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Fetal and Neonatal Exposure to Nicotine Augments Hepatic Fatty Acid Synthesis in Rat Offspring Long-Term

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A thesis submitted in partial fulfillment of the requirements for the Master of Science degree in Pharmacology and Toxicology

FETAL AND NEONATAL EXPOSURE TO NICOTINE AUGMENTS HEPATIC FATTY ACID SYNTHESIS IN RAT OFFSPRING LONG-TERM

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by

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ABSTRACT

While nicotine replacement therapy (NRT) is presumed to be a safer alternative to smoking in pregnancy, the long-term consequences in offspring are still largely unknown. Animal studies now suggest that maternal nicotine exposure during pregnancy and lactation (MNE-PL) leads to a wide variety of adverse outcomes for the offspring, including increased adiposity. The focus of this study was to investigate how MNE-PL in rats may lead to liver dysfunction long-term in offspring through alterations in gene expression and epigenetic modifications. Postnatal day 180 (PND180) offspring exposed to nicotine during pregnancy and lactation (1mg/kg/day) exhibited increased circulating and hepatic triglycerides concomitant with increased expression of fatty acid synthase (FAS), an enzyme involved in hepatic *de novo* fatty acid synthesis. Furthermore, we demonstrate that MNE-PL offspring displayed increased protein expression of the Liver X Receptor α (LXRα), a key regulator of FAS. Chromatin immunoprecipitation revealed enriched binding of LXRα to the putative LXRE element on the *FAS* promoter in PND180 male offspring. This was associated with enhanced acetylation of histone H3 [K9,14] surrounding the *FAS* promoter, a hallmark of chromatin activation. Collectively, these findings suggest that nicotine exposure during pregnancy and lactation leads to increased circulating and hepatic triglyceride levels long-term via changes in transcriptional and epigenetic regulation of the hepatic lipogenic pathway**.**

Keywords: Nicotine Replacement Therapy (NRT), Triglycerides, Fatty Acid Synthase (FAS), Liver, Liver X Receptor (LXR), Obesity, Fetal Programming

CO-AUTHORSHIP STATEMENT

All work presented in this thesis was performed by Noelle Ma, with the exception of Figures 3.2.3A and 4.5. Catherine J. Nicholson and Dr. Alison C. Holloway at McMaster University, Hamilton, Ontario, carried out all animal care and drug administration. Blood measurements were performed with the assistance of Cynthia Sawyez at the Metabolic Phenotype Laboratory, Robarts Research Institute, London, Ontario.

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LIST OF ABBREVIATIONS

ACCα	Acetyl-coA carboxylase α

ADP	Adenosine diphosphate
BMI	Body mass index
bp	Base pairs
Cyp2a6	Cytochrome P450 2A6
ChIP	Chromatin immunoprecipitation
ChoRE	Carbohydrate response element
ChREBP	Carbohydrate-responsive element-binding protein
CO_2	Carbon dioxide
Cyp7a1	Cholesterol 7 α-hydroxylase
CV	Cardiovascular
DGAT	Diglyceride acyltransferase
DNA	Deoxyribonucleic acid
DOHaD	Developmental origins of health and disease
EDTA	Ethylenediaminetetraacetic acid
eIF2α	Eukaryotic initiation factor 2α
ER	Endoplasmic reticulum
EtOH	Ethanol
FAS	Fatty acid synthase
FWD	Forward
g	Gram

G6Pase	Glucose-6-phosphatase
GK	Glycerol kinase
GLP	G9a-like protein
GPAT	Glycerol-3-phosphate acyltransferase
GPO	Glycerol phosphate oxidase
GRP 78	Glucose regulated protein 78
GRP94	Glucose regulated protein 94
H_2O_2	Hydrogen peroxide
HAT	Histone acetyltransferase
HCl	Hydrochloride
HDAC	Histone deacetylase
HepG2	Hepatocellular carcinoma cell line
HMT	Histone methyltransferase
IUGR	Intrauterine growth restriction
kg	Kilogram
LiCl	Lithium chloride
LXR	Liver X Receptor α and Liver X Receptor β
LXRE	LXR binding element
M	Molar
Mlx	Max-like protein X

mg	Milligram
mins	Minutes
ml	Milliliter
mM	Millimolar
MNE	Maternal nicotine exposure
MNE-PL	Maternal nicotine exposure during pregnancy and lactation
MPR	Maternal protein restriction
mRNA	Messenger ribonucleic acid
MUFA	Monounsaturated fatty acids
nAChR	Nicotinic acetylcholine receptor
$NaHCO_3$	Sodium bicarbonate
NaCl	Sodium cholride
ng	Nanogram
no.	Number
p-ACCα	Phosphorylated acetyl-coA carboxylase α
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PGC-1	Peroxisome proliferator-activated receptor gamma coactivator 1
PND	Postnatal day
qRT-PCR	Quantitative real-time polymerase chain reaction

REV	Reverse
RPM	Revolutions per minute
SCD-1	Stearoyl-CoA desaturase-1
SDS	Sodium dodecyl sulfate
SEM	Standard error of the mean
Sirt-1	Sirtuin 1
SREBP	Sterol regulatory element-binding protein
TE	Tris-EDTA
µg	Microgram
µl	Microliter
XBP-1	X-box binding protein

CHAPTER 1 : INTRODUCTION AND LITERATURE REVIEW

1.1 Nicotine

1.1.1 Maternal smoking and obesity

Despite the well-established links between smoking during pregnancy and increased risk of placental complications (1), impaired fetal growth (2, 3) and perinatal mortality (2, 3), approximately 10-20% of Canadian mothers continue to smoke during pregnancy (4, 5). This translates to approximately ~75,000 babies born each year in Canada alone who were exposed to first hand smoke *in utero* (2, 5). Unfortunately, the global prevalence of tobacco smoking in young mothers continues to rise in low- and middle-income countries (6). In addition, over half of the women who are able to quit smoking during pregnancy will relapse within four months of delivery (7). If women cannot abstain from smoking during pregnancy, a recent meta-analysis of thirty prospective studies now suggests that their children have a 47% increase in the odds of becoming overweight (8). Since smoking during pregnancy is a highly modifiable risk factor, women are willingly predisposing their children to obesity at birth (9). It is noteworthy to mention that some studies have identified a higher risk of children being overweight if their mother was overweight prepregnancy (10). However, the association between smoking and childhood obesity was found to be unaffected by maternal diet, parental body size or gestational weight gain(11, 12). Therefore pathways independent of a mother's lifestyle may be playing a role in the long-term health and disease risk in children. Numerous clinical studies have now found that adults exposed to smoking *in utero* have increased plasma triglycerides, a characteristic often associated with obesity

and the metabolic syndrome (13-16). This is of great concern considering that elevated plasma triglycerides levels are an independent risk factor that is significantly associated with cardiovascular (CV) disease (17, 18). Given that nearly one-third of Canadian children and youth (5- to 17- years old) are either overweight or obese (19), and the risk that elevated triglycerides pose (17, 18), it is clear that these situations warrant strategies for the prevention or reduction of hypertriglyceridemia.

1.1.2 Nicotine replacement therapy (NRT)

Although cigarette smoking is one of the most important and modifiable risk factors leading to adverse obstetrical outcomes (2, 9), nicotine dependence still remains the driving force behind continued smoking behaviour (20-22). Consequently, nicotine replacement therapy (NRT) has been widely developed as an effective therapy for smoking cessation (20, 22). NRT provides a substitute source of nicotine that significantly reduces the symptoms of cigarette withdrawal including irritability, hunger and sleepiness (23, 24). NRT exists in various forms including gums, transdermal patches, nasal sprays, inhalers and lozenges that differ in route of administration and dose of nicotine (25). All NRT formulations are considered more effective as either the main or supportive therapy used, compared to placebo or no treatment in smoking cessation (26). The efficacy of NRT is largely based on the individual and will differ between people based on factors such as the setting in which NRT is administered, source of motivation for quitting and a smoker's level of dependency on nicotine (27). A systematic review of studies evaluating the commercially available forms of NRT found that NRT alone increases the odds of smoking cessation 1.5 to 2-fold independent of additional support and setting (28, 29).

It is important to give special consideration to pregnant women using NRT because of the harm nicotine exposure may pose for the developing fetus. Although NRT appears to be a safer alternative to smoking in pregnancy as the number of chemicals is reduced from 4000 to 1, there is little evidence validating this claim (30, 31). To date, there is insufficient data available on the effectiveness of NRT during pregnancy or on the potential risks it may pose (32). Despite this oversight, obstetricians continue to prescribe NRT in pregnancy, highlighting the urgent need to study both safety and efficacy of NRT in this population (33, 34). Moreover, understanding the long-term consequences of maternal nicotine exposure (MNE) in offspring can provide insight into determining a safe dose and management of NRT use in pregnancy.

In addition to the numerous health benefits of abstaining from smoking in pregnancy, prenatal smoking cessation therapies provide additional economic benefits. For instance, approximately every dollar invested in smoking cessation therapies can result in up to three dollars saved in costs from the prevention of neonatal complications and neonatal intensive care unit expenses (35).

1.1.3 Pharmacology of nicotine

Nicotine ($C_{10}H_{14}N_2$) is a weak base and a relatively lipophilic compound, which facilitates its ability to cross cell membranes in its unionized form (36-38). Accordingly, various forms of NRT are buffered in an alkaline pH for absorption by the body (36). In contrast to nicotine absorption from cigarette smoke, NRT results in a gradual absorption of nicotine and reduces the likelihood of abuse by users (36, 39). Nicotine is absorbed with high affinity in the brain, muscle, liver, lung, spleen and kidney (40, 41). Due to the biological nature of nicotine, it is able to cross the placenta and mammary glands leading

to the accumulation of nicotine in fetal serum and breast milk, respectively (42-45). Studies have demonstrated that human fetuses are exposed to higher levels of nicotine in the placenta, amniotic fluid and serum compared to levels measured in the maternal serum (46).

1.1.4 Pharmacokinetics of nicotine

Nicotine is primarily metabolized in the liver and to a small degree in the lung and brain (36, 47-49). Phase I metabolism of nicotine is mainly via the C-oxidation pathway, which converts nicotine to cotinine. The C-oxidation pathway accounts for approximately 72% of total nicotine metabolism (50). C-oxidation begins with the conversion of nicotine to a nicotine $\Delta^{1'(5')}$ iminium ion catalyzed by the liver cytochrome P450 2A6 (CYP 2A6) enzyme (51-53). Genetic variations of this gene are postulated to be the cause of inter-individual variability in nicotine metabolism (54). The nicotine $\Delta^{1'(5')}$ iminium ion is converted by an aldehyde oxidase, the main enzyme involved in the final step of the metabolism to cotinine (52, 55-57). Cotinine may be further metabolized into a variety of metabolites, including the largest single metabolite 3'-trans-hydroxycotinine via CYP 2A6 (53). Nicotine and its metabolites undergo phase II metabolism through a variety of glucoronidation reactions that convert the parent substrates into more water-soluble compounds for excretion (36, 58-60). Finally, nicotine and its metabolites are eliminated predominantly through renal excretion and to a small degree in sweat and breast milk (61-64).

It is important to consider the changes in nicotine and cotinine clearance rates that occur during pregnancy. In pregnancy, nicotine metabolism increases by approximately 50%, while nicotine absorption does not differ between pregnancy and postpartum (41,

65). Correspondingly, cotinine metabolism increases by 140% (65). The elevations to nicotine and cotinine metabolism in pregnant women are postulated to be a result of increased hepatic metabolism by CYP 2A6 concomitant with faster levels of glucuronidation (65). Although an increase in blood flow to the liver could explain an increase in nicotine metabolism, nearly no difference is found in blood flow to the liver during pregnancy (65). Therefore, increased clearance rates are likely due to an induction of drug metabolizing enzymes in the liver during pregnancy (65, 66). Further investigation is warranted to determine if the effective dosage of NRT should be increased in order to accommodate the physiological changes in pregnancy (65).

1.1.5 Action of nicotine

Nicotine is a tertiary amine that binds to nicotinic acetylcholine receptors (nAChRs) found mainly in the brain, autonomic ganglia and at neuromuscular junctions (67). Upon binding, nAChRs undergo allosteric changes that increase their permeability to calcium ions and trigger the release of neurotransmitters such as acetylcholine, norephinephrine, dopamine, and serotonin (68). Specifically, the binding of nicotine to the $\alpha 4\beta 2$ nAChR likely mediates nicotine dependency (69). The addictive nature of nicotine has been linked to the subsequent release of dopamine that leads to pleasurable experiences, mood modulation and stimulation (69). Nicotine addiction develops due to its positive reinforcing effects while avoiding withdrawal symptoms such as anxiety and stress (68).

1.2 Lipogenesis

1.2.1 Overview of the development and functions of the liver

Liver development begins in endodermal cells in the ventral foregut that are specified for a hepatic fate. Through the coordination of a network of transcription factors, these cells express new genes such as albumin and α-fetoprotein that commit cells to develop into hepatoblasts. Hepatoblasts in the liver will bud and eventually differentiate into hepatocytes or bile duct cells. Hepatocytes, the primary cells of the liver, undergo extensive morphology and maturational changes before and after birth in both rodents and humans (70, 71).

The cells within the liver are highly organized in order to carry out a myriad of functions. In brief, blood enters the liver through the hepatic artery and leaves through the hepatic vein. Blood travels through the liver via sinusoids, which consist of small capillaries, lined with highly fenestrated endothelial cells to allow for the exchange between the bloodstream and hepatocytes. The structure of the liver allows for the compartmentalization of function depending on the location of hepatocytes. As a result, hepatocytes are able to carry out various metabolic pathways in distinct regions of the liver (71).

The liver possesses several vital functions in the body including the production of bile, detoxification of xenobiotic agents, breakdown of toxic endogenous compounds, maintenance of cholesterol homeostasis, regulation of gluconeogenesis and the synthesis and metabolism of lipids (71).

1.2.2 The role of the liver and adipose tissue in lipogenesis

Lipogenesis is the synthesis of fatty acids and the subsequent formation of triglycerides, which mainly occurs in the liver and adipose tissue (72). Fatty acids can become oxidized or undergo esterification reactions to form triglycerides (73). The liver maintains lipid homeostasis in part through a balance between lipid storage and lipid metabolism. The liver obtains free fatty acids from either lipolysis of adipose tissue or through *de novo* fatty acid synthesis (73). Interestingly, it has been shown that both *de novo* lipogenesis and reesterification of peripheral fatty acids contribute to the elevated levels of triglycerides observed in patients with abnormal lipid accumulation in the liver (74).

In addition to the liver, adipose tissue also plays a fundamental role in lipogenesis. Adipose tissue constitutes a large energy reservoir in the body that stores energy in the form of triglycerides that are absorbed from the circulation (75, 76). Almost 90% of adipocyte cell volume is made up of lipid droplets consisting of fats, triglycerides, fatty acids, phospholipids and cholesterol (75). In response to lipid requirements by other tissues, triglycerides in adipose tissue are hydrolyzed and released into the bloodstream as free fatty acids and glycerol molecules (77). Although adipose tissue can undergo *de novo* lipogenesis, studies have shown that it does not greatly contribute to the maintenance of excess fat in obese patients (78). In contrast, enhanced hepatic lipogenesis has been found to contribute to increased triglyceride pools and maintenance of excess fat in obese patients (78).

1.2.3 Hepatic de novo lipogenesis

Lipid synthesis in the liver begins with the uptake of glucose through the glucose transporter 2 (Glut2) (79). Once glycogen stores have been maximized, excess glucose is converted into triglycerides (80). Glucose enters the glycolytic pathway to form pyruvate, which then undergoes decarboxylation reactions by pyruvate dehydrogenase to form acetyl-CoA(81-84). Acetyl-CoA carboxylase (ACCα), a cytoplasmic enzyme, converts acetyl-CoA to malonyl-CoA, a committed step in fatty acid synthesis (85). Malonyl-CoA is essential for the production of long chain fatty acids such as palmitate and is a substrate for the fatty acid synthase (FAS) enzyme (85, 86). FAS catalyzes the conversion of palmitate to its major product palmitic acid through successive additions of malonyl-CoA to an acetyl-CoA molecule (87). Subsequently, stearoyl-CoA desaturase-1 (SCD-1) located in the endoplasmic reticulum (ER) converts palmitoyl- and stearoyl-CoA into monounsaturated fatty acids (MUFAs), palmitoleate and oleate, respectively (88). MUFAs undergo further desaturation and elongation reactions before becoming incorporated into various lipid products including triglycerides (Fig 1.2.3) (88).

