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# Maternal Nicotine Exposure Leads to Augmented Expression of the Antioxidant Adipose Tissue Triglyceride Lipase Long-Term in the White Adipose of Female Rat Offspring.

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# Maternal nicotine exposure (MNE) leads to augmented expression of the anti-oxidant adipose tissue triglyceride lipase (ATGL) long-term in the white adipose of female rat offspring

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Maternal Nicotine Exposure (MNE) leads to augmented expression of the anti-oxidant adipose

tissue triglyceride lipase (ATGL) long-term in the white adipose of female rat offspring

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**RUNNING TITLE:** The effect of MNE on white adipose tissue

# ABSTRACT

Globally, approximately 10-25% of women smoke during pregnancy. Since nicotine is highly addictive, women may use nicotine containing products like nicotine replacement therapies for smoking cessation, but the long-term consequences of early life exposure to nicotine remain poorly defined. Our laboratory has previously demonstrated that maternal nicotine exposed (MNE) rat offspring exhibit hypertriglyceridemia due to increased hepatic *de novo lipogenesis*. Hypertriglyceridemia may also be attributed to impaired white adipose tissue (WAT) lipid storage; however, the effects of MNE on WAT are not completely understood. We hypothesize that nicotine-induced alterations in adipose function (*e.g.* lipid storage) underlie dyslipidemia in MNE adults. Female 6 month old rats exposed to nicotine during gestation and lactation exhibited significantly decreased visceral adipocyte cell area by 40%, attributed, in part, to a 3-fold increase in adipose triglyceride lipase (ATGL) protein expression compared to vehicle. Given ATGL has antioxidant properties and *in utero* nicotine exposure promotes oxidative stress in various tissues, we next investigated if there was evidence of increased oxidative stress in MNE WAT. At both 3 weeks and 6 months, MNE offspring expressed 37-48% higher protein levels of SOD1 and SOD2 in WAT. Since oxidative stress can induce inflammation, we examined the inflammatory profile of WAT and found increased expression of cytokines (IL-1β, TNFα, and IL-6) by 44-61% at 6 months. Collectively, this suggests that the expression of WAT ATGL may be induced to counter MNE-induced oxidative stress and inflammation. However, higher levels of ATGL would further promote lipolysis in WAT, culminating in impaired lipid storage and long-term dyslipidemia.

**KEY WORDS:** White adipose tissue, maternal nicotine exposure, oxidative stress, adipocytes

# **INTRODUCTION**

Evidence now demonstrates that cigarette smoke plays a critical role in the development of dyslipidemia and obesity in children exposed during perinatal life (Wen *et al.*, 2010; Weng *et al.*, 2012). This is especially concerning given that approximately 10-25% of women still smoke during pregnancy, with rates as high as 59% in certain Indigenous communities (Cui *et al.*, 2014; Roman-Galvez *et al.*, 2017; Tappin *et al.*, 2010; Tong *et al.*, 2013). To date, nicotine replacement therapies (NRT) for smoking cessation (*i.e.* transdermal patches, gums, e-cigarettes) are thought to benefit pregnant women who are highly addicted and unable to quit smoking by other means (Oncken and Kranzler, 2003). However, the consequences of fetal and neonatal exposure to nicotine alone on the long-term metabolic health of the offspring have yet to be fully defined.

Maternal nicotine use during gestation and lactation leads to significant exposure to the fetus and neonate since it is present in fetal blood and transferred through amniotic fluid, placental tissue, and breast milk (Luck and Nau, 1987; Luck *et al.*, 1985). Numerous animal studies demonstrate that nicotine use during pregnancy leads to adverse neurobehavioral, pulmonary, cardiovascular, and metabolic outcomes in the offspring (Barra *et al.*, 2017; Chou and Chen, 2014; Dasgupta *et al.*, 2012; Ma *et al.*, 2014; Pauly and Slotkin, 2008). Specifically, we and others have shown in rats that perinatal exposure to nicotine alone leads to increased blood pressure, adiposity, decreased glucose tolerance, and impaired pancreatic beta cell development (Fox *et al.*, 2012; Gao *et al.*, 2008; Gao *et al.*, 2005; Holloway *et al.*, 2008). Moreover, MNE during gestation and lactation leads to increased hepatic *de novo* lipogenesis and increased hepatic and circulating triglycerides in postnatal life (Ma *et al.*, 2014). This reciprocates human studies which have demonstrated that children exposed to maternal smoke *in*

*utero* have higher triglyceride levels later in life (Cupul-Uicab *et al.*, 2012). Since increased circulating plasma triglycerides is strongly associated with cardiovascular disease (CVD) (Bansal *et al.*, 2007), elucidating the underlying molecular mechanisms promoting dyslipidemia will undoubtedly uncover therapeutic targets to reduce unwarranted CVD risk to these nicotine-exposed individuals.

Aside from the liver, the augmented circulating and hepatic triglyceride accumulation observed in these MNE offspring could also be attributed to impairments in other metabolic tissues, such as white adipose tissue (WAT). The major role of WAT is to store excess circulating triglycerides to prevent glucose and lipid toxicity, and moreover, ectopic fat deposition (Abate, 2012). Excess circulating triglycerides promote adipocytes to undergo differentiation, hypertrophy, and/or hyperplasia to process and store lipids. Once the maximum triglyceride storage capacity is reached, fatty acids can spillover in the plasma, increasing substrate availability for hepatic triglyceride synthesis (Abate, 2012). Ultimately, hypertriglyceridemia contributes to various systemic abnormalities like dyslipidemia, insulin resistance, and CVD (Abate, 2012). Currently, the effects of MNE on white adipose tissue function are not completely understood. Studies by Somm *et al.* indicated that MNE during gestation resulted in adipocyte hypertrophy and increased expression of adipogenic transcription factors in 3 week male offspring, but the long-term effects were not examined (Somm *et al.*, 2008). A more recent study by Fan *et al.* (2016) demonstrated that maternal exposure of nicotine (from gestational day 9 to weaning) led to decreased adipocyte size in 26 week old male offspring, suggesting an impaired capacity for lipid storage. Furthermore, this study also found an increase in steady-state mRNA levels of adipocyte differentiation and lipogenic markers in MNE WAT compared to vehicle (Fan *et al.*, 2016). However, given most women addicted to

smoking would be exposed to nicotine (*i.e.* NRT or cigarettes) in both prenatal and perinatal life, the goal of this study was to determine if this longer, more relevant, window of nicotine exposure impacts adipose function in *both* male and female offspring. Moreover, this window covers the entire period of rat adipose tissue differentiation (Greenwood and Hirsch, 1974). We hypothesize that *in utero* nicotine exposure will adversely impact WAT in the offspring, leading to dyslipidemia in adulthood. To examine this further, we will investigate if WAT function is affected due to altered adipocyte size, proliferation (*i.e.* Akt-1), differentiation (*i.e.* C/ebpα/β, Srebp-1c), lipogenesis (*i.e.* Lpl, Accα, Fas, Acsl1), fatty acid transport (*i.e.* Fatp1, Fatp4), and/or lipolysis (Atgl) in our well-established rat model of maternal nicotine exposure.

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# MATERIALS AND METHODS

## *Maternal Nicotine Exposure (MNE) Rat Model*

Nulliparous 200-250g female Wistar rats (Harlan, Indianapolis, IN, USA) were injected daily subcutaneously with either saline (vehicle) or nicotine bitartrate at 1 mg/kg/day (Sigma-Aldrich, St. Louis, MO, USA) two weeks prior to mating, during gestation, and until weaning (PND21). This nicotine dose results in maternal serum cotinine concentrations of $135.9 \pm 7.86$ ng/mL, which is comparable to "moderate" female smokers (80 ng/mL) or NRT users (169.9 ng/mL) (Holloway *et al.*, 2006;  Shahab *et al.*, 2016). Litters were culled to eight at birth and following weaning, rats were housed as sibling pairs until 6 weeks of age, then subsequently housed individually. Male and female offspring were sacrificed via carbon dioxide inhalation at 3 weeks and 6 months of age. Gonadal white adipose tissues were extracted, either fixed in formalin or frozen in liquid nitrogen and stored at -80°C for histological and molecular analyses, respectively. All rats were conventionally housed in polycarbonate microisolator cages with ad libitum access to water and standard chow diet (Teklad 22/5 rodent diet; Envigo) under controlled lighting (12:12 L:D), humidity (40-50%), and temperature (22˚C). In accordance with the Canadian Council for Animal Care guidelines, animal experiments were approved by the Animal Research Ethics Board at McMaster University.

## Gonadal Adipocyte Cell Area

A portion of gonadal WAT was fixed in 10% (v/v) neutral buffered formalin overnight, washed in water, and embedded in paraffin. Cross sections were stained with hematoxylin and eosin (H&E) and photographed using the Olympus BX50 microscope under a 10x objective. Cell areas were quantified from a minimum of 160 adipocytes from two cross sections/rat using Northern

Eclipse software (Empix Imaging Inc.).

**RNA Extraction and Real Time-Polymerase Chain Reaction (RT-PCR)**

Total RNA was extracted from homogenized 3 week and 6 month WAT samples using TRIzol reagent according to the manufacturer's instructions (Invitrogen). Two micrograms of RNA were reversed-transcribed to cDNA (high-capacity cDNA Reverse Transcription Kit, Applied Biosystems). Forward and reverse primer sets used for RT-PCR, listed in Table 1, were designed with the National Center for Biotechnology Information's primer designing tool. Relative transcript abundance was determined using SensiFAST No-ROX SYBR Green Supermix (FroggaBio) and the Bio-Rad CFX384 Real Time System. Samples were assayed in triplicate and relative fold change was calculated using comparative cycle times (Ct) method normalized to β-actin. The relative abundance was calculated using the formula $2^{\Delta\Delta Ct}$, where ΔΔCt was the normalized value.

