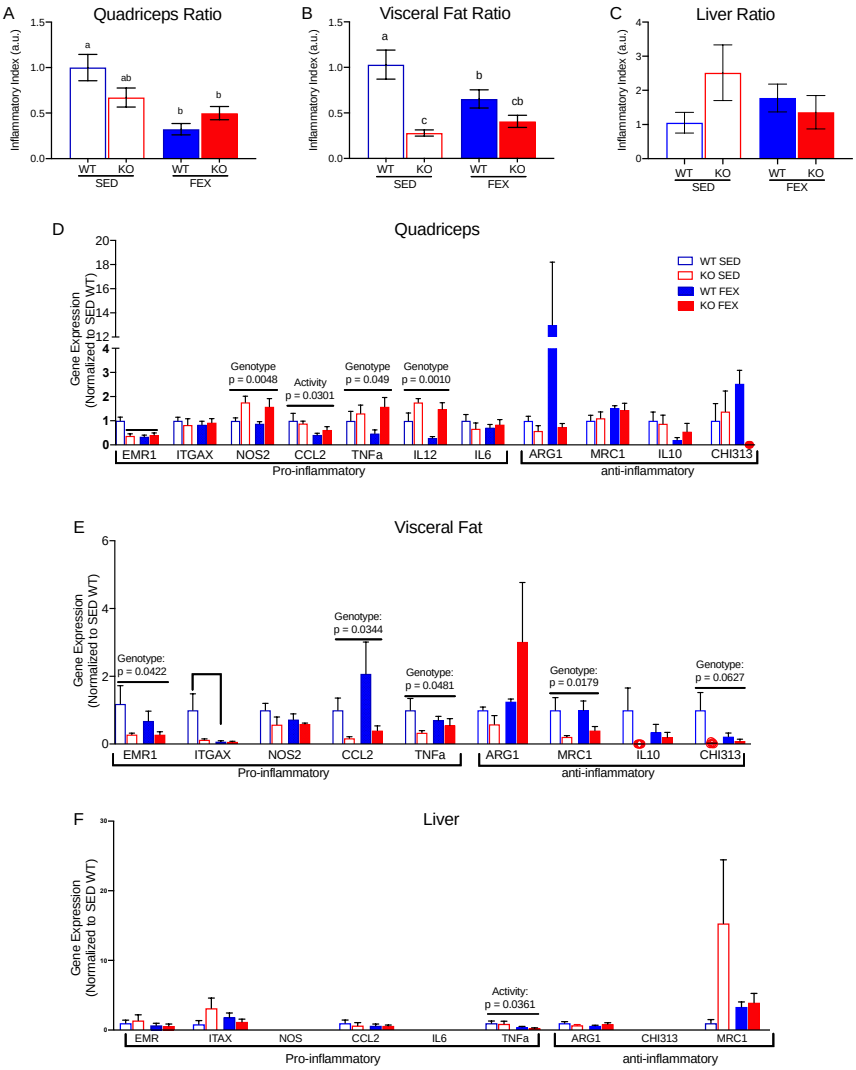


Figure 6

Males