Gene knockdown studies in mice have been used to elucidate the source of MUFAs used in triglyceride incorporation. Mice lacking SCD-1 were found to have approximately 60% lower liver and circulating triglyceride levels compared to heterogeneous mice (89). Notably, an exogenous source of MUFAs was unable to rescue the abnormal triglyceride profile in deficient mice, suggesting that endogenously synthesized MUFAs are the main substrate for hepatic *de novo* triglyceride synthesis (89). Given that endogenous MUFAs are formed in the ER, they are in close proximity to the enzymes involved in triglyceride synthesis. Therefore, endogenous MUFAs are more

readily available at the site of triglyceride synthesis compared to MUFAs derived from dietary sources (89, 90).

Triglycerides consist of a glycerol moiety with fatty acids esterified to all three hydroxyls (91). While studies have implicated enhanced hepatic lipogenesis in patients with augmented plasma triglycerides, the role of the individual fatty acid biosynthetic enzymes in nicotine-induced hypertriglyceridemia is currently unknown (13, 14, 78)

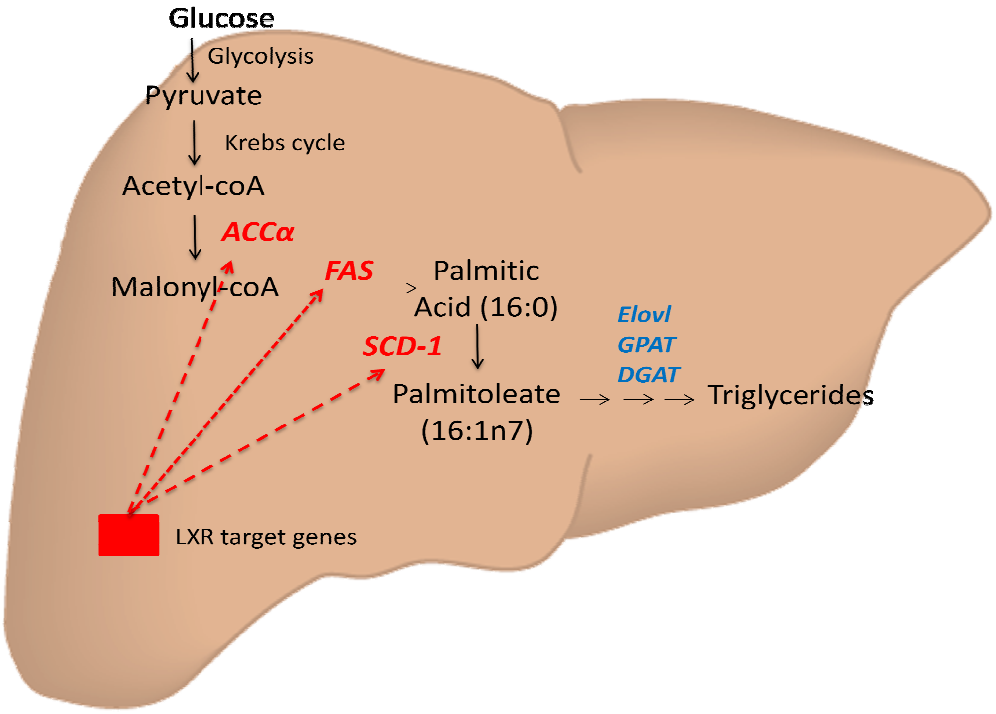

Fig. 1.2.3 Overview of the hepatic *de novo* triglyceride synthesis pathway. The synthesis of fatty acids requires key enzymes including acetyl Co-A carboxylase (ACCα) and fatty acid synthase (FAS). Stearoyl-CoA desaturase 1 (SCD-1) is required for the desaturation of fatty acids, while elongase (Elovl) enzymes, glycerol-3-phosphate acyltransferase (GPAT) and diglyceride acyltransferase (DGAT) are required for triglyceride synthesis.

1.2.4 The regulation of lipogenic genes

Nutritional status and hormones are well known regulators of lipogenesis. Studies examining the effect of nutrition supplementation in rats, have found that fasted animals have significantly lower FAS enzymatic activity in the liver compared to fed animals (92, 93). Moreover, refeeding was shown to lead to an increase in absolute protein content of hepatic FAS by 30- to 50-fold in rats (93). Two major mechanisms have been considered linking nutritional status and the transcriptional regulation of lipogenesis. Firstly being that glucose itself is a substrate for lipogenesis, and secondly that glucose stimulates the release of insulin (72, 94).

Insulin has been identified as an important regulator of lipogenesis. Streptozotocin (STZ)-induced diabetic mice show that treatment with insulin resulted in a rapid restoration of FAS enzymatic activity and mRNA levels. Although the rates of FAS activity decreased over time, the induction of steady-state mRNA was maintained three days after insulin administration, demonstrating that insulin primarily stimulates the transcription of the *FAS* gene (92). Insulin is able to exert its regulatory effect through the action of the transcription factor, sterol regulatory element-binding protein -1c (SREBP-1c). Studies in primary cultured rodent hepatocytes found that in the absence of insulin, SREBP-1c was not expressed. However, the addition of insulin led to an induction of SREBP-1c protein expression (95, 96). Furthermore, *in vivo* studies have demonstrated that SREBP-1c deficient mice, exposed to either fasting-refeeding treatments or a prolonged high carbohydrate diet, situations during which SREBP-1c would be expected to be expressed, exhibited lower induction of lipogenic genes (97).

Therefore, SREBP-1c is crucial for mediating the regulatory effects of insulin on hepatic lipogenesis.

A multitude of regulatory mechanisms are necessary to maintain lipid homeostasis. Nuclear receptors such as the liver X receptor (LXR) are unique ligand activated transcription factors that are able to sense lipids and directly influence transcriptional regulation. Interestingly, LXR has been demonstrated to regulate SREBP-1c and thus likely contributes to the control of hepatic lipogenesis as well. LXR deficient mice were found to have decreased hepatic SREBP-1c expression. When treated with the LXRα agonist, T0901317, LXR deficient mice did not exhibit an induction in hepatic lipogenic genes compared to control mice (98). LXR is considered an intermediary component of the insulin-sensing pathway as insulin treatment resulted in the induction of LXRα mRNA as observed in both *in vitro* and *in vivo* studies (99). LXR has a comprehensive regulatory role over many components of the lipogenesis pathway and will therefore be the primary focus of my thesis.

1.3 The liver X receptor

1.3.1 Overview of the liver X receptor

The liver X receptors (LXRα and LXRβ) are nuclear receptors that are involved in the regulation of cholesterol, lipid and glucose homeostasis (100). LXR is located within the nucleus and heterodimerizes with the retinoid X receptor (RXR) upon activation (101). LXR is regarded as a permissive nuclear receptor as either LXR or RXR ligands can activate the dimer (102-104). Although LXRα and LXRβ isoforms share approximately 77% homology, they differ in their expression profiles (105). LXRα is

predominantly expressed in the liver, intestines, kidney, spleen and testes (106), while LXRβ is ubiquitously expressed in the body (101, 106, 107). LXR/RXR heterodimers bind to specific LXR binding element motifs (LXRE) in the promoter of genes, which consist of direct repeats (DR-4) of the **AGGTCA** sequence separated by 4 nucleotides (101).

1.3.2 LXR and cholesterol synthesis

The discovery of the endogenous activators of LXRα helped to elucidate the biological role of this nuclear receptor. Early studies demonstrated that oxysterols such as 24(*S)*-hydroxy-cholesterol and 22*(R)*-hydroxycholesterol lead to the activation of LXRα (104, 108). Upon ligand binding, LXRα was observed to bind to the *cholesterol 7α-hydroxylase* promoter (*CYP7A*), the rate-limiting enzyme in cholesterol catabolism. These data provided early indication of the role of LXRα in cholesterol homeostasis, specifically the conversion of cholesterol to bile acid (104, 108, 109). Gene knockdown studies in mice have since demonstrated that when challenged with a cholesterol rich diet, LXRα deficient mice are unable to catabolize excess cholesterol and experience less bile acid synthesis compared to control mice. In addition, there was no compensatory effect by LXRβ, further confirming differential gene expression regulation by the LXR isoforms (110). Oxysterol binding to LXRα leads to the activation of other targets such as ATP-binding cassette transporter 1 (ABC1) and inhibits low-density lipoprotein receptor (LDLR) involved in cholesterol efflux and uptake, respectively (111-113). Therefore, LXRα was originally characterized as a cholesterol sensor in the maintenance of cholesterol homeostasis.

Interestingly, LXRα deficient mice also exhibited changes related to other hepatic pathways including fatty acid synthesis. Key enzymes and factors in the fatty acid biosynthetic pathway such as SCD-1, FAS and SREBP-1c, were down regulated in the liver of knockout mice. Furthermore, increased lipid deposits in the liver observed in the liver of deficient mice implicate a broader role of LXRα (110).

1.3.3 LXR and fatty acid synthesis

In addition to SREBP-1c, LXRα has been implicated to play a direct role in hepatic fatty acid synthesis (114, 115). LXR deficient mice exhibited decreased plasma triglyceride levels and free fatty acids concomitant with depressed fatty acid synthesis rates between 60-80% (100). Studies using potent nonsteroidal LXRα agonists such as T09013177 and GW3965 have helped characterize the regulatory role of LXRα (115, 116). Notably, both synthetic agonists are highly selective for LXR α and β over several other nuclear receptors including, RXR and constitutive androstane receptor (CAR) (115, 116). Small animal studies have demonstrated that treatment with T0901317 led to significant increases in plasma triglyceride levels, which was associated with an induction in the activity of FAS, ACCα, SCD-1 and SREBP-1c in mice and hamsters. To further elucidate the role of LXRα in fatty acid synthesis, LXR deficient mice treated with LXRα agonist found that animals exhibited blunted expression of fatty acid biosynthetic genes compared to wild type mice (115). Thus, another layer of fatty acid regulation by LXR was uncovered.

However, it remained unknown whether LXRα had a direct and separate regulatory role from SREBP-1c. An *in vitro* model demonstrated that the treatment of

TPH-1 macrophages with LXR agonists T0901317 or GW3965 led to the activation of LXR and increased FAS mRNA expression despite the active suppression of SREBP-1c expression (117). Moreover, a temporal relationship was observed in LXRα agonist treated mice as triglyceride levels increased with rising FAS gene expression. Therefore, it was determined that LXRα was able to directly activate FAS and contribute to increased triglyceride production in a SREPB-1c independent manner (117). From these experiments, an LXRE was identified between -669 and -655 base pairs (bp) in the rat *FAS* promoter, which is also highly conserved in humans (118). Similarly, SREBP-1c has also been shown to bind directly to highly conserved tandem sites between -71 and -54 bp in the rat *FAS* promoter (119). With the existence of both binding sites, luciferase activity assays have demonstrated that the activation of both LXRα/RXR and SREBP-1c leads to the additive activation of FAS (117). LXRα has been established to directly and indirectly control hepatic fatty acid synthesis.

Likewise, LXR is able to directly target additional enzymes along the fatty acid synthesis pathway. Studies eliminating SREBP-1c expression, found that treatment with an LXR agonist in mice results in an increase in MUFAs and triglycerides via the activation of SCD-1 expression. Furthermore, researchers have identified an LXR binding site in the mouse promoter of *SCD-1* between positions -1263 and -1248 bp (120). LXR can also directly interact with the promoter of ACCα between -101 and -71 bp. Similarly, LXR agonist treatment led to an increase in ACCα mRNA expression independent of SREBP-1c binding in chick embryos (121). This is of importance as changes in ACCα expression are vital in overall triglyceride levels (121). In summary,

LXR-mediated changes in fatty acid synthesis can lead to significant downstream alterations in triglyceride levels.

1.3.4 LXR and glucose regulation

In contrast to the activating role of LXR in cholesterol and fatty acid homeostasis, LXR plays a suppressive role in the regulation of hepatic glucose metabolism (122). Studies have demonstrated in mice treated with LXR agonist GW3965, resulted in the upregulation of hepatic lipogenic targets while repressing peroxisome-proliferator activate-receptor coactivator-1 (PGC-1), a transcriptional coactivator necessary for the activation of a plethora of hepatic gluconeogenic genes (123, 124). While my thesis focuses mainly on fatty acid synthesis, LXR activation can lead to coordinated glucose regulation in adipose tissue and skeletal muscle (122, 125). Studies demonstrate that GW3965 treated mice exhibit increased Glut4 mRNA levels in adipose tissue, while T0901317 treatment in human myotubes leads to increased glucose uptake (122, 125). Together, these studies suggest that LXR activation helps to maintain glucose levels by promoting peripheral glucose uptake while suppressing gluconeogenesis in the liver. LXR may be the key link in the control of lipid and glucose metabolism whereby LXR limits the production of glucose in the liver while promoting glucose uptake and stimulating *de novo* lipogenesis for storage of excess energy as triglycerides in adipose tissue (122). Furthermore, treatment with an LXR agonist has been shown to improve glucose tolerance in mouse models of obesity and insulin resistance (122). Although modulation of LXR activity would appear to be a promising therapy for the treatment of diabetes and hyperglycemia, it is important to resolve and balance the impact of LXR regulation on fatty acid and cholesterol homeostasis as well.

1.4 Epigenetics

1.4.1 Overview of epigenetics

In recent years epigenetics has emerged as a dynamic form of regulation that relies on the information that is held in how the genome is packaged. Although various definitions of epigenetics have been put forth, it is broadly defined as the structural adaptations of chromosomal regions that can signal altered states of activity (126). Therefore, transcription can be turned "on" or "off", depending on the packaging of genes (127).

DNA is wrapped around an octamer of histones, consisting of two copies of histones H2A, H2B, H3 and H4 to form a nucleosome, the main packing element of genomic DNA (128, 129). DNA binding to histones is facilitated through an electrostatic interaction between the positively charged peptides and negatively charged DNA backbone (130). Nucleosomes are linked together by 10 to 60 bp of DNA that are associated with histone H1 to form higher order chromatin structures (131). Histones possess N- (NH2-) and C-terminus (COOH-) tails that emanate out of the nucleosome and are susceptible to covalent modifications such as methylation, acetylation, phoshorylation, ubiquinitination and ADP-ribosylation (127, 131, 132). Alterations to histones can lead to large functional modifications in transcription, revealing a "histone code" that confers states of transcriptionally silent heterochromatin and active euchromatin (127, 133, 134).

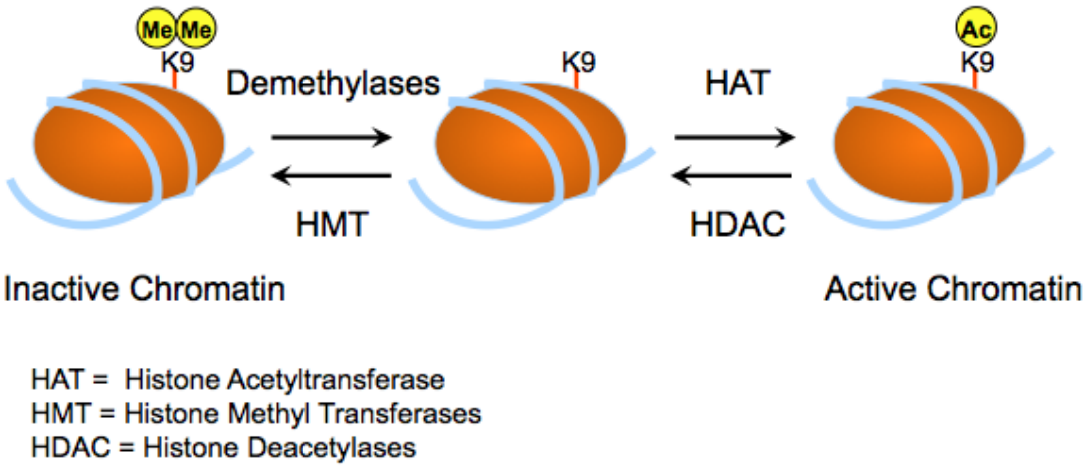

Fig. 1.4.1 Overview of the role of posttranslational histone modifications (acetylation and methylation) in transcriptional regulation

1.4.2 Histone acetylation

One of the first pieces of evidence of the histone code was demonstrating that histone acetylation results in an increase in RNA synthesis (133). Early studies observed that an increase in histone acetylation preceded an increase in RNA and protein synthesis in human lymphocytes (135). Histone acetylation has since been found to dictate the transition to transcriptionally active chromatin by increasing the accessibility of gene templates to transcription factors, which are inhibited by the structure of the nucleosome in the absence of acetylation (136). Histone acetylation increases accessibility by reducing the number of positive charges and disrupting the uniform charge distribution of

positively charged lysine and arginine residues of histone amino tails (130). For that reason, the stability of DNA wrapped around peptides has been found to be five times less stable and possess a greater number of uncoiled base pairs compared to non-acetylated peptides (130, 137). Histone acetylation commonly occurs on the N-terminal of histones H3 and H4, as studies have demonstrated that replacement of N-terminal residues silences the effects of histone deacetylases (HDAC) (138-140). Histone deactylases such as the family of sirtuin enzymes facilitate the removal of acetyl groups and contribute to the formation of heterochromatin (141).