**Protein extraction and Western blot**

WAT was homogenized in RIPA buffer (50 mM Tris-HCL, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Nonidet P40, 0.25% $C_{24}H_{39}NaO_4$) supplemented with a protease inhibitor cocktail (Roche) and phosphatase inhibitors (20 mM NaF, 40mM Na-pyrophosphate, 40mM $Na_3VO_4$, 200mM β-glycerophosphate disodium salt hydrate). The solution was sonicated, mixed in a rotator for 1hr at 4 °C, and centrifuged at 300g for 15 min at 4 °C. The total cellular protein extract in the collected supernatant was quantified by colorimetric DC protein assay (BioRad). Loading samples were heated at 50 °C for 10 min to denature the proteins. Proteins (20μg/well) were separated by size via gel electrophoresis and transferred onto polyvinylidene difluoride membrane (Millipore). Membranes were blocked in 1x Tris-buffered saline-Tween 20 buffer

with 5% non-fat milk or 5% BSA (blocking solutions), and then probed using primary antibodies diluted in the blocking solution (Table 2). A mouse or rabbit secondary antibody was used to detect primary antibody diluted in the blocking solution at 1:5,000 or 1:10,000 dilution, respectively (Table 2). Immuno-reactive bands were visualized, and relative band intensity was calculated using ImageLab software (BioRad) and normalized to β-actin, as previously performed (Ma *et al.*, 2014).

**Statistical Analysis**

Statistical analyses were performed using Graphpad Prism 6 software. Results were presented as the mean of arbitrary values ± SEM. Grubbs' test was used to determine significant outliers and data were tested for normality and equal variance. Comparisons between vehicle and nicotine exposed offspring were assessed using an unpaired Student's t-test, with a p-value of less than 0.05 deemed as significant.

## **RESULTS**

**Maternal nicotine exposure leads to decreased visceral adipocyte size in 6 month old offspring.**

Using our well-established rat MNE model leading to dyslipidemia (Gao *et al.*, 2005; Ma *et al.*, 2014), we wanted to first confirm whether prenatal nicotine exposure has long term effects on adipocyte size in the offspring. Histological analyses revealed both MNE male and female offspring exhibited smaller gonadal adipocytes compared to vehicle controls at 6 months of age (Figure 1A, B). Given the more pronounced effects of perinatal nicotine exposure on female WAT area (13.2% male vs. 39.7% female decrease in adipocyte size), we continued to examine only female offspring to explore the underlying mechanisms involved. Gondal adipose tissue weight did not differ between MNE and vehicle offspring (data not shown), suggesting a possible increase in adipocyte number due to prenatal nicotine exposure. With decreased adipocyte cell area and subsequent increase in cell number, transcript and protein levels of the proliferation marker AKT-1 were subsequently measured. Despite a significant increase in mRNA levels, there were no corresponding changes in AKT-1 protein levels in 6 month female MNE WAT (Figure 1C, D).

**_In utero_ nicotine exposure increases the expression of the differentiation marker C/EBPα in WAT of 6 month old female offspring.**

Given impairments in adipocyte differentiation can also decrease adipocyte size, we next measured the expression of key targets involved in adipogenesis (Abate, 2012), including CCAAT/enhancer-binding protein (C/EBP)-α/β and sterol regulatory element-binding

transcription factor (SREBP)-1c. Protein analyses revealed a significant increase in the 42kDa

band of C/EBP-α in female MNE offspring compared to vehicle at 6 months independent of

changes in transcript levels (Figure 2A, B). Quantitative real time PCR analyses revealed that

WAT from MNE exposed offspring had a significant increase in steady-state transcript levels of

C/EBPβ and SREBP-1c compared to controls (Figure 2C, E). However, immunoblot analyses

revealed no significant differences at the protein level for either target (Figure 2D, F). To

examine if perinatal nicotine exposure had direct effects on C/EBPα, we measured the transcript

and protein levels at 3 weeks age but found no difference in expression from vehicle (Figure 2G,

H).

**Maternal nicotine exposure does not increase the expression of markers involved in lipogenesis and fatty acid transporters in 6 month old female offspring.**

Adipocyte size is influenced by its ability to breakdown triglyceride (TG) molecules from

circulation, transport its constitutive components intracellularly, and re-assemble TGs for storage

(Kersten, 2014). Therefore, we wanted to determine whether perinatal nicotine exposure

impaired triglyceride transport and esterification by measuring the steady state transcript levels

of key targets involved in lipogenesis and fatty acid transport. At six months of age we found no

significant difference between groups in the expression of lipogenic markers including

lipoprotein lipase (LPL), acetyl-coA carboxlyase (ACCα), and fatty acid synthase (FAS), along

with fatty acid transporters FATP1/4, and long-chain acyl CoA synthetase (ASCL)-1 in female

MNE WAT (Figure 3A-F).

***In utero* nicotine exposed female offspring exhibit increased expression of ATGL at 6 months, but not at 3 weeks.**

Since enhanced lipolysis decreases adipocyte size (Zhang *et al.*, 2017), we then examined whether MNE WAT had elevated expression of adipose triglyceride lipase (ATGL), the predominate enzyme involved in intracellular degradation (Zechner *et al.*, 2009). At six months of age, we found a significant increase in both ATGL mRNA expression and protein levels in nicotine exposed female WAT compared to vehicle controls (Figure 4A, B), however this was not observed at 3 weeks (Figure 4C-D). Altogether, these data suggest that augmented ATGL-induced lipolysis may lead to reduced adipocyte cell area in MNE WAT at 6 months.

**Maternal nicotine exposure exhibit enhanced anti-oxidant expression at 6 months and 3 weeks.**

Evidence demonstrates that ATGL deficiency in mice is associated with accumulation of reactive oxygen species (ROS) and decreased SOD2 expression, suggesting this enzyme may act as an antioxidant and suppress oxidative stress (Aquilano *et al.*, 2016; Chen *et al.*, 2017a; Chen *et al.*, 2017b). Moreover, we and others have previously shown that maternal nicotine exposure promotes oxidative stress in the heart, pancreas, and placenta of adult offspring (Barra *et al.*, 2017; Bruin *et al.*, 2008; Sbrana *et al.*, 2011). Therefore, we wanted to determine whether an imbalance between antioxidant defenses occurs in WAT of 6 month female MNE offspring by measuring the transcript and protein levels of the antioxidants superoxide dismustase (SOD)-1 and SOD-2. Both SOD 1 and 2 steady state mRNA and protein levels were significantly elevated in prenatal nicotine exposed WAT in 6 month female offspring compared to vehicle (Figure 5A-D). At 3 weeks of age, there was also a significant increase in SOD-1 protein levels, and a

trending increase in SOD-2, in the nicotine exposed WAT group compared to controls (Figure

5E-H). To determine if there was oxidative stress, we measured protein levels of 4-HNE and

found a non-significant upward trend in nicotine-exposed WAT at both 3 week and 6 month

timepoints (Figure 5I, 5J). Overall, these data suggest that prenatal nicotine exposure directly

increase the expression of the antioxidant SOD-1 and SOD-2 in 6 month WAT which was

enough to prevent lipid oxidative damage.


***In utero* nicotine exposure exhibit inflammation at 6 months, but not at 3 weeks.**

Enhanced oxidative stress can activate nuclear factor-κB (NF-κB), leading the expression of pro-

inflammatory cytokines and inflammation (Li and Karin, 1999; Morgan and Liu, 2011). As well,

activation of this pathway in differentiated 3T3-L1 cells leads to increased lipase expression and

lipolysis (Chi *et al.*, 2014). Since oxidative stress, inflammation, and lipolysis are integrated

pathways, we wanted to determine whether inflammation was present in 6 month *in utero*

nicotine exposed WAT. At 6 months, we found a significant increase in the steady-state mRNA

expression profile of pro-inflammatory cytokines tumor necrosis factor (TNF)-α, interleukin

(IL)-1β, IL-6, and the macrophage marker CD68 (Figure 6A-D). In contrast at 3 weeks of age,

there was no significant difference in steady state mRNA levels of inflammatory markers

between groups (Figure 6E-H).

# **DISCUSSION**

Under normal physiological conditions, WAT serves as the principle lipid storage site to prevent ectopic lipid deposition in peripheral organs. The balance between lipolysis and lipogenesis dictates the overall amount of triglyceride molecules stored as a single large lipid droplet in the cytoplasm of adipocytes, ultimately influencing its size (Bolsoni-Lopes and Alonso-Vale, 2015). Adipocytes have a limited triglyceride storage capacity. Once exceeded, excess lipids will spill-over into circulation resulting in dyslipidemia and increased lipid content at ectopic sites like the liver (Abate, 2012). In our model, we have previously shown that MNE offspring have dyslipidemia with increased levels of circulating triglycerides (Gao *et al.*, 2005; Ma *et al.*, 2014). While we have shown that hepatic de novo lipogenesis is increased in nicotine-exposed offspring, the effect of nicotine exposure on WAT function were not examined. Previous studies show maternal nicotine exposure during gestation or from gestation until weaning altered adipocyte size and increased the expression of adipocyte differentiation markers, but this was only examined in male offspring (Fan *et al.*, 2016; Somm *et al.*, 2008). Moreover, this exposure to nicotine in these studies (gestation only or mid-gestation to weaning only) does not mimic women's smoking habits and/or nicotine use prior to pregnancy. Therefore, our goal was to determine if WAT plays a role in promoting dyslipidemia in both male and female offspring exposed to nicotine in prenatal and perinatal life.

In this study, we demonstrated that both male and female offspring exposed to nicotine during prenatal and perinatal life decreased adipocyte cell area compared to vehicle controls at 6 months of age. Somm *et al.* reported that rats exposed to nicotine during pregnancy alone (from gestational day 4 for 14 days at 3mg/kg/day) had adipocyte hypertrophy compared to vehicle controls in 3 week old male offspring, but the effect on WAT size into adulthood, after nicotine

exposure, was not examined (Somm *et al.,* 2008). Similar to our findings, Fan *et al.* demonstrated that *in utero* nicotine exposure from mid-pregnancy (*e.g.* gestational day 9*)* until weaning decreased adipocyte size in male WAT at 26 weeks of age (Fan *et al.*, 2016). The dose of nicotine (1 mg/kg) administered in this study is relevant as it corresponds to moderate female smokers or NRT users (Holloway, Kellenberger and Petrik, 2006; Shahab *et al.,* 2016), while doses used in other studies (2-3 mg/kg/day) correspond to heavy smokers (Fan *et al.,* 2016; Somm *et al.,* 2008). Examining the effects of MNE on WAT for a longer duration is important given adipose development occurs both pre- and postnatally in rats. In rodent adipose tissue, growth and differentiation occurs from late gestation to 4 weeks in postnatal life, while in humans this occurs from 5 to 29 weeks gestation (Greenwood and Hirsch, 1974; Poissonnet *et al.*, 1984). In both species, adipose tissue expansion happens throughout life (Greenwood and Hirsch, 1974; Spalding *et al.*, 2008). Given the extensive differentiation of adipose tissue during perinatal life, it is vulnerable to alterations by environmental cues (*i.e.* drugs) during this developmental window. Furthermore, both mature adipocytes and adipocyte precursors like mesenchymal stem cells express nicotinic acetyl-choline receptor subunits, suggesting that nicotine may directly alter adipogenesis and adipocyte formation (Gochberg-Sarver *et al.*, 2012; Hoogduijn *et al.*, 2009).

Since the effect on adipocyte size was more pronounced in female offspring, and previous studies focused on males (Fan *et al.,* 2016; Somm *et al.*, 2008), we decided to examine the effects of MNE on female adipose tissue function. One limitation of our study is that we did not evaluate the estrous cycle of the female rats. This is an important consideration since prenatal nicotine exposure increased progesterone levels in offspring compared to vehicle controls, which is reflective of estrous cycle perturbations (Holloway, Kellenberger and Petrik, 2006).

Furthermore, others have reported that oscillations in metabolic related genes in the liver occur with estrous cycle (Villa *et al.*, 2012). Since small adipocyte size results from aberrant adipogenesis, we examined whether MNE impaired adipocyte differentiation, lipid synthesis and transport, and/or lipolysis. We found that female MNE offspring exhibited increased mRNA and protein expression of the adipocyte differentiation factor C/EBPα and the lipolytic enzyme ATGL, without altering the expression of targets involved in lipogenesis and lipid transport. Similar to our results, Fan *et al.* found an increased in steady-state mRNA levels of adipocyte differentiation markers in MNE male offspring, including C/EBPα and SREBP-1c compared to control WAT in adulthood (Fan *et al.*, 2016). Since these studies did not perform protein analyses, our study is the first to demonstrate that MNE increased C/EBPα protein levels in 6 month female WAT. Overall, the increased C/EBPα (42 kDa) expression in WAT suggests that MNE promote adipocyte differentiation and adipogenesis. C/EBPα contains two isoforms (30/42 kDa) alternatively translated from one mRNA transcript (Lin *et al.*, 1993; Ossipow *et al.*, 1993). Constitutive expression of the 42 kDa isoform can induce adipocyte differentiation in 3T3-L1 preadipocytes, suggesting that enhanced C/EBPα expression of this isoform can enhance adipocyte formation (Lin and Lane, 1994). While the steady-state levels of AKT1, C/EBP-α, SREBP 1c mRNA were augmented in 6 month nicotine-exposed offspring, this did not translate to an alteration in protein levels. There are several cases in the literature whereby changes in the mRNA levels do not correlate to an alteration in protein, highlighting the importance of looking at both mRNA and protein (Vogel and Marcotte, 2012). Moreover, it is possible that the mRNA and protein levels of these key proliferation and differentiation markers may be altered earlier in development (i.e. gestation and/or lactation) due to the direct effects of nicotine on their expression. For example, we have demonstrated that nicotine directly enhances the expression of

the proliferation markers GADD45 and Rb1 in *differentiating* 3T3-L1 pre-adipocyte cells (data

not shown). Therefore, in the absence of nicotine exposure after lactation, the levels of these

proliferation and differentiation may have become normalized.

This study was the first to demonstrate that both prenatal and perinatal nicotine exposure

leads to increased ATGL mRNA and protein expression in adult female MNE offspring. This

suggests that MNE may impair the lipid storage capacity of WAT via increased ATGL

expression. Studies demonstrate that augmented ATGL leads to enhanced basal and stimulated

WAT lipolysis and reduced adipocyte size (Ahmadian *et al.*, 2009;   Bezaire *et al.*, 2009;

Kershaw *et al.*, 2006). The primary function of ATGL is to initiate lipolysis, by hydrolyzing the

*sn*-2 ester bond of a triglyceride molecule, resulting in the formation of diacylglycerol and fatty

acid (Zechner *et al.*, 2009). Therefore, decreased adipocyte cell size in gonadal WAT may be

attributed to enhanced lipolysis in MNE offspring.

Recently, evidence supports an additional role for ATGL - as an antioxidant and

suppressor of oxidative stress. Oxidative stress is defined as an imbalance between antioxidant

defenses and ROS production (free radicals) (Sena and Chandel, 2012). Prolonged oxidative

stress can result in free radical damage, ultimately leading to mitochondrial-mediated apoptosis

(Sena and Chandel, 2012). *In vitro* and *in vivo* analyses demonstrate that ATGL deficiency is

associated with ROS accumulation and cellular apoptosis in renal podocytes and proximal

tubules (Chen *et al.,* 2017a;   Chen *et al.*, 2017b). Similarly, ATGL inhibition in C2C12

myoblasts and in murine ATGL deficient skeletal muscle promotes oxidative damage and a

defective cellular antioxidant response, characterized by a significant reduction in antioxidant

SOD-2 expression and glutathione levels (Aquilano *et al.*, 2016). Altogether, these findings

suggest that ATGL may protect against oxidative stress. We and others have previously shown

that maternal nicotine exposure can cause oxidative stress in the offspring in various tissues including the heart, pancreas, and placenta (Barra *et al.*, 2017;  Bruin *et al.,* 2008;  Sbrana *et al.*, 2011). Therefore, we examined the expression of antioxidants SOD-1/2 in WAT of 6 month female MNE offspring to assess whether an imbalance in anti-oxidant expression is present at the same time ATGL levels are elevated. Oxygen can oxidize other molecules to generate ROS, whereby majority of intracellular ROS are derived from superoxide (O2-). Superoxide is formed in the mitochondrial respiratory chain, emitted to the matrix and intermembrane space, and subsequently converted to hydrogen peroxide ($H_2O_2$) by superoxide dismutases (SODs) (Sena and Chandel, 2012). This study is the first to show that MNE WAT had increased transcript expression and protein levels of both SOD-1 and -2 at 6 months with evidence for increased SOD-1 at 3 weeks of age. Although non-significant, we also observed an upward trend for the oxidative marker 4-HNE in nicotine exposed WAT compared to vehicle at both time points. This suggests that perinatal nicotine exposure may instigate WAT oxidative stress, triggering a rapid increase in anti-oxidant expression by weaning which persists into adulthood and may prevent long-term oxidative damage.

Oxidative stress can contribute to altered adipocyte differentiation and lipolysis. Depending on environmental cues, mesenchymal stem cells can differentiate into various cell types derived from the mesodermal lineage including myocytes, adipocytes, osteocytes, and chondrocytes. Evidence suggests that elevated ROS levels, produced during oxidative stress, can stimulate adipogenesis while suppressing osteogenesis (Atashi *et al.*, 2015). During conversion of stem cells to adipocytes, mitochondrial complexes I/III and the NADPH oxidase isoform NOX4 produce ROS to initiate adipocyte signaling cascades, leading to terminal differentiation (Atashi, Modarressi and Pepper, 2015). Therefore, increased SOD-1 expression in fetal and

neonatal nicotine exposed WAT may suggest that augmented ROS production may directly promote the expression of adipogenic transcription factors like C/EBPα (Hu *et al.*, 2005). *In vitro* analyses using both differentiated 3T3-L1 and human primary adipocyte cultures demonstrate that exposure to exogenous 4-HNE elevated lipolysis in basal and stimulated conditions characterized by a dose-dependent significant increase in glycerol and fatty acid release (Zhang *et al.*, 2013). Altogether, these findings suggest that MNE may augment oxidative stress in WAT characterized by increased expression of antioxidants SOD-1/2 and ATGL, leading to altered adipocyte differentiation and lipolysis.