Among the known posttranslational modifications, acetylation and deacetylation have garnered the most attention (142). Enzymes, namely histone acetyltransferases (HAT), are responsible for the acetylation of histone tails. HATs were found to possess domains that were homologous for yeast adaptor proteins indicating that acetyltransferases are also recruited in a gene specific manner (143, 144). HATs are classified based on their function: A-type are involved in transcription whereas B-type are involved in nucleosomal assembly. For example, p300/CBP are A-type HATs that have been found to interact with many DNA-binding transcriptional factors (145).

1.4.3 Histone methylation

Histone methylation is a common posttranslational modification that is commonly associated with the inhibition of gene expression (146). Histone methylation mediates transcriptional silencing by acting as a signal for the recruitment of repressive complexes that leads to deacetylation (147, 148). Methylation commonly occurs at lysine or arginine residues on histones H3 or H4 in a mono-, di- or tri-methylated manner (149). Since the discovery of histone methylation as a regulatory signal, several histone

methyltransferases (HMT) have been characterized. For example, the human SUV39H1 possesses catalytic activity and demonstrates site preference for methylation at the N-terminal of histone H3 (150).

1.4.4 Epigenetics and fetal programming

To date, there is very little data on the epigenetic regulation of LXR target genes. However, studies have begun to reveal evidence of altered gene activation due to posttranslational histone modifications. Treatment of chick embryos with LXR agonist T0901317 led to a direct increase in ACCα mRNA expression increased LXR binding to the LXRE within the 5' upstream region of *ACCα* and enhanced acetylation of histone H3 (151). In a similar fashion, treatment with T0901317 in human hepatocellular carcinoma cell line (HepG2) led to an enrichment of histones H3 and H4 acetylation at the LXRE of the *FAS* promoter. Further experiments showed that histone acetylation was diminished in LXRα deficient cells, suggesting that LXRα expression was required for these histone modifications (152). Although lipogenic genes can be subjected to posttranslational histone modifications, it has yet to be concluded if chromatin remodeling is associated in models of fetal programming. Our laboratory has begun to shed light on permanent epigenetic changes due to insults during perinatal life. We have previously shown that maternal protein restriction (MPR) through pregnancy and weaning leads to long-term hypercholesterolemia in rat offspring as a result of impaired Cyp7a1 expression, an LXR target gene (153). This decreased Cyp7a1 expression was influenced by diminished acetylation and increased methylation at the LXRE of the *Cyp7a1* gene, promoting a repressive chromatin environment that persisted into adulthood (153). Recently, we have also shown that MPR during pregnancy alone leads

to the suppression of LXRα expression long-term due to decreases in the acetylation of histone H3 [K9,14] and RNA polymerase II recruitment surrounding the proximal promoter (-144 to +134 bp) of the *LXR* gene (154). Additionally, our laboratory has explored the posttranslational modifications that occur in response to maternal hypoxia during gestation in rat offspring. We demonstrated that maternal hypoxia led to decreased glucose-6-phosphatase (G6Pase) mRNA and protein expression, concomitant with increased methylation of histone H3 [K9] at the LXRE of the *G6Pase* promoter in 12 month old rat offspring (155). Our work supports that multiple models of fetal programming are associated with posttranslational histone modifications of hepatic LXR target genes and will continue to be explored in our model of MNE.

1.5 Developmental origins of health and disease (DOHaD)

1.5.1 History of DOHaD

Epidemiological data has demonstrated that poor fetal growth is strongly correlated with various long-term developmental alterations in offspring including increased risk for cardiovascular disease (156), high blood pressure (157), type two diabetes (158, 159), and raised fasting serum triglyceride levels (160). It is important to note that these relationships were found independent of confounding factors such as alcohol consumption and social class of mothers, strengthening the studies supporting DOHaD.

Early evidence of DOHaD in humans was observed during the Dutch famine, an acute period of exposure to suboptimal nutrition. Studies examining this period of history discovered that children exposed to poor nutrition *in utero* experienced decreased glucose tolerance in later life (161). Similar studies in England found that low birth weight was

associated with an increase in mortality from CV disease in adulthood (156, 162). Growing evidence has supported the idea that developmental adaptations made *in utero*, may lead to permanent changes or "programming" of the body (163). To help characterize this phenomenon, Hales and Barker put forth "The Thrifty phenotype hypothesis". They proposed that the *in utero* environment provides cues of the postnatal environment for the fetus, which causes the fetus to make thrifty adaptations for survival. However, these once advantageous changes become detrimental if the postnatal environment differs from the cues established *in utero* (158).

Since the conception of DOHaD, a wide range of adverse intrauterine environments including alterations in oxygenation and hormone levels have been linked to increased incidence of CV and metabolic disease in adult life (164).

1.5.2 Models of fetal programming

Animal models have helped to elucidate the mechanisms underlying DOHaD, and shed light on the nature of the insult, timing and duration that lead to specific physiological outcomes (164). Rodent models have demonstrated that even minor changes in protein intake throughout pregnancy and lactation can lead to long-term hypercholesterolemia in rat offspring (153). Furthermore, our laboratory has demonstrated that MPR during gestation alone results in glucose intolerance in later life (165). These studies revealed that the manifestation of features of the metabolic syndrome in offspring does not solely rest on the type of insult, but the duration of the insult in perinatal life. Other nutritional insults explored in animal models of fetal programming include iron deficiency, high fat diet and micronutrient deficiencies (166-168).

Stress-related increases in glucocorticoids during pregnancy have been linked to low birth weight and higher fasting plasma glucose levels in adult rat offspring (169, 170). Long-term programming, studies have demonstrated that 20 year old individuals, who were all low birth weight, had higher cortisol levels compared to individuals who were deemed appropriate weight for gestational age (171). This suggests that the hypothalamaus-pituatary–adrenal axis could be susceptible to programming changes *in utero* (171).

Hypoxia is a common consequence of placental insufficiency and has also been implicated in the fetal programming of adult disease (172). Chronic maternal hypoxia exposure in a rodent model has been shown to lead to decreased relaxation of mesenteric arteries and increased risk for ischemic injury in adult life (173, 174). In concordance with hypoxia-induced CV dysfunction, maternal hypoxia can also lead to permanent impairments in glucose homeostasis. Livers of adult male rat offspring exposed to hypoxia during gestation alone exhibited decreased G6Pase expression, an enzyme necessary for the progression of the glycolytic pathway (155, 175). Consequently, exposure to chronic maternal hypoxia can predispose offspring to a variety of disorders in adulthood.

Although catch-up growth may not be a tangible *in utero* insult, it is nonetheless a contributor to the fetal programming phenomenon. Catch-up growth is defined as a child's return to their genetic growth trajectory after experiencing a period of slowed growth (176). For example, following MPR in rats, offspring that are fed a high caloric diet in postnatal life have a propensity to catch up in weight and then continue to become overweight compared to control rats (177). Similarly, children who experienced catch up

growth between the ages of 0 and 2 years old, were more likely to have increased body mass index (BMI) and fat mass at 5 years of age compared to other children their age (176). Although it is generally accepted that regardless of age, an association exists between rapid growth and being overweight as an adult (178), the underlying molecular mechanisms are not well characterized.

In summary, human and animal studies have clearly established that physiological changes that occur in response to the maternal environment in fetal and neonatal life can impact an individual's health long-term. Further understanding of the critical windows of DOHaD could lead to novel therapies for the prevention of a variety of adult onset diseases.

1.5.3 Fetal programming models of MNE

Animal models of MNE have been critical in understanding the effect of nicotine exposure alone. Numerous studies on both smoking and nicotine have explored alterations in brain, lung and reproductive outcomes in offspring (179-184). More recently, MNE studies indicate that nicotine alone could be linked to the development of features of the metabolic syndrome such as obesity (185).

Given the neurological risks observed in children of smoking mothers, animal models have been imperative in investigating this underlying association. Rats exposed to the equivalent amount of nicotine as smoking 3 packs per day via an osmotic pump, exhibited a decrease in the number of rat fetal brain cells. Furthermore, it was found that exposure to nicotine resulted in a delay in cerebellum maturation in rat offspring (186). Using a similar method of nicotine delivery during pregnancy, one study observed an

increase in the stimulation of cholinergic activity in the cerebral cortex of rat offspring (187). This increased activity may lead to premature stimulation and disruptions to the normal timing of postnatal brain development (187). In conjunction with developmental impairments, prenatal nicotine exposure has been shown to influence behavioural outcomes in offspring as well. Rat offspring exposed to nicotine *in utero*, were observed to be more anxious and poorer adaptors compared to control offspring at PND45 (188). These studies link nicotine as a critical component of cigarette smoke to the development of neurological impairments in children exposed to smoke during pregnancy.

Children of smoking mothers also share strikingly similar compromises in lung development and function observed in animal models of MNE (189). In rhesus monkey and rodent studies of MNE during pregnancy, offspring were observed to have increased collagen deposition around large airways, a potential precursor to lung fibrosis, in newborn and 3 month old offspring, respectively (189, 190). Newborn monkeys exposed to nicotine *in utero* also suffered significant impairment to lung development, decreased function capacity and increased pulmonary resistance.

Interestingly, epidemiological evidence has now linked *in utero* exposure to tobacco and the development of obesity in children (191). A variety of windows of nicotine exposure have resulted in a spectrum of obese-related outcomes in offspring. Nicotine exposure during pregnancy via the implantation of nicotine osmotic minipumps in rats led to both early- and long-term alterations in rat offspring. During early neonatal life, MNE rat offspring exhibited increased white adipose tissue and adipocyte hypertrophy. Subsequently, these offspring continued to develop insulin resistance in adulthood (185).

Notably, our rat model of maternal nicotine exposure during pregnancy and lactation (MNE-PL) has already been found to lead to increased bodyweight, adiposity, blood pressure, β-cell loss, impaired fecundity and glucose homeostasis long-term in offspring (192-196). Importantly, this animal model is highly relevant to the human population as the amount of nicotine used is equivalent to the amount of nicotine children of average smokers are exposed to (192). In our rat model of MNE-PL, the dose of nicotine results in maternal cotinine concentrations comparable to moderate smokers (80-163ng/ml) and concentrations of cotinine measured in offspring that are within the range (5-30ng/ml) observed in infants nursed by smoking mothers (192, 197, 198). Furthermore, previous studies of this model of MNE-PL found there was no effect on maternal food intake, eliminating maternal obesity as a confounding variable in the long-term programming of metabolic dysfunction in rat offspring (10, 192).

Surprisingly, some nicotine-induced developmental changes have been transgenerational. Second-generation offspring of dams exposed to nicotine during pregnancy and lactation, developed perturbations in blood pressure and insulin levels despite the fact that these offspring were not directly exposed to nicotine themselves (194, 199). In humans, limited data exists on the impact of a mother's exposure to smoking *in utero* on her children. One study has demonstrated that a smoking mother exposed to smoke *in utero*, is more likely to have a lighter child compared to a smoking mother who had not been exposed to smoke *in utero* (200).

1.6 Thesis hypothesis and objectives

Smoking during pregnancy is associated with numerous fetal and neonatal complications short-term, along with increased risk of adult-onset diseases. While

nicotine replacement therapy is widely prescribed in pregnancy as a pharmacotherapy for smoking cessation, there is little information to date on the effects of maternal nicotine exposure on long-term diseases processes. Animal studies suggest that nicotine exposure alone during fetal and neonatal life may increase the risk of dyslipidemia and obesity in postnatal life. This study aimed to investigate the permanent changes in liver function, which may mediate in part the etiology of metabolic dysfunction rat offspring exposed to nicotine during pregnancy and lactation.

Hypothesis:

We hypothesize that maternal nicotine exposure during pregnancy and lactation will lead to long-term liver dysfunction in the offspring.

Objectives:

1) To determine whether maternal nicotine exposure during pregnancy and lactation leads to changes in expression of hepatic fatty acid biosynthetic enzymes in rat offspring.

2) To examine the underlying transcriptional mechanisms and posttranslational histone modifications that may be involved.

CHAPTER 2 : MATERIALS AND METHODS

2.1 Maternal nicotine exposure during pregnancy and lactation (MNE-PL) animal model

2.1.1 Animal care and drug administration

All animal experiments were performed at McMaster University and were approved by the Animal Research Ethics Board at McMaster University, in accordance with the guidelines of the Canadian Council for Animal Care. Nulliparous female Wistar rats (200-250g, Harlan, Indianapolis, IN, USA) were randomly assigned to receive daily subcutaneous injections of saline (vehicle) or nicotine bitartrate (1mg/kg/day, Sigma-Aldrich, St. Louis, MO, USA) for 2 weeks prior to mating, during pregnancy until weaning (PND21) as previously described (195, 201). This dose of nicotine has been previously shown to lead to cotinine concentrations in maternal serum (135.9±7.86 ng/ml) that fall within the range reported of "moderate" female smokers (80-163ng/ml) and in serum cotinine concentrations (26.2±1.78 ng/ml) in offspring, that are comparable to the range (5-30 ng/ml) found in infants nursed by smoking mothers (45, 192, 195, 198). Dams were allowed to deliver normally and at PND1 all litters were culled to eight. At PND1 and PND21, subsets of male offspring were sacrificed by CO_2 inhalation for body weight measurements and liver tissue collection. All animals were weighed at necropsy. Livers were snapped frozen in liquid nitrogen and stored at -80°C until molecular analysis.

A third subset of offspring were caged as sibling pairs and at PND180, male and female rats were fasted overnight and sacrificed by CO_2 inhalation for body weight measurements. Livers were snapped frozen in liquid nitrogen and stored at -80°C until molecular analysis. Blood was collected, allowed to clot, spun and serum was stored at -80°C until analysis (Fig. 2.1.1).

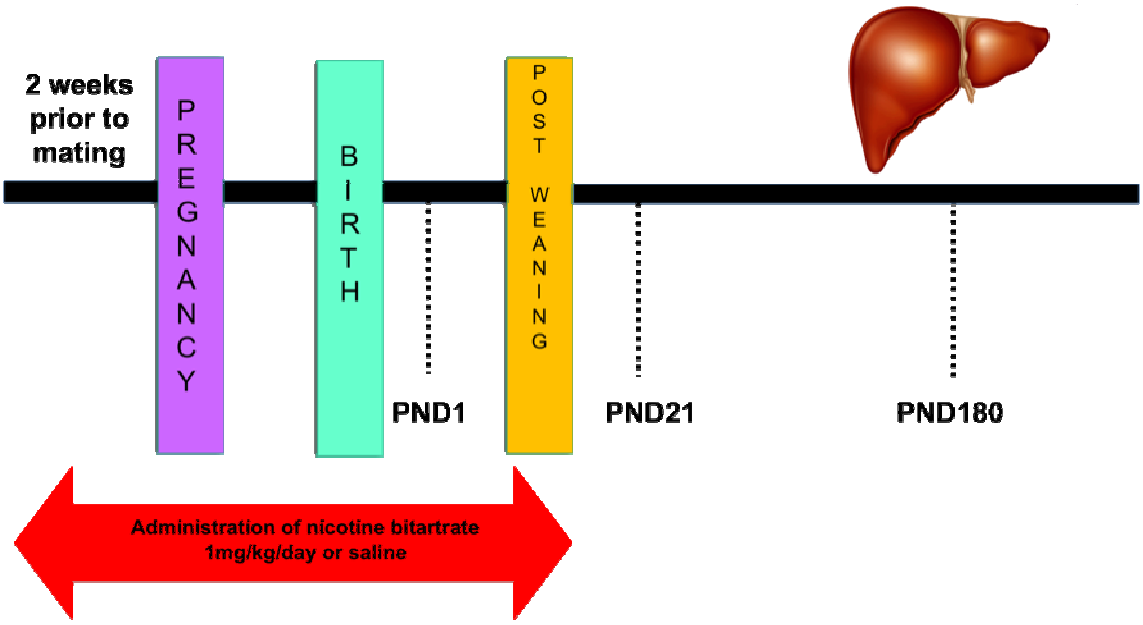

Fig. 2.1.1 Overview of animal model of maternal nicotine exposure during pregnancy and lactation. Pregnant rats were administered nicotine bitartrate or saline (vehicle) for two weeks prior to mating until weaning. Offspring were sacrificed at PND1, 21 and 180 for analysis.