Enhanced oxidative stress can promote pathways, like inflammation, that also influence adipose function. Exposure to hydrogen peroxide or ROS accumulation activates the transcription factor NF-κB, leading the expression of pro-inflammatory cytokines such as TNF-α or IL-1 (Lawrence, 2009; Li and Karin, 1999; Morgan and Liu, 2011). Intriguingly, treatment with either TNF-α or IL-6 in differentiated adipocytes can increase the expression of ATGL and enhance glycerol production (a marker of lipolysis). Moreover, *in vitro* studies reveal that TNF-α induces lipolysis in differentiated human and murine adipocytes through multiple distinct pathways (i.e. mitogen-activated protein kinase kinase, (MAPKK); extracellular signal-related kinase 1/2 (ERK); and elevation of cyclic adenosine monophosphate (cAMP); NH2-terminal kinase (JNK)) (Ryden *et al.*, 2002; Yang *et al.*, 2011; Zhang *et al.*, 2002). Similarly, treatment with IL-6 induces lipolysis through increased expression of ATGL in soleus muscles *ex vivo* (Macdonald *et al.*, 2013) and enhanced glycerol production in 3T3-L1 cells (Ji *et al.*, 2011). Our results reveal that fetal and neonatal nicotine exposure culminates in increased steady-state mRNA levels of proinflammatory mediators, including TNF-α and IL-6 by 6 months of age. These results coincide with previous findings demonstrating that *in utero* nicotine exposure

resulted in increased circulating pro-inflammatory cytokines throughout development and in 11 week old offspring (Mohsenzadeh *et al.*, 2014;  Orellana *et al.*, 2014). Since inflammation was present in 6 month female WAT and not at 3 weeks, this suggests that nicotine does not have a direct effect on inflammation but that augmented oxidative stress may mediate the expression of pro-inflammatory cytokines. One research group demonstrated that CD-1 mice fed a nicotine-containing diet for 14 weeks postnatally decreased adipocyte cell size without affecting white adipose inflammation (Liu *et al.*, 2018). In fact, treatment with 4-HNE in 3T3-L1 cells induced inflammation by activating p38, subsequently increasing the expression of cyclooxygenase-2 (COX) (Zarrouki *et al.*, 2007). Further studies are warranted to determine whether inflammation may in part promote lipolysis in prenatal nicotine exposed WAT long term.

In summary, our findings demonstrate that maternal nicotine exposure during pregnancy and lactation leads to decreased WAT adipocyte size and impaired lipid storage in postnatal life characterized by augmented lipolysis and inflammation triggered possibly by a pro-oxidative stress environment. This impairment in WAT lipid storage can contribute to increased circulating triglycerides, leading to enhanced hepatic *de novo* lipogenesis, high blood pressure, and increased CVD risk as we have previously shown (Gao *et al.* 2008;  Ma *et al.*, 2014). These findings provide insight into the consequence of prolonged oxidative stress driving WAT dysfunction long-term. Postnatal intervention strategies targeting oxidative stress may benefit children exposed to nicotine *in utero* through cigarette smoke and/or NRT. In fact, studies demonstrate that the use of antioxidants (i.e. anthocyanin, quercetin, bottle gourd) can significantly reduce dyslipidemia (Li *et al.*, 2015;  Talirevic and Jelena, 2012). Overall, results from this study suggests that therapeutic strategies targeting oxidative stress may effectively protect nicotine exposed offspring from WAT dysfunction and dyslipidemia.

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## REFERENCES

Abate, N. (2012). Adipocyte maturation arrest: a determinant of systemic insulin resistance to glucose disposal. *J Clin Endocrinol Metab* **97**(3), 760-3.

Ahmadian, M., Duncan, R. E., Varady, K. A., Frasson, D., Hellerstein, M. K., Birkenfeld, A. L., Samuel, V. T., Shulman, G. I., Wang, Y., Kang, C.*, et al.* (2009). Adipose overexpression of desnutrin promotes fatty acid use and attenuates diet-induced obesity. *Diabetes* **58**(4), 855-66.

Aquilano, K., Baldelli, S., La Barbera, L., Lettieri Barbato, D., Tatulli, G., and Ciriolo, M. R. (2016). Adipose triglyceride lipase decrement affects skeletal muscle homeostasis during aging through FAs-PPARalpha-PGC-1alpha antioxidant response. *Oncotarget* **7**(17), 23019-32.

Atashi, F., Modarressi, A., and Pepper, M. S. (2015). The role of reactive oxygen species in mesenchymal stem cell adipogenic and osteogenic differentiation: a review. *Stem Cells Dev* **24**(10), 1150-63.

Bansal, S., Buring, J. E., Rifai, N., Mora, S., Sacks, F. M., and Ridker, P. M. (2007). Fasting compared with nonfasting triglycerides and risk of cardiovascular events in women. *JAMA* **298**(3), 309-16.

Barra, N. G., Lisyansky, M., Vanduzer, T. A., Raha, S., Holloway, A. C., and Hardy, D. B. (2017). Maternal nicotine exposure leads to decreased cardiac protein disulfide isomerase and impaired mitochondrial function in male rat offspring. *J Appl Toxicol* doi: 10.1002/jat.3503.

Bezaire, V., Mairal, A., Ribet, C., Lefort, C., Girousse, A., Jocken, J., Laurencikiene, J., Anesia, R., Rodriguez, A. M., Ryden, M.*, et al.* (2009). Contribution of adipose triglyceride lipase and hormone-sensitive lipase to lipolysis in hMADS adipocytes. *J Biol Chem* **284**(27), 18282-91.

Bolsoni-Lopes, A., and Alonso-Vale, M. I. (2015). Lipolysis and lipases in white adipose tissue - An update. *Arch Endocrinol Metab* **59**(4), 335-42.

Bruin, J. E., Petre, M. A., Lehman, M. A., Raha, S., Gerstein, H. C., Morrison, K. M., and Holloway, A. C. (2008). Maternal nicotine exposure increases oxidative stress in the offspring. *Free Radic Biol Med* **44**(11), 1919-25.

Chen, W., Jiang, Y., Han, J., Hu, J., He, T., Yan, T., Huang, N., Zhang, Q., Mei, H., Liao, Y.*, et al.* (2017a). Atgl deficiency induces podocyte apoptosis and leads to glomerular filtration barrier damage. *FEBS J* **284**(7), 1070-1081.

Chen, W., Zhang, Q., Cheng, S., Huang, J., Diao, G., and Han, J. (2017b). Atgl gene deletion predisposes to proximal tubule damage by impairing the fatty acid metabolism. *Biochem Biophys Res Commun* **487**(1), 160-166.

Chi, W., Dao, D., Lau, T. C., Henriksbo, B. D., Cavallari, J. F., Foley, K. P., and Schertzer, J. D. (2014). Bacterial peptidoglycan stimulates adipocyte lipolysis via NOD1. *PLoS One* **9**(5), e97675.

Chou, H. C., and Chen, C. M. (2014). Maternal nicotine exposure during gestation and lactation induces cardiac remodeling in rat offspring. *Reprod Toxicol* **50**, 4-10.

Cui, Y., Shooshtari, S., Forget, E. L., Clara, I., and Cheung, K. F. (2014). Smoking during pregnancy: findings from the 2009-2010 Canadian Community Health Survey. *PLoS One* **9**(1), e84640.

Cupul-Uicab, L. A., Skjaerven, R., Haug, K., Travlos, G. S., Wilson, R. E., Eggesbo, M., Hoppin, J. A., Whitworth, K. W., and Longnecker, M. P. (2012). Exposure to tobacco smoke in utero and subsequent plasma lipids, ApoB, and CRP among adult women in the MoBa cohort. *Environ Health Perspect* **120**(11), 1532-7.

Dasgupta, C., Xiao, D., Xu, Z., Yang, S., and Zhang, L. (2012). Developmental nicotine exposure results in programming of alveolar simplification and interstitial pulmonary fibrosis in adult male rats. *Reprod Toxicol* **34**(3), 370-7.

Fan, J., Zhang, W. X., Rao, Y. S., Xue, J. L., Wang, F. F., Zhang, L., and Yan, Y. E. (2016). Perinatal Nicotine Exposure Increases Obesity Susceptibility in Adult Male Rat Offspring by Altering Early Adipogenesis. *Endocrinology* **157**(11), 4276-4286.

Fox, K. A., Longo, M., Tamayo, E., Gamble, P., Makhlouf, M., Mateus, J. F., and Saade, G. R. (2012). Sex-specific effects of nicotine exposure on developmental programming of blood pressure and vascular reactivity in the C57Bl/6J mouse. *Am J Obstet Gynecol* **207**(3), 208 e1-9.

Gao, Y. J., Holloway, A. C., Su, L. Y., Takemori, K., Lu, C., and Lee, R. M. (2008). Effects of fetal and neonatal exposure to nicotine on blood pressure and perivascular adipose tissue function in adult life. *Eur J Pharmacol* **590**(1-3), 264-8.

Gao, Y. J., Holloway, A. C., Zeng, Z. H., Lim, G. E., Petrik, J. J., Foster, W. G., and Lee, R. M. (2005). Prenatal exposure to nicotine causes postnatal obesity and altered perivascular adipose tissue function. *Obes Res* **13**(4), 687-92.

Gochberg-Sarver, A., Kedmi, M., Gana-Weisz, M., Bar-Shira, A., and Orr-Urtreger, A. (2012). Tnfalpha, Cox2 and AdipoQ adipokine gene expression levels are modulated in murine adipose tissues by both nicotine and nACh receptors containing the beta2 subunit. *Mol Genet Metab* **107**(3), 561-70.

Greenwood, M. R., and Hirsch, J. (1974). Postnatal development of adipocyte cellularity in the normal rat. *J Lipid Res* **15**(5), 474-83.

Holloway, A. C., Kellenberger, L. D., and Petrik, J. J. (2006). Fetal and neonatal exposure to nicotine disrupts ovarian function and fertility in adult female rats. *Endocrine* **30**(2), 213-6.