2.1.2 Plasma and hepatic lipid measurements

Total cholesterol, triglyceride and glucose levels were measured from blood and hepatic tissue samples using Cobas® analyzer at the Metabolic Phenotype Laboratory at Robarts Research Institute (London, Ontario, Canada). Briefly, triglycerides are hydrolyzed by lipoprotein lipase to glycerol and fatty acids. Glycerol is then phosphorylated to glycerol-3-phosphate by ATP in a reaction catalyzed by glycerol kinase (GK). The oxidation of glycerol-3-phosphate is catalyzed by glycerol phosphate oxidase (GPO) to form dihydroxyacetone phosphate and hydrogen peroxide (H_2O_2). In the presence of peroxidase, H_2O_2 alters the oxidative coupling of 4-chlorophenol and 4-aminsophenazone to form a red-colored quinoneiminse dye, which is measured at 512 nm. The increase in absorbance is directly proportional to the concentration of triglycerides in the sample.

2.2 Molecular analysis

2.2.1 Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA from male and female liver tissue was extracted at PND1, PND21 and PND180 by the one-step method described by Chomczynski and Sacchi (202). RNA was treated with deoxyribonuclease to remove any contaminating DNA. 4µg of the total RNA was reverse transcribed to cDNA using random primers and Superscript II RNase H-reverse transcriptase (Invitrogen, Carlsbad, CA, USA). Primer sets directed for the genes of interest (FAS, ACCα, SCD-1, SIRT-1, p300) were generated using OligoPerfect™Designer (Invitrogen, Carlsbad, CA, USA) (Table 2.2.1). The Bio-Rad CFX384 Real Time System was employed to determine quantitative mRNA expression using the DNA binding dye SsoFast™ EvaGreen® Supermix (Bio-Rad, Mississauga,

Ontario, Canada). The cycling conditions were as follows: 50°C for 2 mins, 95°C for 10 mins, followed by 45 cycles of 95°C for 15 sec, and 60°C for 1 mins. The cycle threshold was set at a level where the exponential increase in PCR amplification was roughly corresponding between all samples. The relative fold changes were calculated using the comparative cycle times (Ct) method with β-actin as the reference gene. All primer sets were demonstrated to have good linear correlation (slope≈-3.4) strongly suggesting equal priming efficiency (data not shown). ΔCt values for each primer set were calibrated to the experimental samples with the lowest transcript abundance (highest Ct value). The relative abundance of each primer set compared with calibrator was determined by the formula, $2^{\Delta\Delta Ct}$, in which ΔΔCt is the calibrated Ct value.

Table 2.2.1 Primer sequences for quantitative real-time PCR analysis

Gene	Primer (5'-3')	Reference No.
FAS	**FWD** GGA CAT GGT CAC AGA CGA TGA C **REV** CGT CGA ACT TGG ACA GAT CCT T	X62889.1
ACCα	**FWD** TCC GTA TGT GAC CAA AGA CC **REV** TAC GTT GTT CCC AAG GAC TG	NM_022193.1
SCD-1	**FWD** GCT TGT GGA GCC ACA GGA CTT AC **REV** ATC CCG GGC CCA TTC ATA TAC	NM_031841.1
p300	**FWD** AGC GAG CTT ATG CTG CTC TC **REV** GGC ACT CAT GTT GTT CAT GG	NW_001084859.1

Sirt1	**FWD** AGC TGG GGT TTC TGT TTC CT **REV** CTG GTT ATG CTC TTG GTG TCT TTC	NC_005119.3
β-actin	**FWD** CAG CCT TCC TTC CTG GGT AT **REV** AGG AGC CAG GGC AGT AAT TCT	NM_031144.3

2.2.2 Protein extraction and western immunoblotting analysis

Tissue protein was extracted from snap frozen liver samples using RIPA lysis buffer solution (50 mM Tris-HCl, pH 7.4, NP-40 1%, Na-deoxycholate 0.25%, 150 mM NaCl, 1mM EDTA, 50 mM NAF, 1mM NaV, 25 mM β-glycerophosphate) and a protease inhibitor (Roche). The liver sample was placed in 600 µl of RIPA lysis buffer and homogenized with an IKA T10 Basic S1 Dispersing Tool (IKA Works Inc, Wilminsgton, NC). The homogenates were placed on ice for 5 mins before rotation at 4°C for 10 mins. The homogenates were subsequently centrifuged at 300 g for 15 mins at 4°C. The supernatant was transferred to fresh tubes and centrifuged at 14 000 RPM for 20 mins at 4°C. The supernatant was retained as the protein preparation. Equal concentrations of total protein were normalized using a colorimetric BCA Protein Assay (Pierce Corp., Madison, WI, USA). Each loading sample contained 30 µg of protein. Samples were fractionated in gradient polyacrylamide gels (Invitrogen, Carlsbad, CA, USA) and transferred onto polyvinylidenefidluoride membrane (Millipore, Etobicoke,

Ontario, Canada). Blots were probed with FAS (catalog no. 3180S 1:1000, Cell Signaling), ACCα (catalog no. 3662S 1:1000, Cell Signaling), LXRα (cat. no.sc-13068 1:5000, Santa Cruz Biotechnology), SREBP-1c (catalog no. sc-366, Santa Cruz Biotechnology), p-ACCα (catalog no.3661P 1:1000, Cell Signaling) antibodies, and monoclonal horseradish peroxidase-conjugated β-actin was used as the housekeeping protein (catalog no. A3854 1:50000, Sigma-Aldrich). All antibodies were diluted in 5-7% milk-1xTris-buffered saline-Tween 20 (0.01%) buffer. Horseradish peroxidase conjugated donkey anti-rabbit IgG (catalog no. 711035152 1:10000, Jackson ImmunoResearch Laboratories, West Grove, PA) or horseradish peroxidase conjugated donkey anti-mouse IgG (catalog no. 715001003 1:50000) diluted in 5-7% milk-1xTris-buffered saline-Tween 20 (0.01%) were used as the secondary antibodies. Immunoreactive bands were detected using an enhanced chemiluminescence detection system (Thermo Scientific, Waltham, MA) and imaged with a VersaDoc Imaging System (BioRad). Densitometry analysis was performed using Image Lab Software (BioRad).

2.2.3 Chromatin immunoprecipitation (ChIP)

Chromatin was extracted from liver tissues excised from PND180 male offspring as previously described (153). In brief, a small piece of snap frozen liver was homogenized in 0.5 mL of 1% formaldehyde and fixed for 10 mins at room temperature to cross-link proteins and DNA. Glycine (0.125M, final concentration) was added to all samples to terminate cross-linking. Samples were microfuged at 3000 RPM at room temperature for 5 mins and supernatant was subsequently discarded. Samples were then washed once with cold PBS and spun at 3000 RPM. Supernatant was discarded again and 500 µl of SDS lysis buffer (Millipore, Etobicoke, Ontario, Canada) with protease

inhibitor cocktail (Roche, Mississauga, Ontario, Canada) was added to each sample. Samples were incubated for 20 mins at 4°C and then sonicated to produce sheared, soluble chromatin. The lysates were diluted ten times with ChIP dilution buffer (Millipore, Etobicoke, Ontario, Canada) and aliquoted to volumes of 300 µl. Each of the aliquots were precleared with protein A/G Plus agarose beads (20 µl, Millipore, Etobicoke, Ontario, Canada) and rotated for 2 hours at 4°C. In order to pellet the beads, samples were microfuged at 14000 RPM at 4°C, and the supernatant containing the sheared chromatin was retained and placed in new tubes. The aliquots were incubated with 3 µg of antibodies against LXRα (cat# sc-13068x, Santa Cruz Biotechnology, Santa Cruz, California) or acetylated histone H3 (lysine 9,14, cat #05-399, Millipore, Etobicoke, Ontario, Canada) and rotated overnight at 4°C. Two aliquots were reserved as 'controls' – one incubated without antibody ('input') and another with non-immune IgG (Millipore, Etobicoke, Ontario, Canada). Protein A/G Plus agarose beads (60 µl) were added to each tube and then rocked for 1 hour at 4°C. The immune complexes were collected by centrifugation. The beads containing the immunoprecipitated complexes were washed sequentially for 5 mins in wash buffer I (20 mM Tris-HCl, pH 8.1, 2 mM EDTA, 0.1% SDS, 1% Triton X-100, 150 mM NaCl), wash buffer II (same as I, except containing 500 mM NaCl), wash buffer III (10 mM Tris-HCl, pH 8.1, 1 mM EDTA, 1% NP-40, 1% deoxycholate, 0.25 M LiCl), and twice in Tris-EDTA (TE) buffer. The beads were eluted with 250 µl elution buffer (1% SDS, 0.1mM $NaHCO_3$ + 20 µg salmon sperm DNA (Sigma-Aldrich, Oakville, Ontario, Canada) at room temperature. The elution step was repeated once and eluates were combined. Samples were heated at 65°C for 4 hours to reverse the crosslinking of the immunoprecipitated chromatin complexes and 'input

controls' (10% of the total soluble chromatin). Proteinase K buffer was added to each sample (50 mM Tris-Hydrochloride (HCl), pH 8.5, 1% SDS, 10 mM EDTA) and incubated for 1 hour at 45°C. The DNA was purified by phenol-chloroform extraction and DNA was precipitated overnight at -20°C in 100% EtOH containing 10% sodium acetate (pH 5.6). The supernatant was removed and remaining pellets were dried. All samples and 'input' controls' were resuspended in 50 µl TE buffer prior to PCR analysis. Real-time PCR was employed using forward (5'-GCCACGATGACCGGTAGTAA-3') and reverse (5'-GCGTTGCTAGGCAATAGGGT-3') primers (PE Applied Biosystems, Boston, MA, USA) that amplify a -690 to -561 bp region encompassing the published rat *FAS* LXRE site (118). Using serial dilutions of rat liver chromosomal DNA, the primers were demonstrated to have equal efficiency in priming to their target sequences (data not shown).

2.3 Statistics

All results are expressed as the mean of arbitrary values ± the standard error of the mean (SEM). All results from quantitative qRT-PCR, ChIP and western immunoblot analysis were evaluated using an unpaired Student's t test, where a p value of less than 0.05 was considered significant.

CHAPTER 3 : RESULTS

3.1 Characterization of offspring exposed to maternal nicotine exposure during pregnancy and lactation (MNE-PL)

3.1.1 Weight responses in male offspring exposed to MNE-PL

The birth weights of male MNE offspring were recorded throughout development. At PND1, the weights of male MNE offspring were significantly lower compared to control ($p < 0.05$) (Fig. 3.1.1A). At PND21, there was no significant difference in birth weight between male offspring exposed to nicotine and control offspring (Fig. 3.1.1B). By PND180, an increase in body weight from 563±5g to 605±4g ($p<0.001$) was observed in nicotine-exposed male offspring (Fig. 3.1.1C). This increase in weight is noteworthy considering that previous studies in this MNE-PL animal model have demonstrated that the growth trajectory of nicotine-exposed offspring was significantly enhanced compared to control, while nicotine administration had no effect on gestational length, maternal food intake, maternal weight gain or litter size (196, 201).

3.1.2 Glucose, cholesterol and triglyceride measurements in control and nicotine exposed male offspring

MNE-PL led to a significant increase in fasting serum triglycerides in male but not female offspring at PND180 (Fig. 3.1.2C). Similarly, hepatic triglyceride levels were significantly elevated in male offspring compared to control, although female offspring were not examined (Fig. 3.1.2D). Analysis of circulating levels of fasting glucose and cholesterol were not significantly altered between control and MNE-PL male offspring at PND180 (Fig 3.1.2A-B).

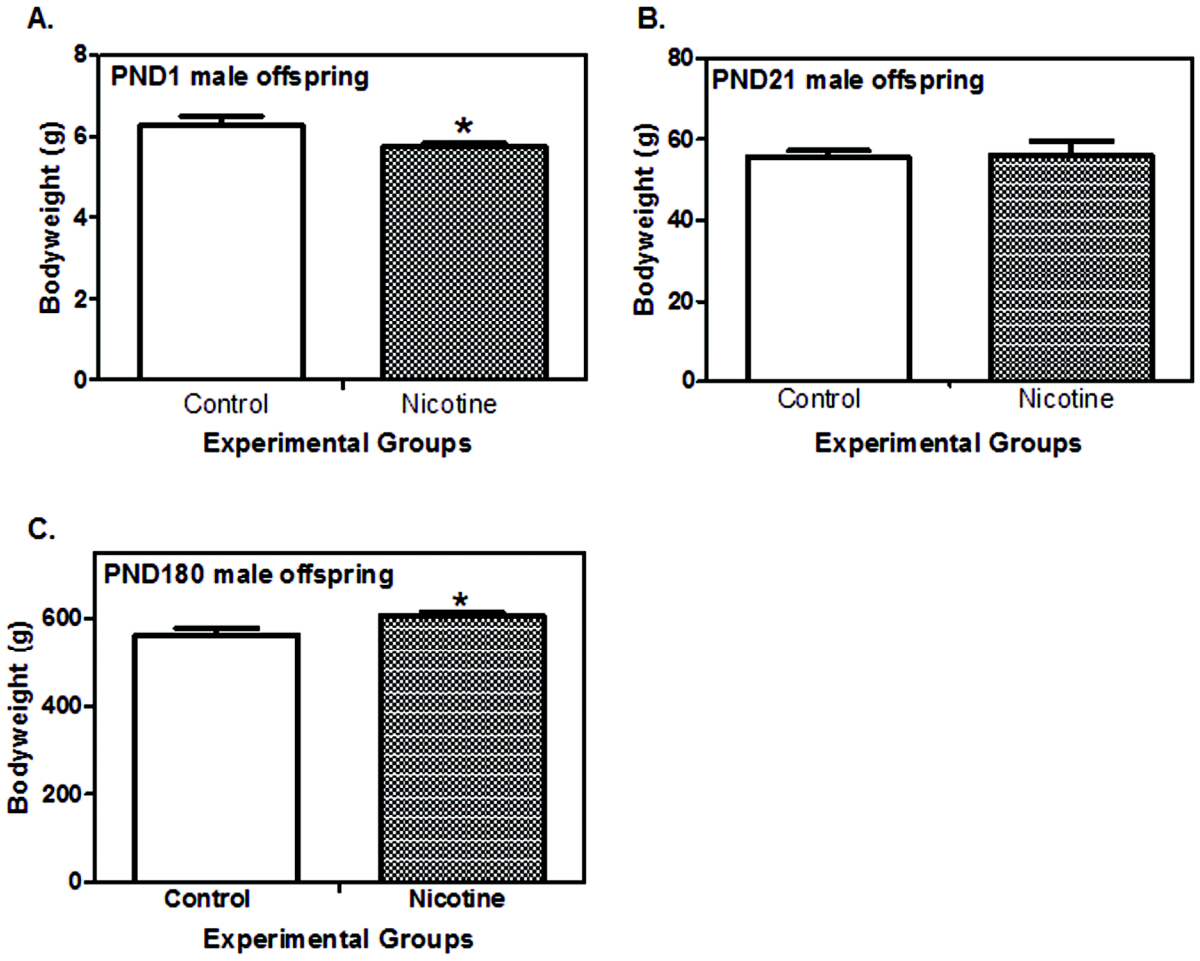

Fig. 3.1.1 The effect of maternal nicotine exposure during pregnancy and lactation on body weight in male PND1 (A), PND21 (B) and PND180 (C) rat offspring. Results are expressed as the mean ± SEM. Nicotine effects were determined using a Student's t test. * = ($p < 0.05$); n = 6/group.