Holloway, A. C., Petrik, J. J., Bruin, J. E., and Gerstein, H. C. (2008). Rosiglitazone prevents diabetes by increasing beta-cell mass in an animal model of type 2 diabetes characterized by reduced beta-cell mass at birth. *Diabetes Obes Metab* **10**(9), 763-71.

Hoogduijn, M. J., Cheng, A., and Genever, P. G. (2009). Functional nicotinic and muscarinic receptors on mesenchymal stem cells. *Stem Cells Dev* **18**(1), 103-12.

Hu, Y., Rosen, D. G., Zhou, Y., Feng, L., Yang, G., Liu, J., and Huang, P. (2005). Mitochondrial manganese-superoxide dismutase expression in ovarian cancer: role in cell proliferation and response to oxidative stress. *J Biol Chem* **280**(47), 39485-92.

Ji, C., Chen, X., Gao, C., Jiao, L., Wang, J., Xu, G., Fu, H., Guo, X., and Zhao, Y. (2011). IL-6 induces lipolysis and mitochondrial dysfunction, but does not affect insulin-mediated glucose transport in 3T3-L1 adipocytes. *J Bioenerg Biomembr* **43**(4), 367-75.

Kershaw, E. E., Hamm, J. K., Verhagen, L. A., Peroni, O., Katic, M., and Flier, J. S. (2006). Adipose triglyceride lipase: function, regulation by insulin, and comparison with adiponutrin. *Diabetes* **55**(1), 148-57.

Kersten, S. (2014). Physiological regulation of lipoprotein lipase. *Biochim Biophys Acta* **1841**(7), 919-33.

Lawrence, T. (2009). The nuclear factor NF-kappaB pathway in inflammation. *Cold Spring Harb Perspect Biol* **1**(6), a001651.

Li, D., Zhang, Y., Liu, Y., Sun, R., and Xia, M. (2015). Purified anthocyanin supplementation reduces dyslipidemia, enhances antioxidant capacity, and prevents insulin resistance in diabetic patients. *J Nutr* **145**(4), 742-8.

Li, N., and Karin, M. (1999). Is NF-kappaB the sensor of oxidative stress? *FASEB J* **13**(10), 1137-43.

Lin, F. T., and Lane, M. D. (1994). CCAAT/enhancer binding protein alpha is sufficient to initiate the 3T3-L1 adipocyte differentiation program. *Proc Natl Acad Sci U S A* **91**(19), 8757-61.

Lin, F. T., MacDougald, O. A., Diehl, A. M., and Lane, M. D. (1993). A 30-kDa alternative translation product of the CCAAT/enhancer binding protein alpha message: transcriptional activator lacking antimitotic activity. *Proc Natl Acad Sci U S A* **90**(20), 9606-10.

Liu, M., Chuang Key, C. C., Weckerle, A., Boudyguina, E., Sawyer, J. K., Gebre, A. K., Spoo, W., Makwana, O., and Parks, J. S. (2018). Feeding of tobacco blend or nicotine induced weight loss associated with decreased adipocyte size and increased physical activity in male mice. *Food Chem Toxicol* **113**, 287-295.

Luck, W., and Nau, H. (1987). Nicotine and cotinine concentrations in the milk of smoking mothers: influence of cigarette consumption and diurnal variation. *Eur J Pediatr* **146**(1), 21-6.

Luck, W., Nau, H., Hansen, R., and Steldinger, R. (1985). Extent of nicotine and cotinine transfer to the human fetus, placenta and amniotic fluid of smoking mothers. *Dev Pharmacol Ther* **8**(6), 384-95.

Ma, N., Nicholson, C. J., Wong, M., Holloway, A. C., and Hardy, D. B. (2014). Fetal and neonatal exposure to nicotine leads to augmented hepatic and circulating triglycerides in adult male offspring due to increased expression of fatty acid synthase. *Toxicol Appl Pharmacol* **275**(1), 1-11.

Macdonald, T. L., Wan, Z., Frendo-Cumbo, S., Dyck, D. J., and Wright, D. C. (2013). IL-6 and epinephrine have divergent fiber type effects on intramuscular lipolysis. *J Appl Physiol (1985)* **115**(10), 1457-63.

Mohsenzadeh, Y., Rahmani, A., Cheraghi, J., Pyrani, M., and Asadollahi, K. (2014). Prenatal exposure to nicotine in pregnant rat increased inflammatory marker in newborn rat. *Mediators Inflamm* **2014**, 274048.

Morgan, M. J., and Liu, Z. G. (2011). Crosstalk of reactive oxygen species and NF-kappaB signaling. *Cell Res* **21**(1), 103-15.

Oncken, C. A., and Kranzler, H. R. (2003). Pharmacotherapies to enhance smoking cessation during pregnancy. *Drug Alcohol Rev* **22**(2), 191-202.

Orellana, J. A., Busso, D., Ramirez, G., Campos, M., Rigotti, A., Eugenin, J., and von Bernhardi, R. (2014). Prenatal nicotine exposure enhances Cx43 and Panx1 unopposed channel activity in brain cells of adult offspring mice fed a high-fat/cholesterol diet. *Front Cell Neurosci* **8**, 403.

Ossipow, V., Descombes, P., and Schibler, U. (1993). CCAAT/enhancer-binding protein mRNA is translated into multiple proteins with different transcription activation potentials. *Proc Natl Acad Sci U S A* **90**(17), 8219-23.

Pauly, J. R., and Slotkin, T. A. (2008). Maternal tobacco smoking, nicotine replacement and neurobehavioural development. *Acta Paediatr* **97**(10), 1331-7.

Poissonnet, C. M., Burdi, A. R., and Garn, S. M. (1984). The chronology of adipose tissue appearance and distribution in the human fetus. *Early Hum Dev* **10**(1-2), 1-11.

Roman-Galvez, R. M., Amezcua-Prieto, C., Olmedo-Requena, R., Lewis-Mikhael Saad, A. M., Martinez-Galiano, J. M., and Bueno-Cavanillas, A. (2017). Partner smoking influences whether mothers quit smoking during pregnancy: A prospective cohort study. *BJOG* doi: 10.1111/1471-0528.14986.

Ryden, M., Dicker, A., van Harmelen, V., Hauner, H., Brunnberg, M., Perbeck, L., Lonnqvist, F., and Arner, P. (2002). Mapping of early signaling events in tumor necrosis factor-alpha - mediated lipolysis in human fat cells. *J Biol Chem* **277**(2), 1085-91.

Sbrana, E., Suter, M. A., Abramovici, A. R., Hawkins, H. K., Moss, J. E., Patterson, L., Shope, C., and Aagaard-Tillery, K. (2011). Maternal tobacco use is associated with increased markers of oxidative stress in the placenta. *Am J Obstet Gynecol* **205**(3), 246 e1-7.

Sena, L. A., and Chandel, N. S. (2012). Physiological roles of mitochondrial reactive oxygen species. *Mol Cell* **48**(2), 158-67.

Shahab, L., Dobbie, F., Hiscock, R., McNeill, A., and Bauld, L. (2016). Prevalence and Impact of Long-term Use of Nicotine Replacement Therapy in UK Stop-Smoking Services: Findings From the ELONS Study. *Nicotine Tob Res* doi: 10.1093/ntr/ntw258.

Somm, E., Schwitzgebel, V. M., Vauthay, D. M., Camm, E. J., Chen, C. Y., Giacobino, J. P., Sizonenko, S. V., Aubert, M. L., and Huppi, P. S. (2008). Prenatal nicotine exposure alters early pancreatic islet and adipose tissue development with consequences on the control of body weight and glucose metabolism later in life. *Endocrinology* **149**(12), 6289-99.

Spalding, K. L., Arner, E., Westermark, P. O., Bernard, S., Buchholz, B. A., Bergmann, O., Blomqvist, L., Hoffstedt, J., Naslund, E., Britton, T*., et al.* (2008). Dynamics of fat cell turnover in humans. *Nature* **453**(7196), 783-7.

Talirevic, E., and Jelena, S. (2012). Quercetin in the treatment of dyslipidemia. *Med Arh* **66**(2), 87-8.

Tappin, D. M., MacAskill, S., Bauld, L., Eadie, D., Shipton, D., and Galbraith, L. (2010). Smoking prevalence and smoking cessation services for pregnant women in Scotland. *Subst Abuse Treat Prev Policy* **5**, 1.

Tong, V. T., Dietz, P. M., Morrow, B., D'Angelo, D. V., Farr, S. L., Rockhill, K. M., England, L. J., Centers for Disease, C., and Prevention (2013). Trends in smoking before, during, and after pregnancy--Pregnancy Risk Assessment Monitoring System, United States, 40 sites, 2000-2010. *MMWR Surveill Summ* **62**(6), 1-19.

Villa, A., Della Torre, S., Stell, A., Cook, J., Brown, M., and Maggi, A. (2012). Tetradian oscillation of estrogen receptor alpha is necessary to prevent liver lipid deposition. *Proc Natl Acad Sci U S A* **109**(29), 11806-11.

Vogel, C., and Marcotte, E. M. (2012). Insights into the regulation of protein abundance from proteomic and transcriptomic analyses. *Nat Rev Genet* **13**(4), 227-32.

Wen, X., Triche, E. W., Hogan, J. W., Shenassa, E. D., and Buka, S. L. (2010). Birth weight and adult hypercholesterolemia: subgroups of small-for-gestational-age based on maternal smoking status during pregnancy. *Epidemiology* **21**(6), 786-90.

Weng, S. F., Redsell, S. A., Swift, J. A., Yang, M., and Glazebrook, C. P. (2012). Systematic review and meta-analyses of risk factors for childhood overweight identifiable during infancy. *Arch Dis Child* **97**(12), 1019-26.

Yang, X., Zhang, X., Heckmann, B. L., Lu, X., and Liu, J. (2011). Relative contribution of adipose triglyceride lipase and hormone-sensitive lipase to tumor necrosis factor-alpha (TNF-alpha)-induced lipolysis in adipocytes. *J Biol Chem* **286**(47), 40477-85.

Zarrouki, B., Soares, A. F., Guichardant, M., Lagarde, M., and Geloen, A. (2007). The lipid peroxidation end-product 4-HNE induces COX-2 expression through p38MAPK activation in 3T3-L1 adipose cell. *FEBS Lett* **581**(13), 2394-400.

Zechner, R., Kienesberger, P. C., Haemmerle, G., Zimmermann, R., and Lass, A. (2009). Adipose triglyceride lipase and the lipolytic catabolism of cellular fat stores. *J Lipid Res* **50**(1), 3-21.

Zhang, H. H., Halbleib, M., Ahmad, F., Manganiello, V. C., and Greenberg, A. S. (2002). Tumor necrosis factor-alpha stimulates lipolysis in differentiated human adipocytes through activation of extracellular signal-related kinase and elevation of intracellular cAMP. *Diabetes* **51**(10), 2929-35.

Zhang, X., Wang, Z., Li, J., Gu, D., Li, S., Shen, C., and Song, Z. (2013). Increased 4-hydroxynonenal formation contributes to obesity-related lipolytic activation in adipocytes. *PLoS One* **8**(8), e70663.

Zhang, Y., Li, J., and Wen, X. (2017). Jueming prescription and its ingredients, semen cassiae and Rhizoma Curcumae Longae, stimulate lipolysis and enhance the phosphorylation of hormonesensitive lipase in cultured rat white adipose tissue. *Mol Med Rep* **16**(5), 6200-6207.

## FIGURE LEGENDS

**Figure 1: Maternal nicotine exposure leads to decreased visceral adipocyte cell area in 6 month old offspring.** Representative cross-sections of gonadal WAT excised from **(A)** vehicle and *in utero* nicotine exposed 6 month old male and female offspring were stained with hematoxylin and eosin. **(B)** Bar graph representing calculated cell areas analyzed using Northern Eclipse software (n=4/group/sex). **(C)** Transcript and **(D)** protein levels of AKT-1 in six month vehicle and perinatal nicotine exposed WAT were determined via real time PCR and Western blot, respectively. mRNA levels were expressed as means normalized to β-actin ± SEM (n=15/vehicle; n=11/nicotine). Protein levels were expressed as means normalized to β-actin ± SEM (n=8/vehicle; n=7/nicotine). **,*** Significant difference (**$p<0.01$, ***$p < 0.001$).

**Figure 2: *In utero* nicotine exposure increases the expression of the differentiation marker C/EBPα in WAT of 6 month old female offspring.** Transcript and protein levels of targets of interest in 3 week and six month vehicle and perinatal nicotine exposed WAT were determined via real time PCR and Western blot, respectively. Six month **(A)** C/EBPα mRNA and **(B)** protein levels. **(C)** C/EBPβ mRNA and **(D)** protein levels. **(E)** SREBP1-c mRNA and **(F)** protein levels. Three week **(G)** C/EBPα mRNA and **(H)** protein levels. mRNA levels were expressed as means normalized to β-actin ± SEM (n=15/vehicle; n=11/nicotine). Protein levels were expressed as means normalized to β-actin ± SEM (n=8/vehicle; n=7/nicotine). *, Significant difference (*$p<0.05$).

**Figure 3: Maternal nicotine exposure does not increase the expression of markers involved in lipogenesis and fatty acid transporters in WAT of 6 month old female offspring.**

Transcript levels of targets of interest in six month vehicle and perinatal nicotine exposed WAT were determined via real time PCR. Six month **(A)** LPL, **(B)** ACCα, **(C)** FAS, **(D)** ASCL1, **(E)** FATP1, and **(F)** FATP4 mRNA levels. mRNA levels were expressed as means normalized to β-actin ± SEM (n=15/vehicle; n=11/nicotine).

**Figure 4:** *In utero* **nicotine exposed female offspring exhibit increased expression of ATGL at 6 months, but not at 3 weeks.** Transcript and protein levels of ATGL in 3 week and six month vehicle and perinatal nicotine exposed WAT were determined via real time PCR and Western blot, respectively. Six month ATGL **(A)** mRNA and **(B)** protein levels. Three week ATGL **(C)** mRNA and **(D)** protein levels. mRNA levels were expressed as means normalized to β-actin ± SEM (n=15/vehicle; n=11/nicotine). Protein levels were expressed as means normalized to β-actin ± SEM (n=8/vehicle; n=8/nicotine). *,** Significant difference (*$p<0.05$, **$p<0.01$).

**Figure 5:** *In utero* **nicotine exposed female offspring exhibit increased anti-oxidant expression in WAT at 3 weeks and 6 months.** Transcript and protein levels of SOD-1 and SOD-2 in 3 week and six month vehicle and perinatal nicotine exposed WAT were determined via real time PCR and Western blot, respectively. Six month SOD-1 **(A)** mRNA and **(B)** protein levels and SOD-2 **(C)** mRNA and **(D)** protein levels. Three week SOD-1 **(E)** mRNA and **(F)** protein levels and SOD-2 **(G)** mRNA and **(H)** protein levels. Protein levels of 4-HNE at **(I)** 6 months and **(J)** 3 weeks of age. mRNA levels were expressed as means normalized to β-actin ± SEM (n=15/vehicle; n=11/nicotine). Protein levels were expressed as means normalized to β-actin ± SEM (n=8/vehicle; n=8/nicotine). *,** Significant difference (*$p<0.05$, **$p<0.01$).

**Figure 6: Maternal nicotine exposed female offspring exhibit increases in WAT inflammation at 6 months, but not at 3 weeks.** Transcript levels of targets of interest in six month vehicle and perinatal nicotine exposed WAT were determined via real time PCR. Six month **(A)** IL-1β, **(B)** TNFα, **(C)** IL-6, and **(D)** CD68 mRNA levels. Three week **(E)** IL-1β, **(F)** TNFα, **(G)** IL-6, and **(H)** CD68 mRNA levels. mRNA levels were expressed as means normalized to β-actin ± SEM (n=15/vehicle; n=11/nicotine). *,**,*** Significant difference ($*p<0.05$, $**p<0.01$, $**p<0.001$).

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# **TABLES**

Table 1. Forward and reverse primer sequences used for quantitative Real-Time PCR

| GENE | Forward | Reverse | GenBank/Reference |
|------|---------|---------|-------------------|
| AKT-1 | ATGTGTATGAGAAGAAGCTGAGCC | GTTCACTGTCCACACACTCCA | NM_033230.2 |
| C/EBPα | GCCGGGAGAACTCTAACTCC | TCGATGTAGGCGCTGATGTC | NM_001287577.1 |
| C/EBPβ | ACCACGACTTCCTTTCCGAC | TAACCGTAGTCGGACGGCTT | NM_024125.5 |
| SREBP1c | CATGGACGAGCTACCCTTCG | TCTTCGATGTCGGTCAAGAGC | NM_001276707.1 |
| LPL | GGATGCAACATTGGAGAACCC | GCTGGGGTTTTCTTCATTCAGC | NM_012598.2 |
| ACCα | TCCGTATGTGACCAAAGACC | TACGTTGTTCCCAAGGACTG | NM_022193.1 |
| FAS | GGACATGGTCACAGACGATGAC | CGTCGAACTTGGACAGATCCTT | NM_017332.1 |
| ACSL1 | CTACAGGCAACCCCAAAGGA | AATGCACTCTCCGTCGCTT | NM_012820.1 |
| FATP1 | CAGCCTCTGTGGCCTCATT | ACCCACGTACACACCGAAC | NM_053580.2 |
| FATP4 | CTTGGGCAACTTTGACAGCC | AGGACAGGATGCGGCTATTG | NM_001100706.1 |
| ATGL | AACGCCACTCACATCTACGG | TACCAGGTTGAAGGAGGGGT | NM_001108509.2 |
| EGR-1 | CGAGCGAACAACCCTACGA | CGATGTCAGAAAAGGACTCTGTG | NM_012551.2 |
| FOXO-1 | TGCAGCAGACACCTTGCTAT | TTGGGGCTGGGGGAATTTAG | NM_001191846.2 |
| IL-1β | CAGCTTTCGACAGTGAGGAGA | GTCGAGATGCTGCTGTGAGA | NM_031512.2 |
| TNFα | CCGGGCAGGTCTACTTTGGA | AGGCCACTACTTCAGCGTCTCG | NM_012675.3 |
| IL-6 | CTTCCAGCCAGTTGCCTTCTTG | TGGTCTGTTGTGGGTGGTATCC | NM_012589.2 |
| CD68 | ACTGGGGCTCTTGGAAACTACAC | CCTTGGTTTTGTTCGGGTTCA | NM_001031638.1 |
| SOD-1 | ATTGGCCGTACTATGGTGGTC | GCAATCCCAATCACACCACA | NM_017050.1 |
| SOD-2 | ATTGCCGCCTGCTCTAATCA | TCCCACACATCAATCCCCAG | NM_017051.2 |

Table 2. Western Blot antibodies, dilutions used in experiments, and company and catalogue information.