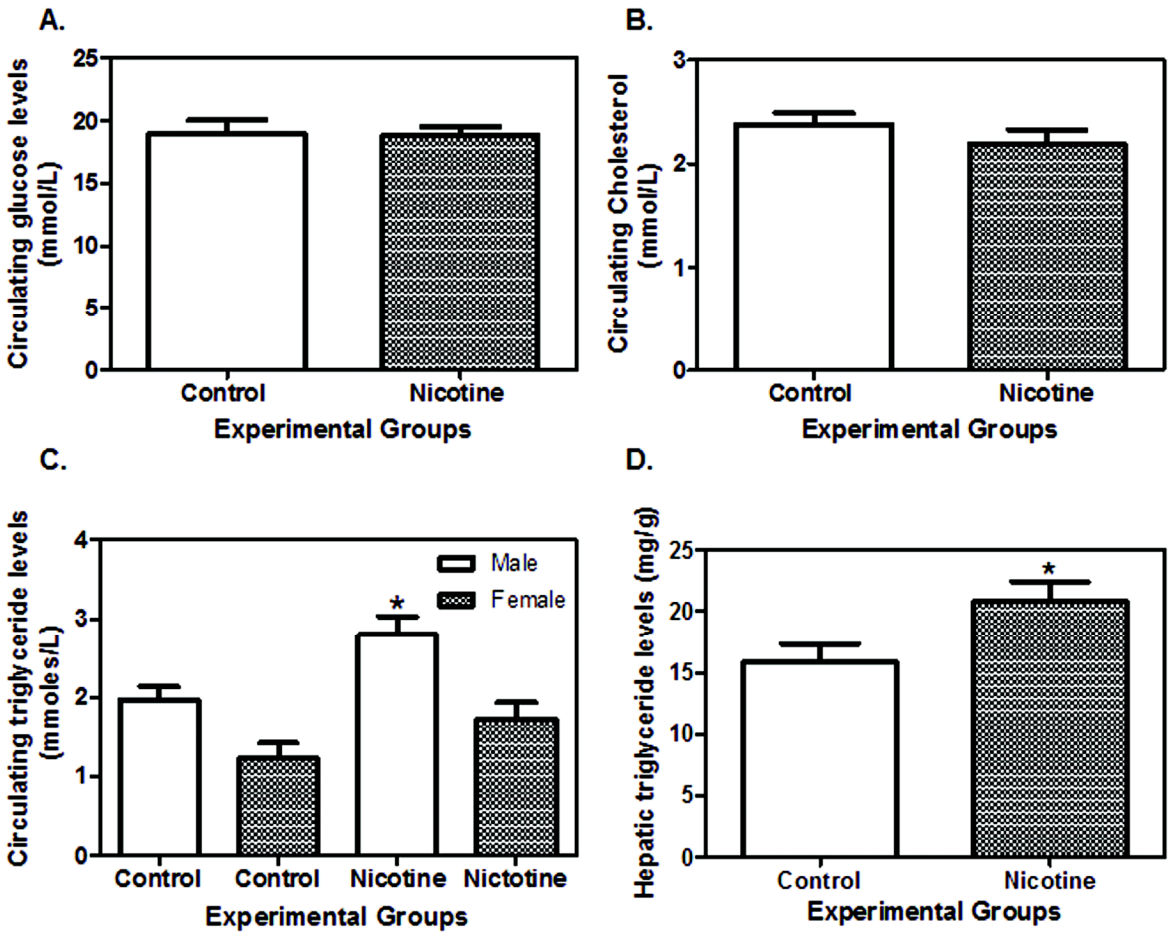

Fig. 3.1.2 The effect of MNE-PL on circulating levels of glucose (A), cholesterol (B), triglycerides (C) and hepatic triglycerides (D) in PND180 male rat offspring. Results are expressed as the mean ± SEM. Nicotine effects were determined using a Student's t test. For data in Figure 3.1.2C, the t test was performed with the data within each gender. * = ($p < 0.05$); n = 7-17/group.

3.2 Long-term effects of MNE-PL on hepatic lipogenic genes in offspring

3.2.1 MNE-PL increased the steady-state mRNA levels of fatty acid synthesis enzymes in the liver of male PND180 offspring

In order to elucidate the molecular mechanisms underlying the elevated triglyceride levels in nicotine-exposed male adult rat offspring, we next examined the hepatic enzymes involved in the fatty acid synthesis pathway leading to *de novo* triglyceride production (85, 87, 88). qRT-PCR revealed significant increases ($p<0.05$) in hepatic fatty acid synthase (FAS) and acetyl-CoA carboxylase (ACCα) mRNA, both enzymes involved in the initial steps of fatty acid synthesis, in male (Fig. 3.2.1.1A-B) and female (Fig. 3.2.1.2A-B) PND180 MNE-PL offspring (85, 87). Interestingly, no significant changes in the hepatic steady-state mRNA levels of stearoyl-CoA dehydrogenase 1 (SCD-1) were observed in male (Fig. 3.2.1.1C) or female offspring (Fig. 3.2.1.2C).

However, due to the lack of significant change in circulating triglyceride levels, PND180 female offspring were not further investigated in this study. More importantly, this model has been previously demonstrated to exhibit early life programming effects in a sexually dimorphic manner, which was not the focus of this investigation (195).

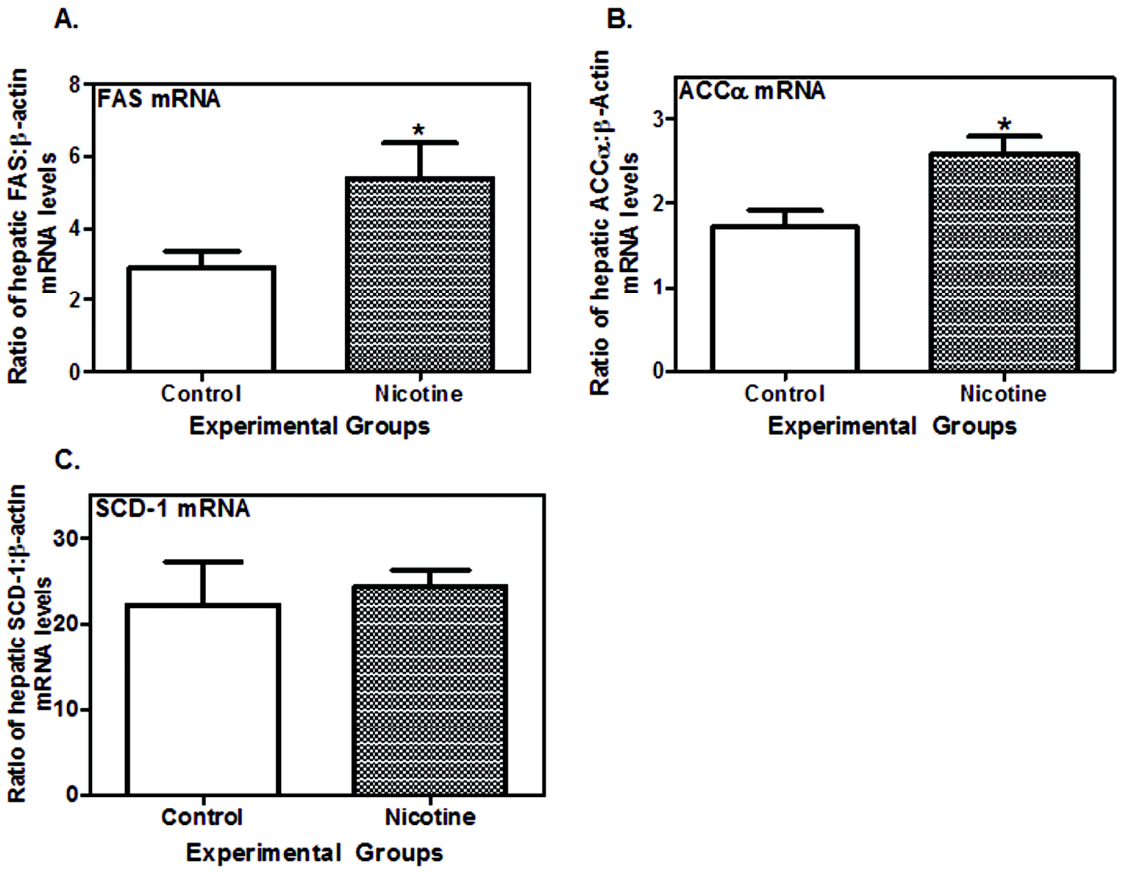

Fig. 3.2.1.1 The effect of MNE-PL on hepatic steady-state mRNA levels of FAS (A), ACCα (B), and SCD-1 (C) in PND180 male rat offspring. RNA was extracted and mRNA levels were assessed using qRT-PCR with primers specific for FAS, ACCα and SCD-1. Results are expressed as the mean ± SEM. Nicotine effects were determined using a Student's t test. * = $p < 0.05$); n = 5-10/group.

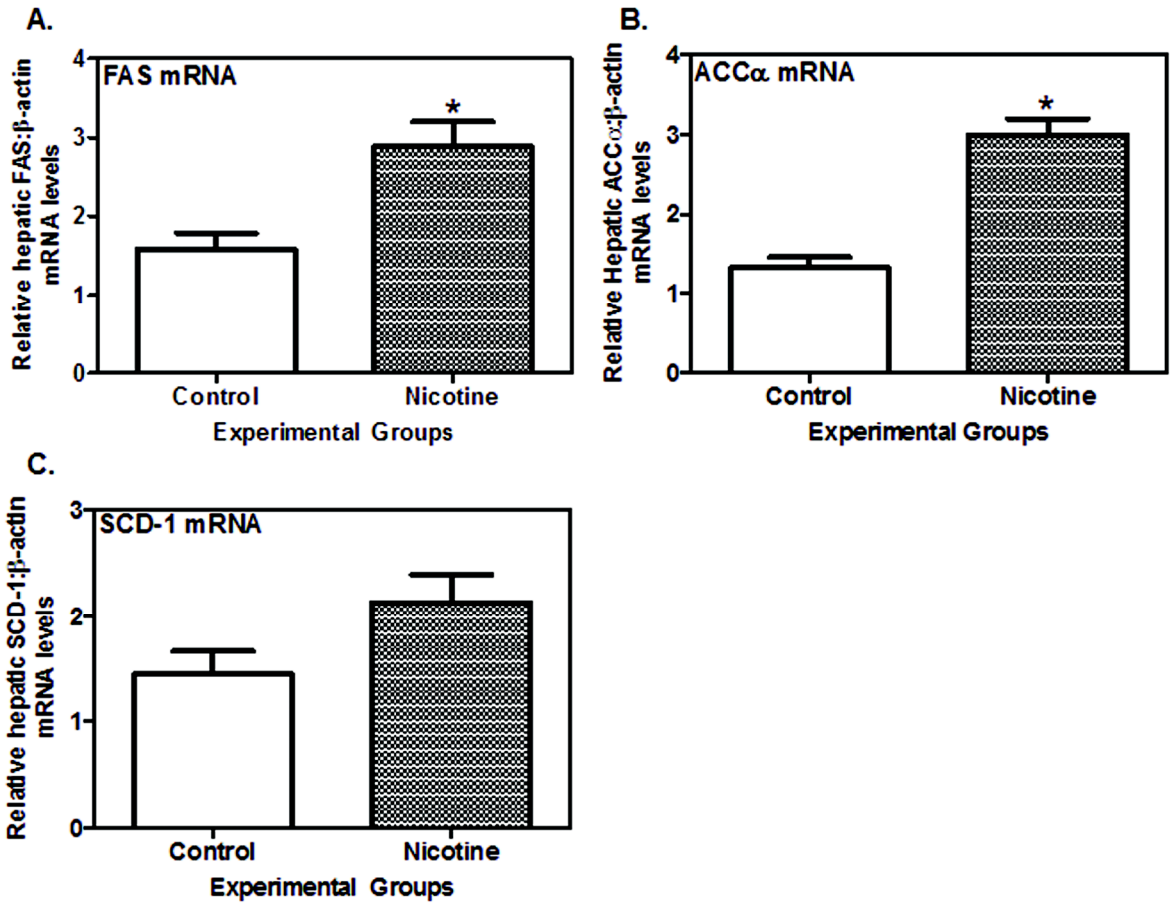

Fig. 3.2.1.2 The effect of MNE-PL on hepatic steady-state mRNA levels of FAS (A), ACCα (B), and SCD-1 (C) in PND180 female rat offspring. RNA was extracted and mRNA levels were assessed using qRT-PCR using primers specific for FAS, ACCα and SCD-1. Results are expressed as the mean ± SEM. Nicotine effects were determined using a Student's t test. * = $p < 0.05$); n = 4-5/group.

3.2.2 MNE-PL correspondingly increased the protein levels of hepatic enzymes involved in fatty acid synthesis in male PND180 offspring

As differences were found in the steady-state mRNA levels of hepatic lipogenic genes (*e.g*. FAS and ACCα), we next performed western immunoblot analysis to determine changes at the protein level. At PND180, MNE-PL led to a significant increase in FAS protein levels (Fig. 3.2.2.1). Despite changes in mRNA levels, there were no corresponding changes in protein levels of ACCα (Fig 3.2.2.2A). Subsequently, the inactivated form of ACCα was explored to determine if levels of inactivated ACCα were altered due to MNE-PL (203). Western immunblotting revealed no difference in phosphorylated-ACCα (Fig. 3.2.2.2B) protein levels between PND180 nicotine exposed and control offspring.

3.2.3 MNE-PL leads to long-term increases in hepatic protein levels of LXRα

To begin to decipher the underlying mechanisms behind the increase in FAS expression in PND180 male MNE-PL offspring, we next investigated hepatic levels of LXRα and SREBP-1c, both regulators of FAS (118, 204). At PND180, MNE-PL led to a significant increase in hepatic LXRα protein levels (Fig. 3.2.3A) in male offspring compared to control, while no difference was observed in SREBP-1c protein expression (Fig. 3.2.3B).

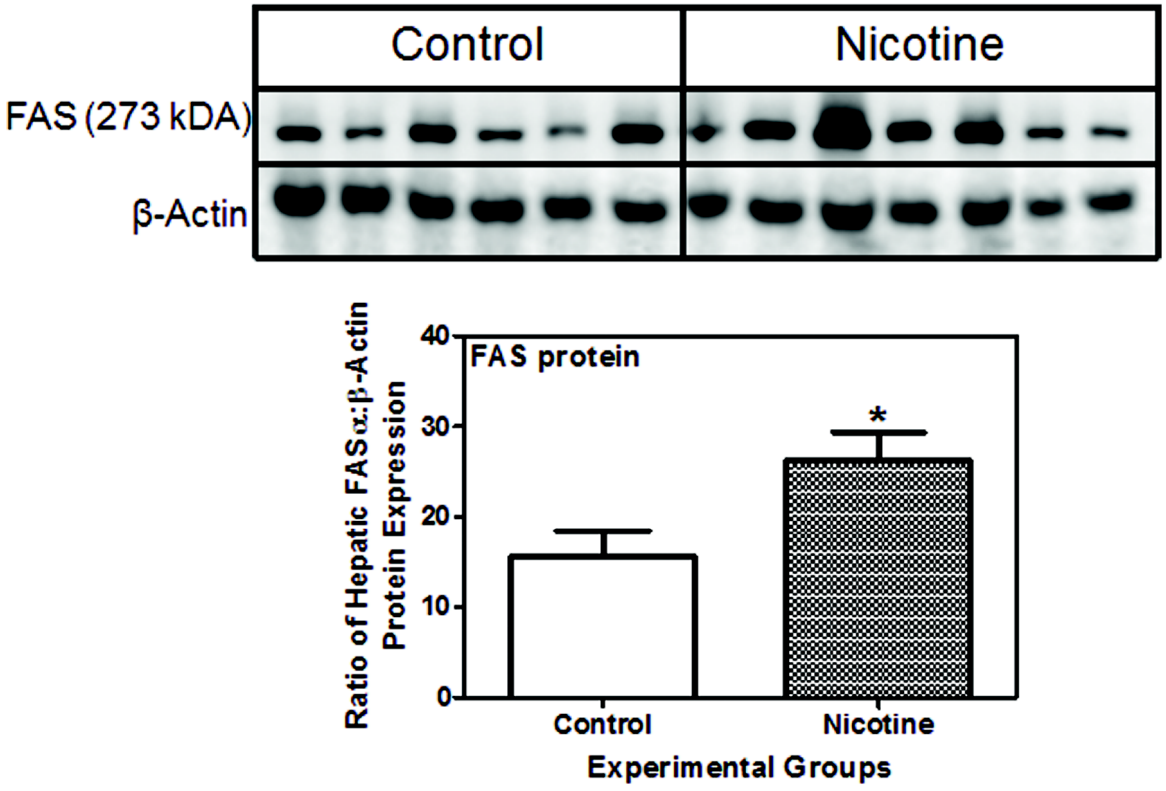

Fig. 3.2.2.1 MNE-PL leads to an increase in FAS protein levels in PND180 male offspring. Protein was extracted and the expression of FAS was measured using western immunoblot analysis. The protein levels were quantified using densitometry and normalized to the protein levels of a housekeeping protein, β-actin. Results are expressed as the mean ± SEM. Nicotine effects were determined using a Student's t test. * = $p < 0.05$); n = 6-7/group.

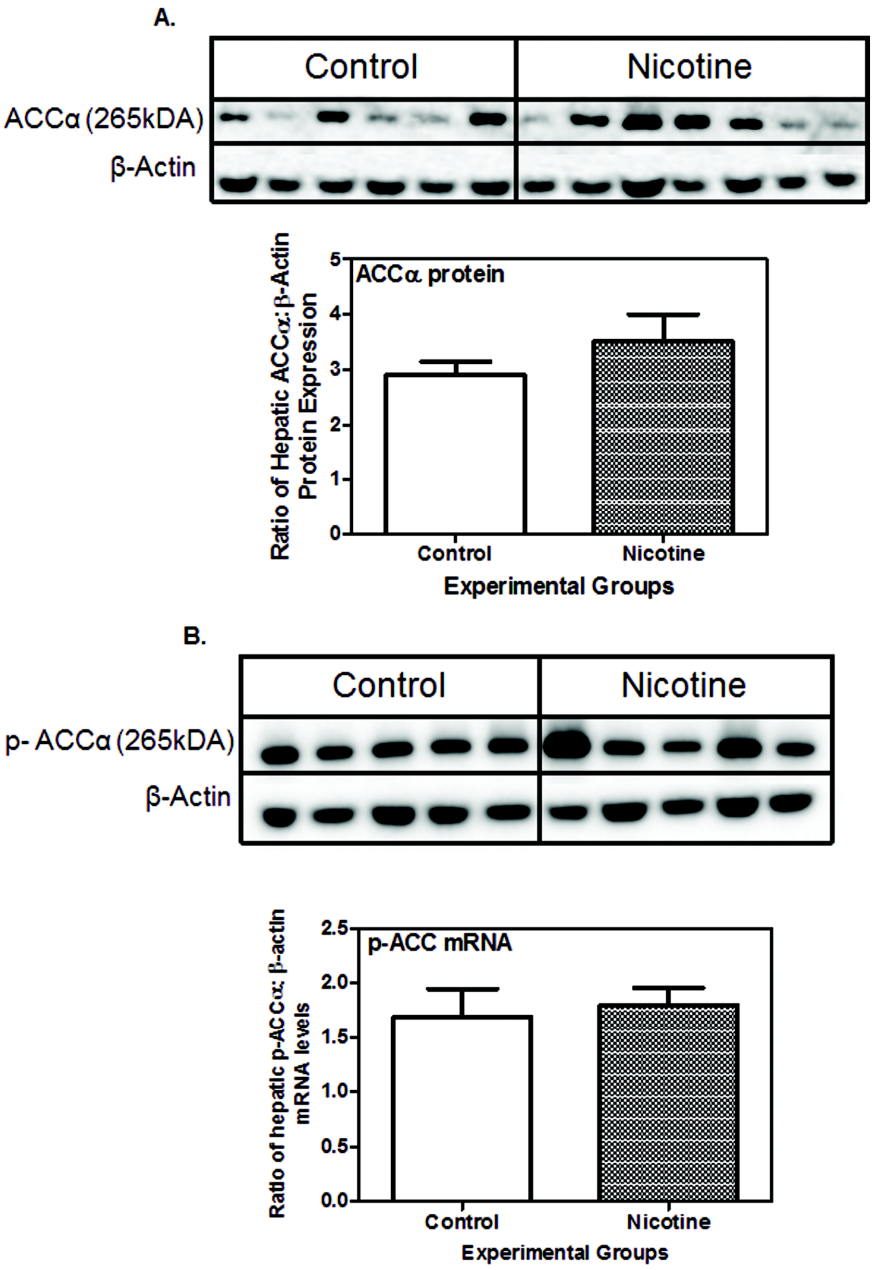

Fig. 3.2.2.2 Exposure to nicotine during pregnancy and lactation does not influence ACCα (A) and p-ACCα (B) protein levels in PND180 male offspring. Protein was extracted and the expression of ACCα and p-ACCα was measured using western immunoblot analysis. The protein levels were quantified using densitometry and normalized to the protein levels of a housekeeping protein, β-actin. Results are expressed as the mean ± SEM. Nicotine effects were determined using a Student's t test. * = $p < 0.05$); n = 5-7/group.

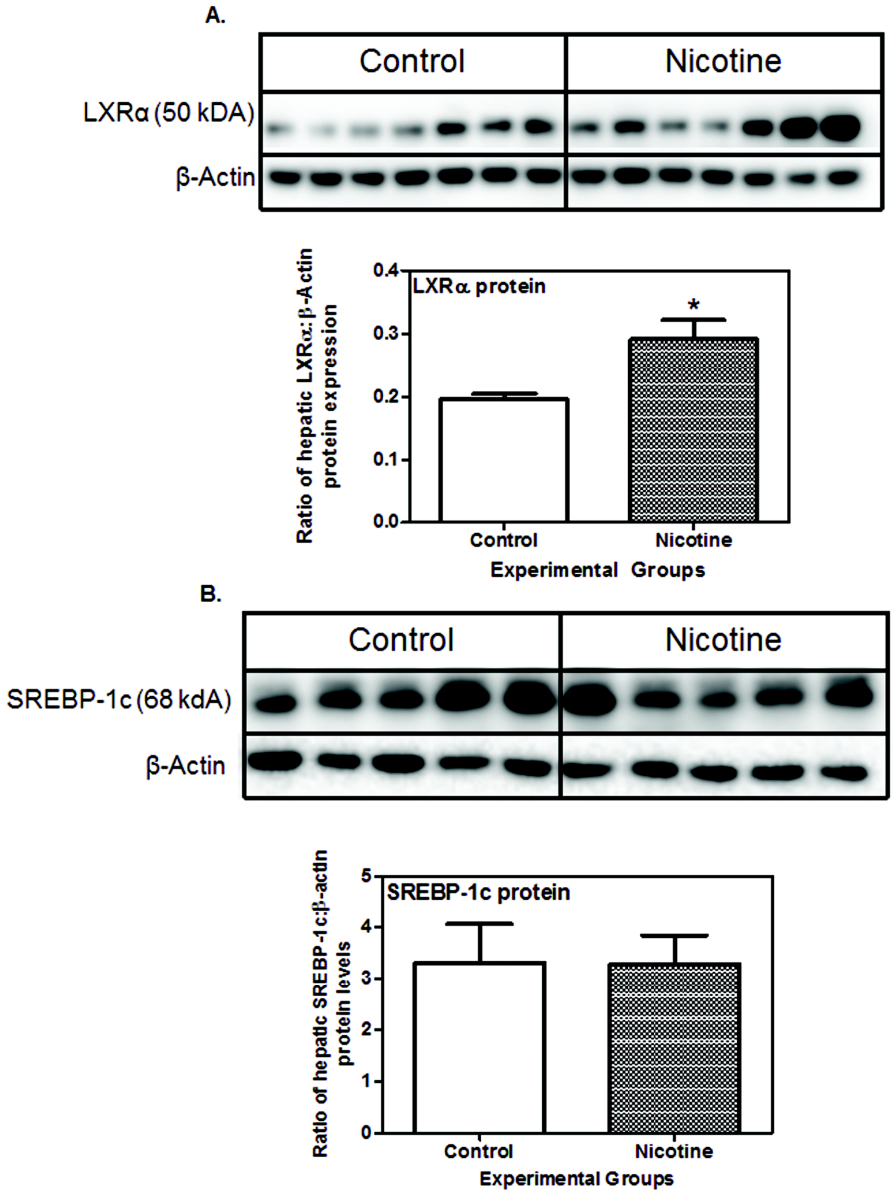

Fig. 3.2.3 MNE-PL leads to an increase in hepatic LXRα protein levels in PND180 male rat offspring. Protein was extracted and the expression of LXRα (A) and SREBP-1c (B) was analyzed using western immunoblot analysis. The protein levels were quantified using densitometry and normalized to a housekeeping protein, β-actin. Results are expressed as the mean ± SEM. * = $p < 0.05$; n= 5-7/group.

3.2.4 MNE-PL leads to increased LXRα binding to the putative LXRE on the FAS promoter in PND180 male rat offspring

The increase in LXRα protein expression suggests that LXRα may be facilitating the increase in FAS protein and mRNA expression in adult male offspring exposed to nicotine during pregnancy and lactation. To explore this further, we employed chromatin immunoprecipitation (ChIP) to examine the *in vivo* binding of LXRα to its putative LXR binding element (LXRE) on the proximal rat promoter (-669 to -665 bp) of *FAS* (118). Real-time primers were designed to surround the LXRE of the *FAS* gene and were demonstrated to equally amplify their target sequences over a range of chromatin concentrations (data not shown). While not significant (p=0.13), male PND180 MNE-PL offspring in this small samples size (n=5) were observed to have increased LXRα binding at the putative LXRE of the *FAS* promoter (Fig. 3.2.4). Nonimmune IgG displayed negligible binding compared to LXRα binding to the *FAS* promoter.

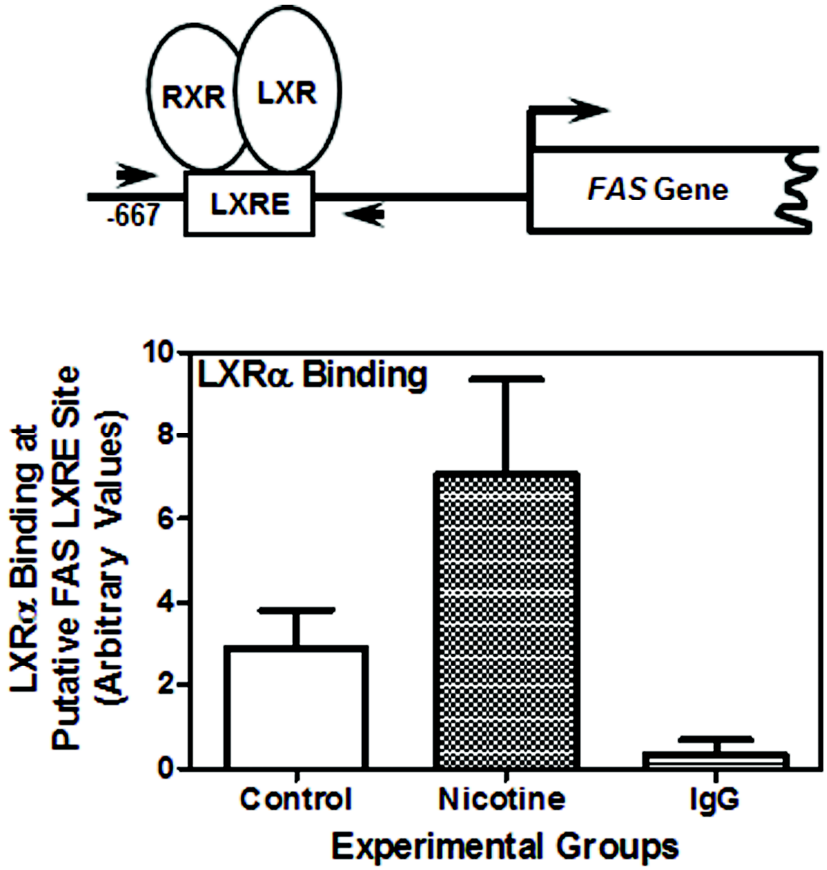

Fig. 3.2.4 MNE-PL increases LXRα binding to the LXRE of the *FAS* promoter in the liver of PND180 male offspring. The *in vivo* binding of LXR to the hepatic LXRE of the *FAS* promoter in male rat offspring at PND180 was assessed by chromatin immunoprecipitation. Briefly, cross-linked chromatin was immunoprecipitated using an antibody specific for LXR and the relative abundance of a region surrounding the LXRE (-669 to -655 bp) of the *FAS* promoter was quantified using qRT-PCR. The relative level of immunoprecipitated DNA was normalized to total genomic DNA for each sample. The effect of nicotine was determined using a Student's t test. Results are expressed as the mean ± SEM. * = $p < 0.05$; n= 5/group.

3.3 Epigenetic alterations of the hepatic fatty acid synthesis pathway in adult rat offspring due to MNE-PL

3.3.1 MNE-PL induced transcriptional activation of hepatic FAS expression is associated with an increase in the acetylation of histone H3 [K9,14] surrounding the LXRE of the FAS promoter region in PND180 male offspring

Since LXRα has been demonstrated to enhance the acetylation of histone H3 [K9,14] to increase hepatic *FAS* transcription (152), we next employed ChIP to investigate if chromatin remodeling could be a factor influencing the observed increase in FAS mRNA and protein levels in MNE-PL offspring. The acetylation of histone H3 [K9, 14] is well known to be associated with chromatin activation (136). ChIP revealed that male MNE-PL offspring in this small samples size (n=5) were trending towards an significant increase (p=0.09) in the acetylation of histone H3 [K9,14] surrounding the putative LXRE (-669 to -655 bp) of the *FAS* promoter (Fig. 3.3.1) (118). Nonimmune IgG displayed negligible binding compared acetylation of histone H3 [K9,14] surrounding the *FAS* promoter.

3.3.2 The effects of MNE-PL on the steady-state mRNA levels of histone modifying enzymes (Sirt-1 and p300) in the livers of PND180 male offspring

As differences were found in the levels of acetylation of histone H3 [K9, 14] surrounding the putative LXRE of the *FAS* promoter, we investigated possible mechanisms responsible for the alterations in the epigenetic status of adult male offspring. qRT-PCR revealed no difference in mRNA expression of Sirt-1 (Fig. 3.3.2A) or p300 (Fig. 3.3.2B), known histone deacetylases and acetyltransferases, respectively (141, 145).