| Antibody name | Source | Dilution | Company (#Catalogue) |
|---|---|---|---|
| 4-HNE | Mouse monoclonal | 1:500 | R&D Systems Minneapolis, MN, USA (#MAB3249) |
| AKT-1 | Rabbit polyclonal | 1:500 | Abcam Inc., Cambridge, MA, USA (#abcam 5919) |
| ATGL (H-144) | Rabbit polyclonal | 1:1000 | Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA (#sc-67355) |
| C/EBPα (14AA) | Rabbit polyclonal | 1:500 | Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA (#sc-61) |
| C/EBPβ (C-19) | Rabbit polyclonal | 1:300 | Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA (#sc-150) |
| SOD-1 (FL-154) | Rabbit polyclonal | 1:1000 | Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA (#sc-11407) |
| SOD-2 (FL-222) | Rabbit polyclonal | 1:1000 | Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA (#sc-30080) |
| SREBP 1c (H-160) | Rabbit polyclonal | 1:500 | Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA (#sc-8984) |
| Mouse IgG (H+L) Secondary | Sheep | 1:5000 | GE Healthcare UK, Pittsburgh, PA, USA (#NA931) |
| Rabbit IgG (H+L) Secondary | Donkey | 1:10000 | Jackson ImmunoResearch Laboratories, West Grove, PA, USA (#711-001-003) |

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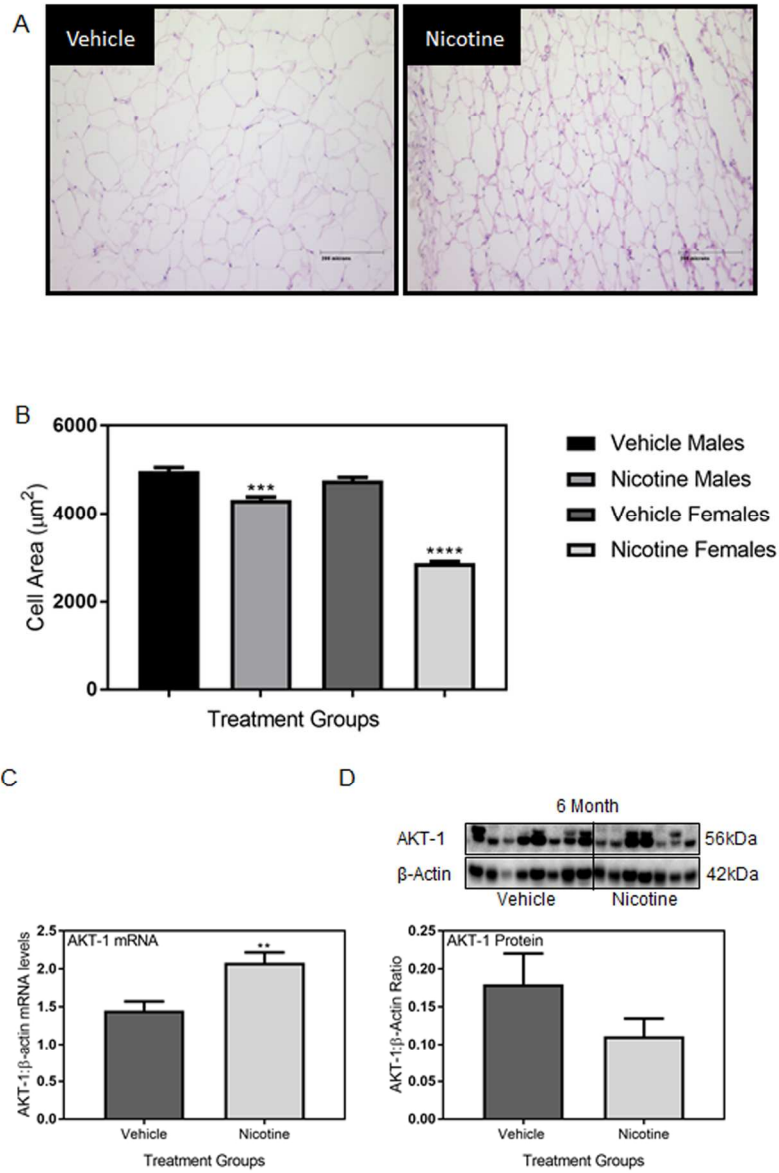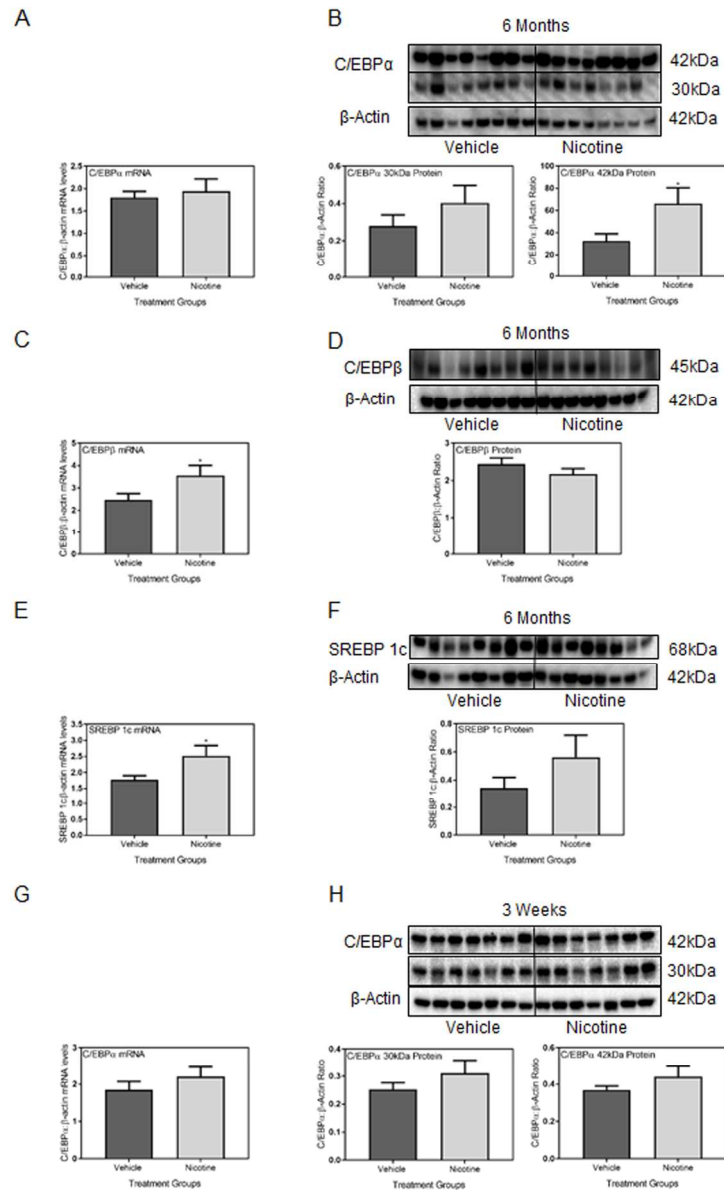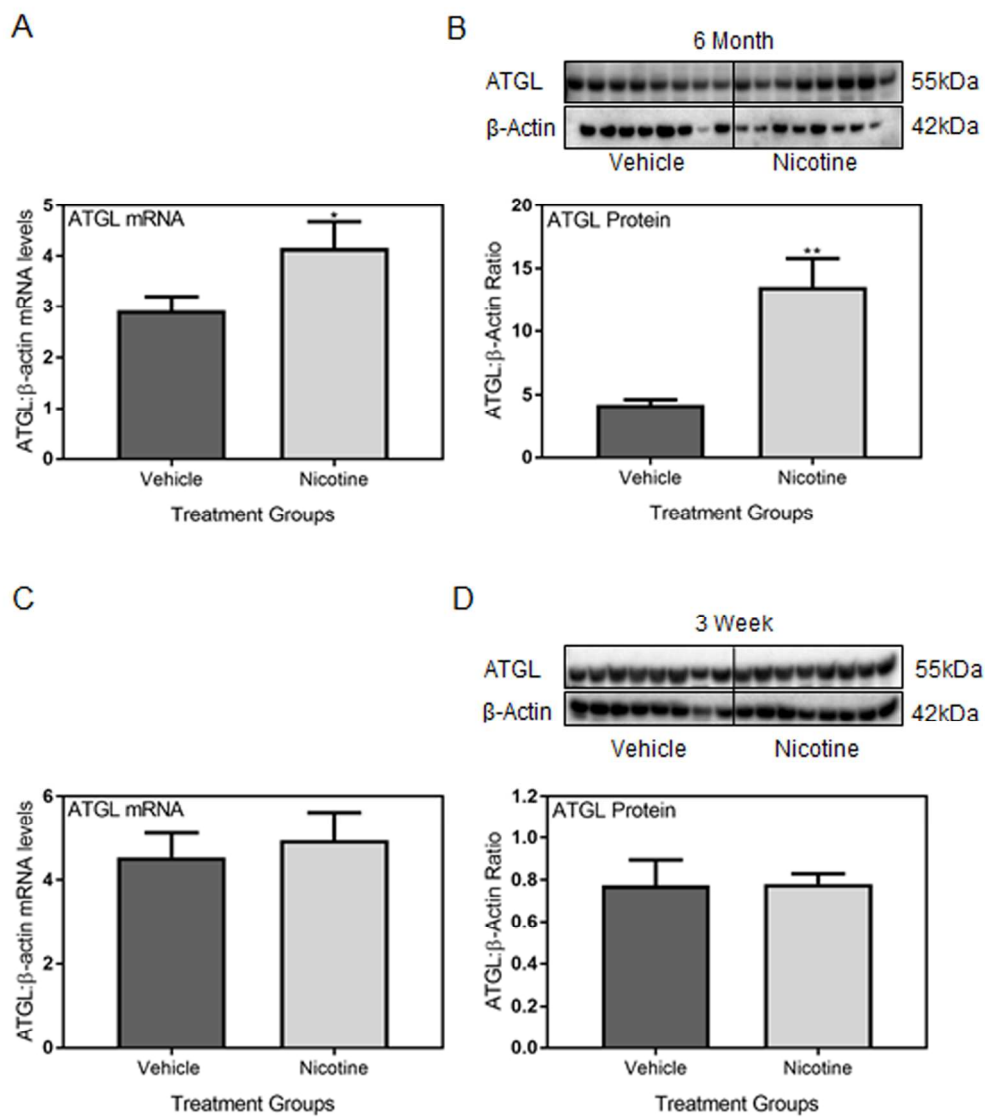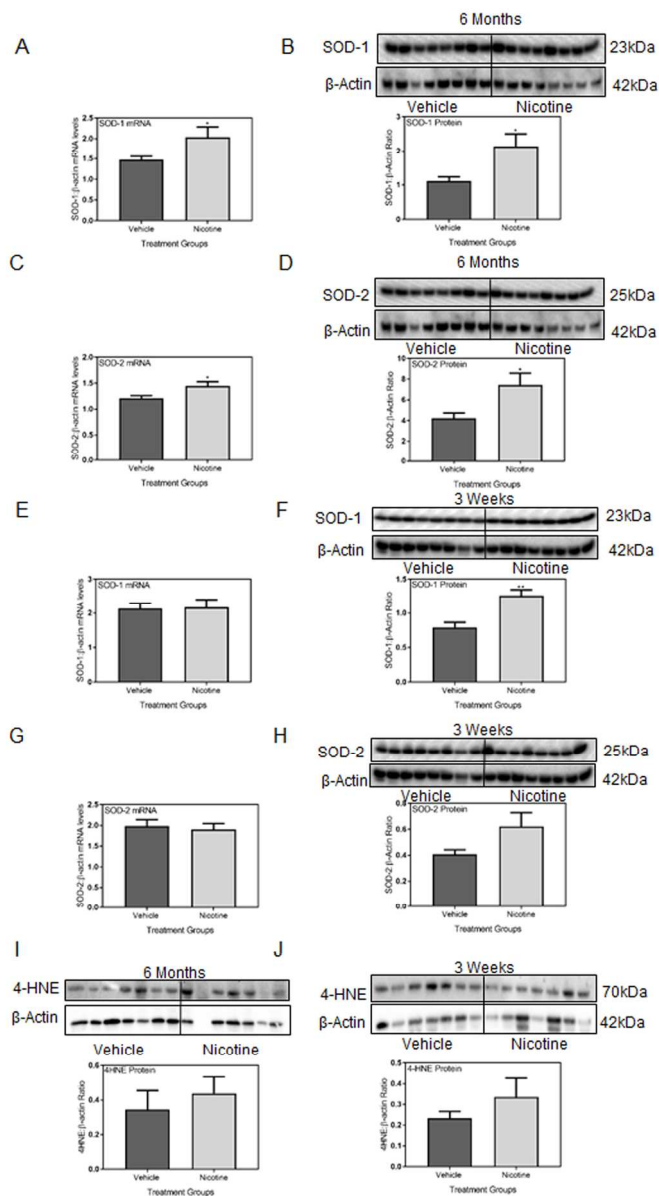48
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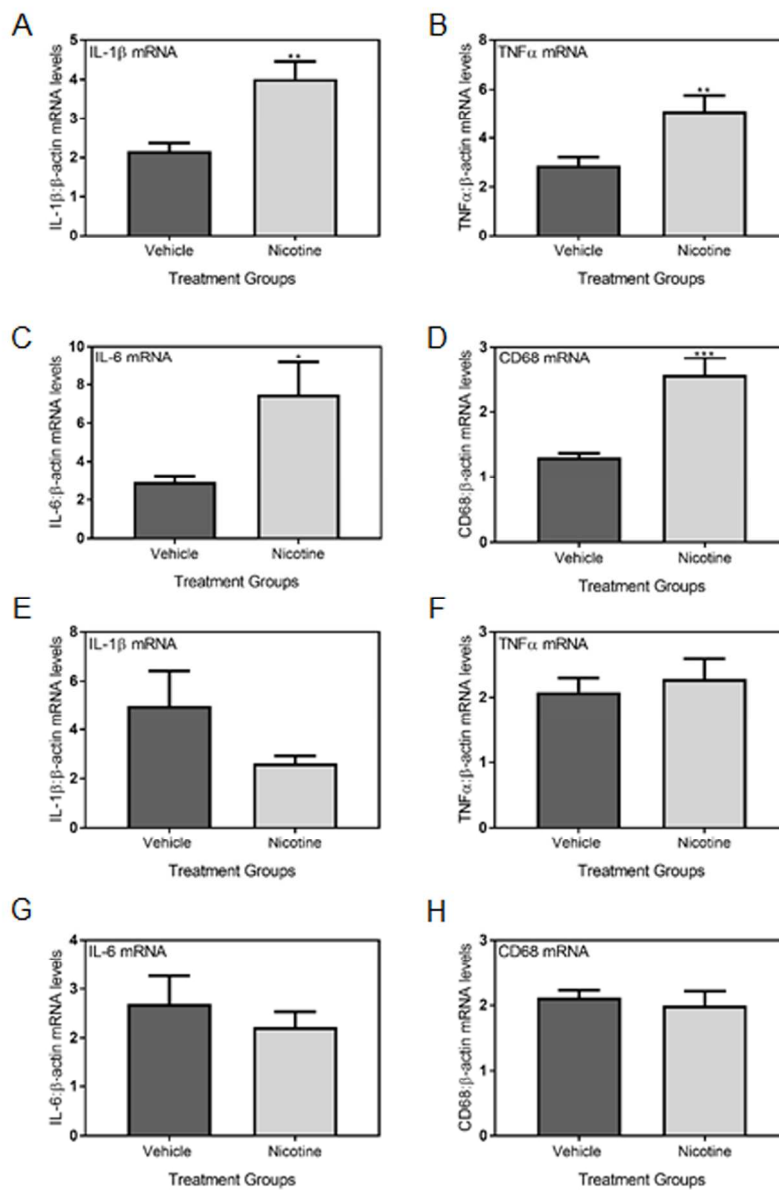58
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Figure 1