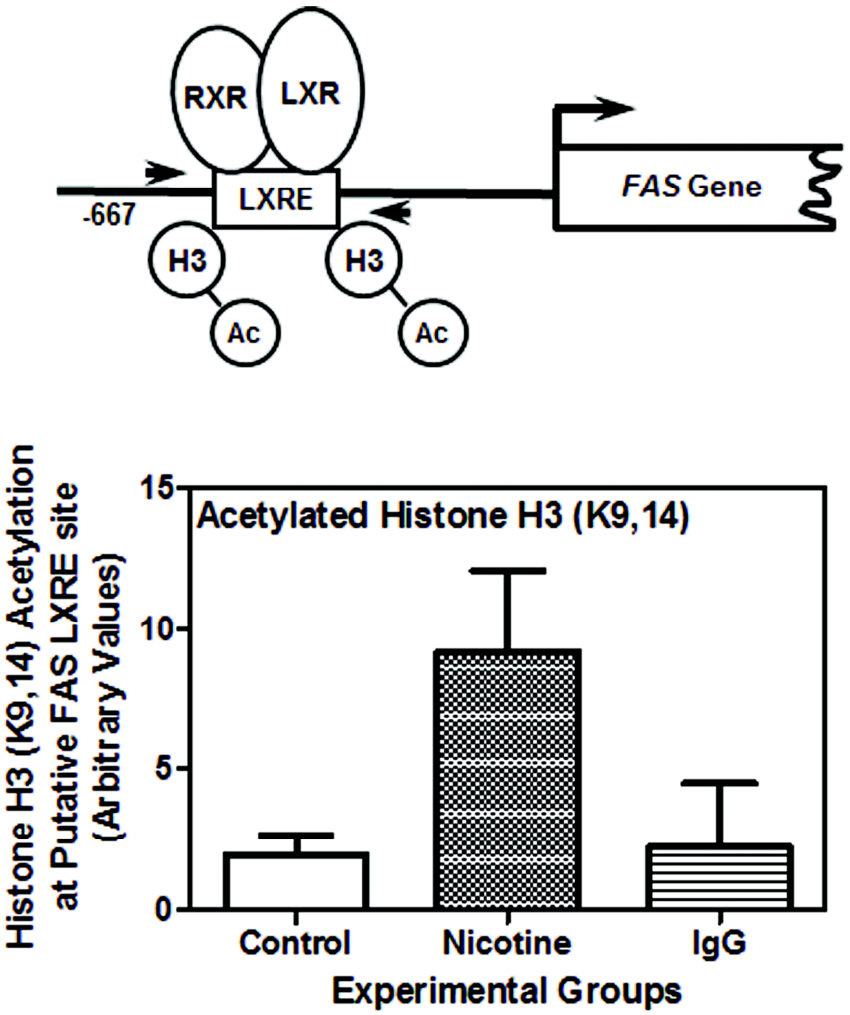

Fig. 3.3.1 MNE-PL increases the acetylation of histone H3 [K9,14] surrounding the LXRE of the *FAS* promoter in the liver of PND180 male offspring. Briefly, cross-linked chromatin immunoprecipicated using an antibody specific for acetylated histone H3 [K9,14] was isolated and the relative abundance of a region surrounding the LXRE (-669 to -655 bp) of the *FAS* promoter was quantified using qRT-PCR. The relative level of immunoprecipitated DNA was normalized to total genomic DNA for each sample. Results are expressed as the mean ± SEM. Nicotine effects were determined using a Student's t test. * = $p < 0.05$; n= 5/group.

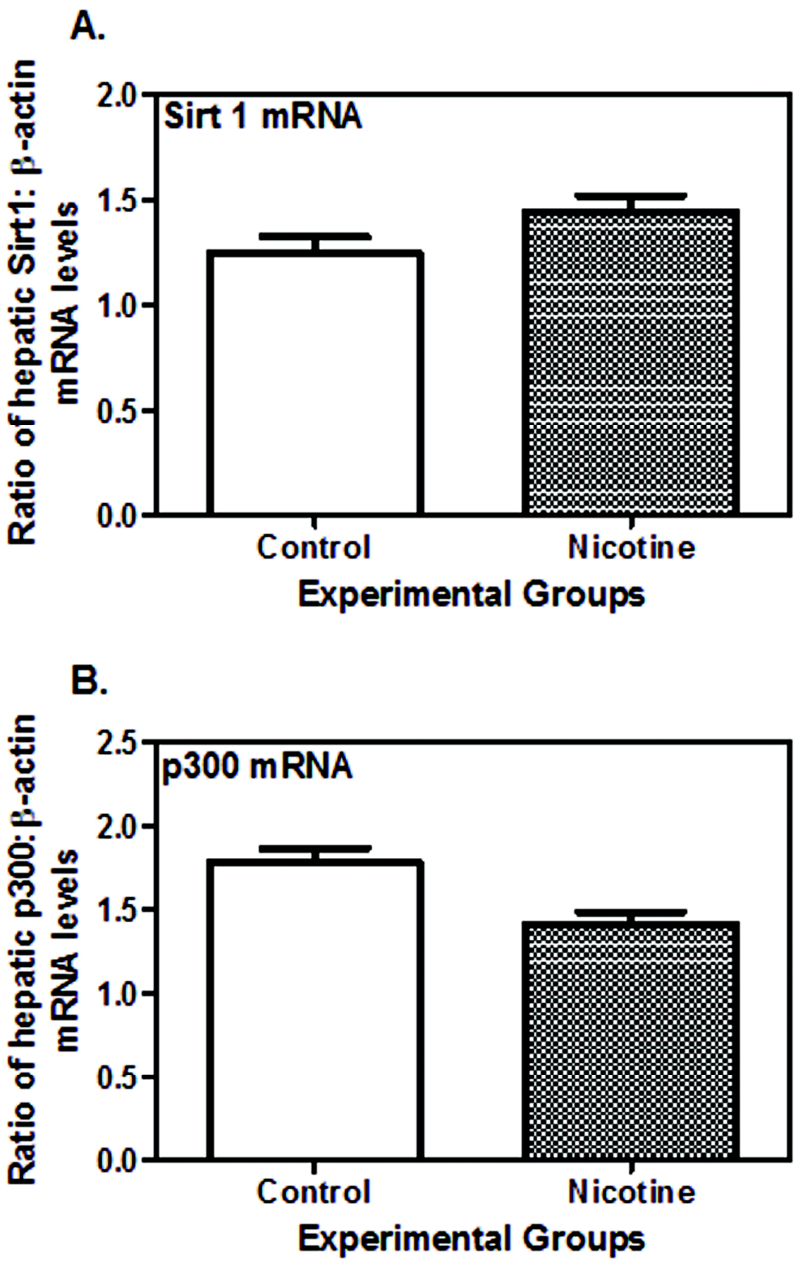

Fig. 3.3.2 MNE-PL does not influence hepatic steady-state mRNA levels of Sirt-1 (A) and p300 (B) in PND180 male rat offspring. RNA was extracted and mRNA levels were assessed using qRT-PCR with primers specific for Sirt-1 and p300. Results are expressed as the mean ± SEM. Nicotine effects were determined using a Student's t test. * = $p < 0.05$); n = 5/group.

3.4 Direct effects of MNE-PL on hepatic fatty acid synthesis in offspring

3.4.1 The effects of MNE-PL on hepatic fatty acid synthesis in PND1 and PND21 male offspring

Earlier developmental time points in offspring were examined in order to determine whether MNE-PL directly leads to the elevations in triglyceride levels observed in male offspring at PND180. qRT-PCR analysis found no differences in FAS, ACCα and SCD-1 mRNA levels between PND1 control and MNE offspring (Fig. 3.4.1.1A-C).

Similarly, analysis of FAS, ACCα and SCD-1 mRNA levels via qRT-PCR revealed no difference in PND21 male control and nicotine exposed offspring (Fig. 3.4.1.2A-C). Importantly, the PND21 time point represents the longest period of exposure (21 days) to nicotine in offspring.

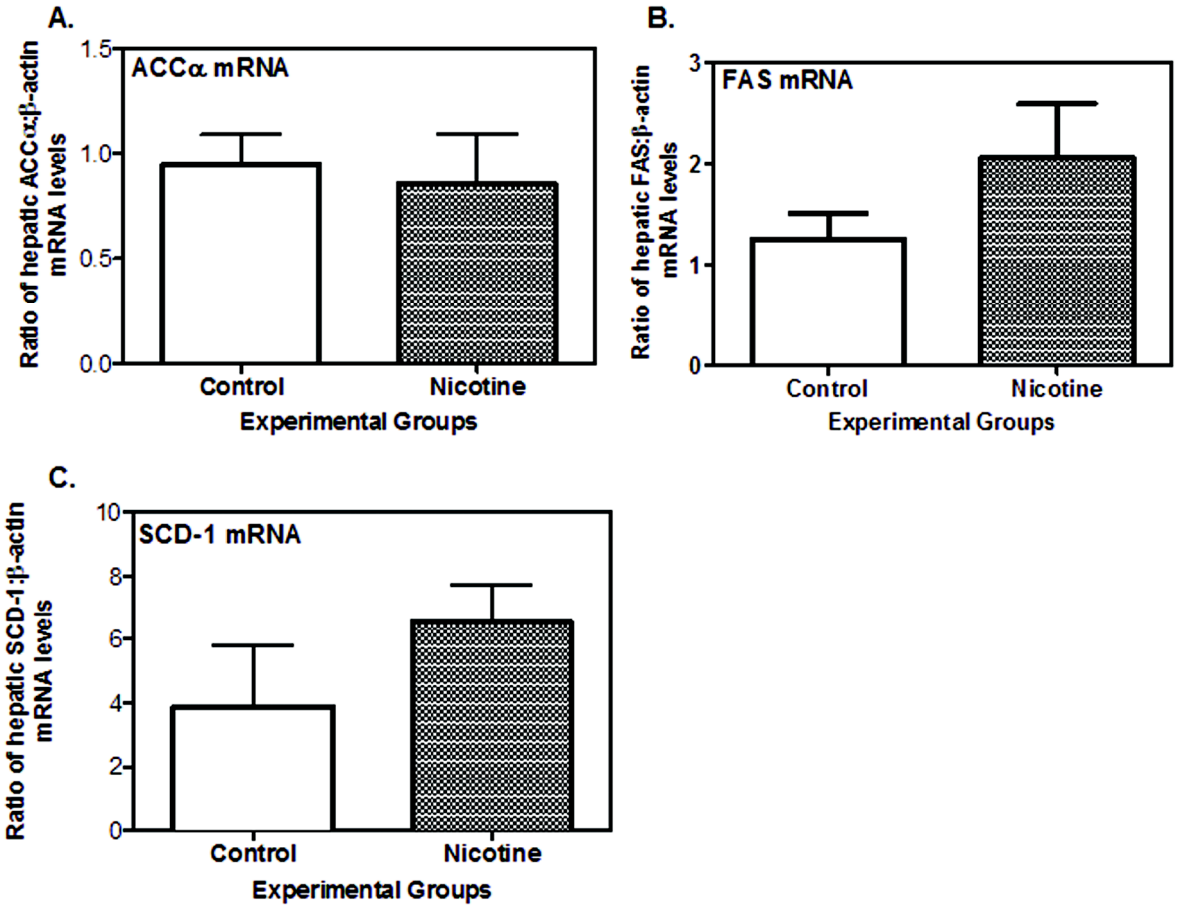

Fig. 3.4.1.1 MNE-PL does not influence hepatic steady-state mRNA levels of ACCα (A), FAS (B), and SCD-1 (C) in PND1 male rat offspring. RNA was extracted and mRNA levels were assessed using qRT-PCR using primers specific for FAS, ACCα and SCD-1. Results were expressed as the mean ± SEM. Nicotine effects were determined using a Student's t test. * = $p < 0.05$); n = 2-5/group.

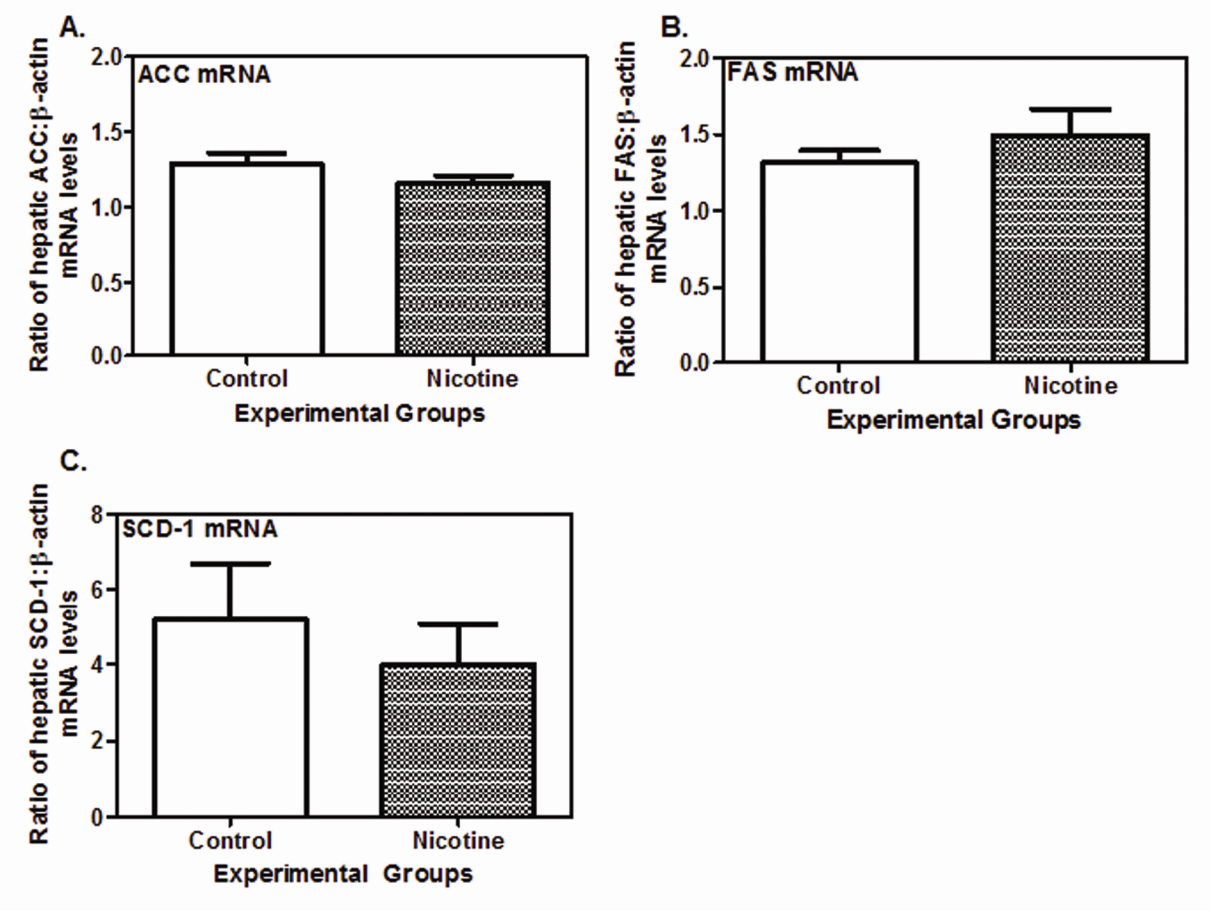

Fig. 3.4.1.2 MNE-PL does not influence hepatic steady-state mRNA levels of ACCα (A), FAS (B), and SCD-1 (C) in PND21 male rat offspring. RNA was extracted and mRNA levels were assessed using q-RT-PR using primers specific for FAS, ACCα and SCD-1. Results are expressed as the mean ± SEM. Nicotine effects were determined using a Student's t test. * = $p < 0.05$); n = 4-5/group.

CHAPTER 4 : DISCUSSION

4.1 Clinical relevance of the study

In this study I present evidence that MNE-PL leads to hypertriglyceridemia in PND180 male rat offspring. My data suggests that this effect is due to increased *de novo* hepatic triglyceride synthesis. Specifically, I demonstrated that FAS expression was enhanced due to increased LXRα protein levels and binding to the LXRE of the *FAS* promoter in PND180 male MNE-PL offspring. This increase in LXRα binding at the *FAS* promoter was associated with enriched histone H3 acetylation [K9,14] at the LXRE site, previously shown to lead to FAS transcriptional activation (152). Given the well-established link between an adverse *in utero* environment and the development of metabolic dysfunction long-term (158, 205), this study sheds light on possible molecular mechanisms that mediate the programming of obesity in children exposed to smoke *in utero* (8). My study also raises questions regarding the safety of NRT during pregnancy and long-term consequences for offspring.

4.2 Examining the mechanisms of nicotine action on hepatic fatty acid synthesis

In this thesis, I investigated the influence of MNE-PL on the hepatic fatty acid synthesis pathway. I present evidence that MNE-PL leads to elevated levels of hepatic and circulating triglyceride levels, concomitant with alterations to fatty acid biosynthetic enzyme expression in PND180 male offspring. MNE-PL did not affect circulating glucose or cholesterol levels in offspring. My findings support human studies that demonstrated adult women exposed to tobacco *in utero* exhibit elevated triglyceride

levels in adulthood (13). Livers of PND1 and PND21 MNE-PL offspring were examined to determine whether nicotine directly augments triglyceride levels. These earlier developmental time points represent different windows of direct nicotine exposure in fetal and neonatal life. PND1 and PND21 MNE-PL offspring did not exhibit any significant differences in the expression of FAS, ACCα, or SCD-1 compared to control offspring. Consequently my results suggest that elevated triglycerides were not a direct result of nicotine exposure, as we would have expected to observe alterations in target gene expression at weaning, following the longest window of direct nicotine exposure. Moreover, only a subset of primary human hepatic stellate cells express the neuronal subunits of nAcHR (α3,α6, α7, β2 and β4) (206). Together, given the lack of nAcHR subunit expression in hepatocytes and unaltered fatty acid synthesis in early life, it is highly conceivable that nicotine induced alterations in long-term fatty acid synthesis and triglyceride levels are a result of indirect mechanisms.