124x188mm (300 x 300 DPI)

Figure 2

124x201mm (300 x 300 DPI)
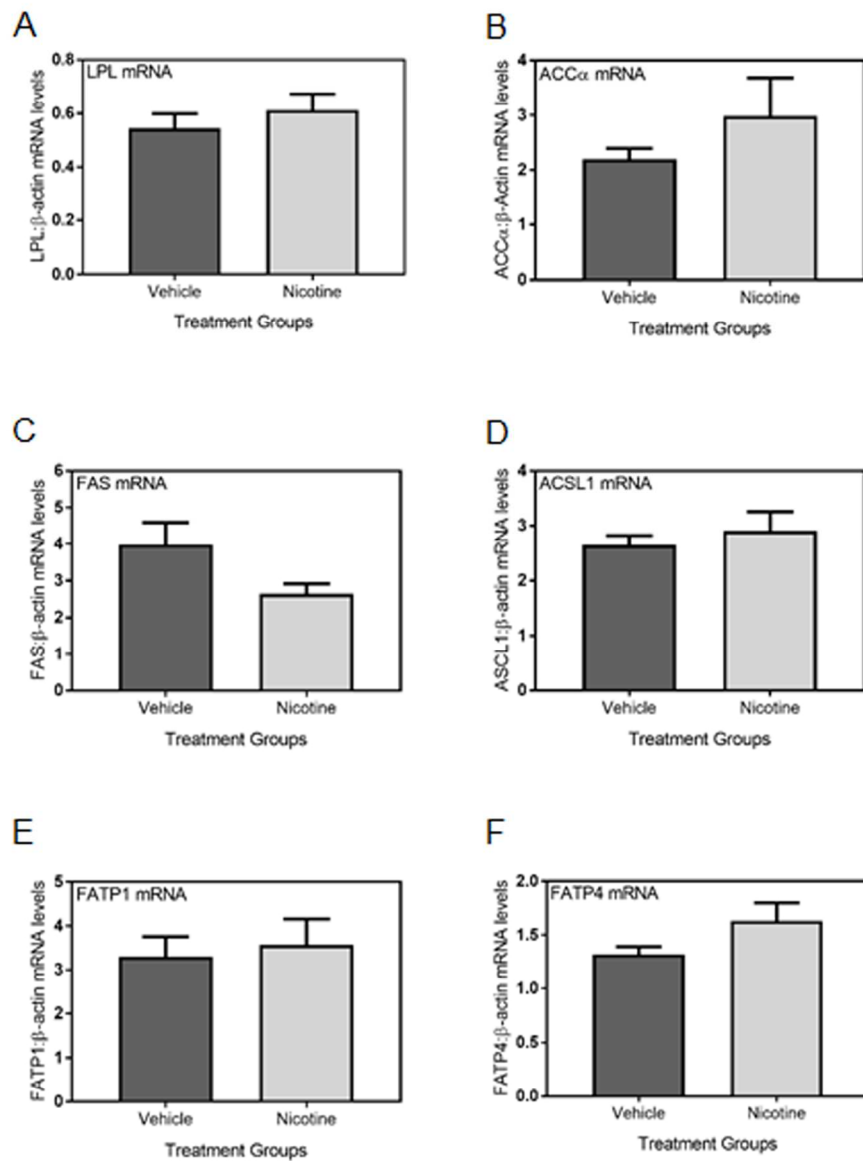
Figure 3

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Figure 4

124x141mm (300 x 300 DPI)

Figure 5

112x202mm (300 x 300 DPI)

Figure 6

124x184mm (300 x 300 DPI)