4.3 Possible mechanisms underlying the effect of MNE-PL on long-term programming of hypertriglyceridemia in offspring

In this study, MNE-PL resulted in significantly heavier male offspring compared to control offspring at PND180. These results support findings from human studies that have repeatedly demonstrated an increase in the risk of obesity in children (ages 2 – 33 years old) exposed to smoking during pregnancy regardless of parental socioeconomic background, infant feeding patterns and gestational weight gain (8, 11, 207-210). My rat model suggests that nicotine alone leads to the development of obesity and elevated triglycerides in children exposed to smoking *in utero* (209). Despite being heavier in later life, I found that MNE-PL offspring at PND1 weigh less compared to control offspring.

Children of smoking mothers also shared this surprising characteristic, as exposed children are at higher risk for being low birth weight compared to unexposed offspring (208, 211, 212). Further studies demonstrate that fetal growth restriction is more likely responsible for low birth weight in children rather than preterm delivery (208, 211-213). Other animal models of MNE have also proposed that nicotine alone can impair growth in offspring. It was found that nicotine injections of 2mg/kg/day during gestation in rats led to lower average fetal weight *in utero* compared to control fetuses (214). Similarly, rat offspring exposed to 6mg/kg/day of nicotine during early postnatal life gained significantly less weight per day compared to control offspring (215). However, these nicotine-exposed pups caught up in size by PND18. While these studies suggest that nicotine alone can impair fetal growth, it is important to note that these animal models utilized higher concentrations of nicotine compared to the model of MNE-PL used in my study. Therefore, future studies should closely monitor the growth of MNE-PL offspring to ascertain whether a moderate dosage of nicotine for a longer period of time lead to comparable growth impairments in offspring.

To resolve the two extreme phenotypes that are observed in response to nicotine exposure in perinatal life, my data suggests that MNE-PL offspring undergo catch up growth. A study has demonstrated that babies of smoking mothers tended to be lighter at birth but caught up in weight to children of non-smoking mothers by 12 months of age (216). Specifically, male children caught up at a faster rate compared to female children (216). Furthermore, children that were born small and were able catch up in bodyweight had higher percentages of body fat and increased BMI in early life compared to other children (176). Therefore, catch up growth may program offspring to overshoot normal

growth trajectories leading to a higher likelihood of developing characteristics that are predictive of adult obesity (176). Although the mechanisms by which children are able to catch up in growth are largely unknown, it has been postulated that low leptin levels in low birth weight babies may signal for greater food intake (176). The connection between catch up growth and differences in nutritional intake has been investigated by comparing breast-fed and formula fed preterm babies. Formula fed babies were found to experience high neonatal growth during the first 2 weeks following birth and this was associated with higher markers of insulin resistance and an atherogenic lipoprotein profile that was observed up to 16 years later (217, 218). Since catch up growth in babies was found irrespective of birth weight, researched proposed that the programming of metabolic outcomes largely occurs antenatally. Therefore in this study, MNE-PL may impair fetal development leading to subsequent catch growth and the programming of hypertriglyceridemia in offspring.

4.4 Catch up growth and ER stress

Interestingly, our laboratory has demonstrated that rat offspring exposed to an adverse *in utero* environment followed by rapid catch up growth in postnatal life, exhibit increased ER stress (219). Various insults such as glucose deprivation, changes in oxidation-reduction balance, infection and development of secretory cells can all lead to the misfolding of proteins and initiate the unfolded protein response (UPR) (220, 221). The UPR response helps cells cope with an overloaded ER by decreasing translation, increasing efflux and stimulating the degradation of proteins (220). The UPR leads to the phosphorylation of the eukaryotic initiation factor 2α (eIF2α), which leads to the attenuation of further translation (220). It also increases chaperone protein levels (*e.g.*

glucose regulated proteins Grp78 and Grp94) to facilitate increased folding and induces the splicing of the X-box binding protein (Xbp-1) to increase protein degradation (220). ER-stress induced apoptosis is activated if a cell is overwhelmed by the amount of misfolded protein (221). Specifically, our laboratory has demonstrated that postnatal catch up growth is associated with elevated levels of ER chaperone protein Grp78 and enhanced phosphorylation of eIF2α long-term (219). Since our MNE-PL model appears to lead to catch up growth in offspring, future studies should investigate whether hepatic ER stress pathways are activated in conjunction with alterations in fatty acid and triglyceride synthesis.

The role of ER stress in models of catch up growth is an attractive mechanism to explain in part, the underlying mechanisms of fetal programming. Indeed, studies have already postulated that obesity induces hepatic insulin resistance through elevated ER stress signaling in the liver of rodent models (222, 223). Notably, my preliminary data in PND180 male offspring exposed to MNE-PL, exhibited increased Grp78 protein levels (Fig. 4.4).

Yet, the involvement of ER stress in the long-term programming of hypertriglyceridemia in nicotine-exposed offspring has not been fully elucidated. Although nicotine did not have a direct effect in my MNE-PL model, it would be interesting to assess if nicotine could directly induce ER stress in PND1 or PND21 offspring, or if ER stress is activated due to catch up growth. It is noteworthy that one study has demonstrated that antagonism of LXRα protects against steatosis by decreasing ER stress (224). This study presents a novel mechanism by which ER stress mediates the

development of liver pathology through LXRα activation. Given the increase in LXRα in MNE-PL offspring and the association between ER stress and catch up growth, future studies will evaluate ER stress markers in the liver of PND180 male offspring. It is plausible that nicotine induced ER stress may be causing permanent changes in hepatic function via the aberrant activation of LXRα in MNE-PL offspring.

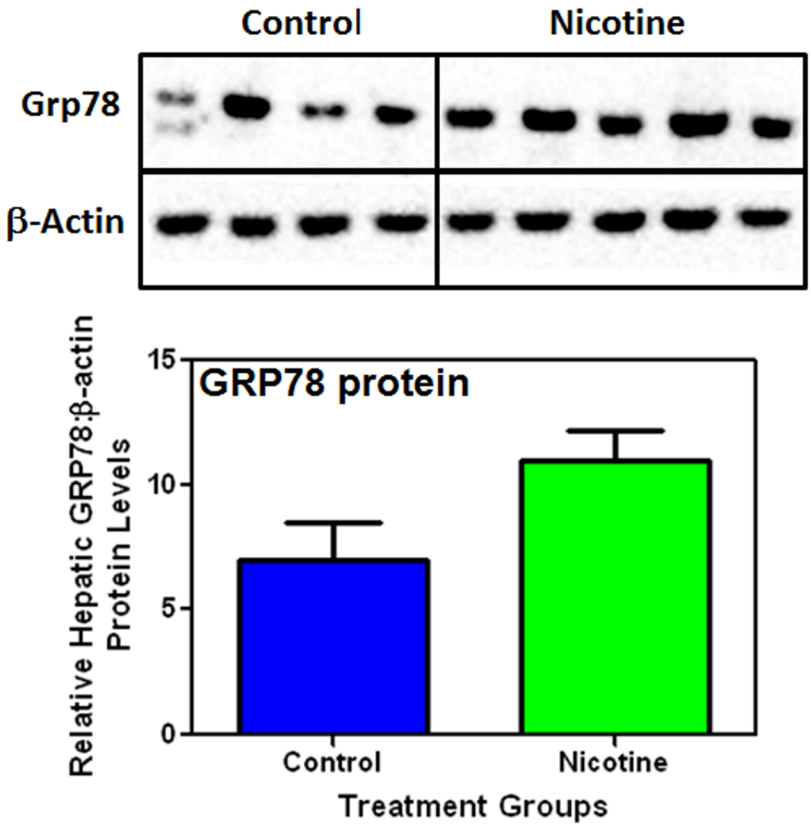

Fig. 4.4 MNE-PL leads to increased Grp78 protein expression in PND180 male offspring. Protein was extracted and the expression of Grp78 was measured using western immunoblot analysis. The protein levels were quantified using densitometry and normalized to the protein levels of a housekeeping protein, β-actin. Results are expressed as the mean ± SEM. Nicotine effects were determined using a Student's t test. * = $p < 0.05$); n = 8/group.

4.5 Molecular mechanisms underlying elevated triglycerides in adult MNE-PL offspring

The three main sources of free fatty acids used for triglyceride synthesis in the liver include *de novo*, circulating, and dietary (87). Human and animal studies have both demonstrated that alterations in fatty acid synthesis lead to impairments in triglyceride homeostasis (87, 115, 118, 225). In the present study, I have demonstrated elevations in the steady-state mRNA levels of FAS and ACCα in the liver of male MNE-PL offspring at PND180. MNE-PL offspring also exhibited a corresponding increase in FAS protein expression suggesting that alterations in FAS play a central role in *de novo* triglyceride synthesis. Studies in rodent models of obesity associated with elevated plasma triglyceride levels, have similarly demonstrated an increase in hepatic FAS protein expression (226, 227). Moreover, *de novo* fatty acid synthesis is considered a dynamic process as human studies have shown that the rate synthesis can be drastically altered in various disease states (228, 229). Taken together, these data suggest that nicotine exposure may permanently increase triglyceride levels, in part, via a FAS-dependent pathway.

To date, I have only examined *de novo* triglyceride synthesis as the main source for elevated triglycerides observed in PND180 MNE-PL offspring. However, other mechanisms could be contributing to the development of hypertriglyceridemia in nicotine exposed offspring including differences in food consumption and adipose tissue distribution. Since high carbohydrate diets have been shown to increase *de novo* fatty acid synthesis and plasma triglyceride levels in both rodents and humans, future studies should closely monitor post-weaning food consumption in nicotine-exposed offspring (230-232). In addition, obtaining adipose tissue from MNE-PL offspring will also be

pursued in later studies, considering elevated amounts of body fat is a predictor of triglyceride levels in humans (233).

Given the increase in expression of fatty acid biosynthetic genes, I next investigated changes in regulatory mechanisms. The expression of FAS is under the direct control of various transcription factors including LXRα (-669 to -655 bp), SREBP-1c (-71 to -54 bp), and ChREBP (-7214 to -7190 bp) (117, 119, 234). My study determined that there were significantly higher levels of LXRα protein in MNE-PL offspring compared to control offspring. In parallel, unpublished data from our laboratory has demonstrated that following short-term neonatal exposure to an LXR agonist leads to higher triglycerides levels in rat offspring (Fig. 4.5). Models involving treatment with LXRα agonists have demonstrated similar elevations in plasma triglycerides along with an induction of hepatic FAS expression (118). Likewise, LXR deficient mice treated with LXRα agonist do not exhibit an increase in triglyceride levels (204). Therefore, LXRα activation is imperative to FAS activation and triglyceride synthesis.

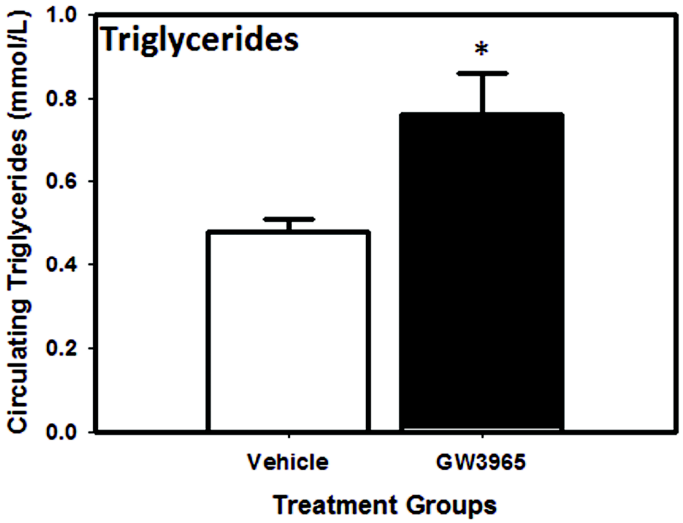

Fig. 4.5 Treatment with LXR agonist, GW3965, during pregnancy in rats led to increased circulating triglyceride levels in offspring. Results are expressed as the mean ± SEM. LXR agonist effects were determined using a Student's t test. * = ($p < 0.05$); n = 6-9/group.

To determine whether the increase in LXRα and its target, FAS, were functionally related, ChIP experiments were carried out to measure *in vivo* binding of hepatic LXRα to the promoter of *FAS* in PND180 male offspring. ChIP studies revealed increased LXRα binding surrounding the putative LXRE (-669 to -655 bp) of the *FAS* promoter (117). My results suggest that an elevation in hepatic LXRα expression mediates the transcriptional activation of FAS. Remarkably, other models of fetal programming have demonstrated that LXR and LXR-target genes are susceptible to permanent programming changes (153, 155, 235). Thus, it is conceivable that nicotine-mediated changes in triglyceride levels are occurring in part through prolonged or even permanent increase in FAS activation. It would be interesting to further ascertain the role of LXRα in modulating permanent elevations in FAS expression by undertaking *in vitro* studies. *In vitro* studies involving the treatment of hepatocytes with LXRα antagonists such as piperine, followed by the analysis of FAS expression and ChIP analysis would help confirm whether permanent FAS activation in MNE-PL offspring is attributed directly to LXRα (224).

While it has been established that LXRα plays a large role in the regulation of FAS, other regulatory pathways should be considered in future studies. SREBP-1c is a transcription factor that is activated in its cleaved form and inhibited in the presence of sterols (119, 236, 237). Similar to LXRα, SREBP-1c can directly activate certain fatty acid biosynthetic enzymes including FAS, ACCα and SCD-1 (119, 238, 239). In my study I found no difference in SREBP-1c protein expression between PND180 MNE-PL and control offspring. My findings support previous studies demonstrating augmented

triglyceride levels in obese mice despite diminished SREBP-1c expression (240). For this reason, SREBP-1c is considered an auxiliary regulator of fatty acid synthesis that is under the direct control of LXRα (115, 236). Future studies could investigate the carbohydrate-responsive element binding protein (ChREBP). ChREBP is a transcription factor from the same family as SREBP-1c that heterodimerizes with the protein Max-like protein X (Mlx) (241). It binds to carbohydrate response elements (ChoRE) of glucose responsive targets found throughout the *de novo* lipogenic pathway (241, 242). ChREBP is ubiquitously expressed, with the highest expression found in the liver, brown and white adipose tissues, small intestine, kidneys and muscle (242). ChREBP knock-out mice exhibited lower levels of hepatic FAS and ACCα and a 65% decrease in fatty acid synthesis rates compared to control mice. Importantly, there were no changes in SREBP levels, meaning that the loss of ChREBP was not mediated via SREBP-1c (242). Consequently, ChREBP is another independent regulatory mechanism that should be considered for future investigation in adult MNE-PL male offspring.

4.6 The role of epigenetics in the fetal programming of hypertriglyceridemia in adult MNE-PL offspring

In this present study, I have demonstrated an increase in acetylation of histone H3 surrounding the LXRE of the hepatic *FAS* gene in PND180 MNE-PL offspring. My findings suggest that maternal nicotine exposure leads to long-term posttranslational histone modifcations that facilitate increased transcription of *FAS*. While my results demonstrate changes in chromatin remodeling due to MNE-PL, the effect of nicotine on epigenetic changes is virtually unknown. *In vitro* studies in primary neuronal cortical cells and primary human lymphocytes have shown that nicotine exposure leads to a more

transcriptionally 'permissive' chromatin environment through alterations in the expression of HMTs such as GLP, G9a and Setdb1 (243). Similar histone modifications were observed *in vivo*. A single intraperitoneal injection of 3mg/kg of nicotine was able to elicit measurable changes in mouse cortical extracts 6 hours following injection (243). It would be interesting to determine whether measurable changes occur in methylation and HMT expression in my model of short-term neonatal exposure to nicotine. Future studies should investigate all developmental time points (PND1, 21 and 180) in MNE-PL offspring, to examine whether MNE results in posttranslational histone modifications that precede the development of long-term hypertriglycerdemia in offspring.

Lastly, mRNA levels of Sirt-1 and p300, known histone acetyltransferases and histone deacetylases, respectively, were measured in order to understand the mechanisms underlying nicotine-induced epigenetic modifications (141, 145). While MNE-PL does not influence Sirt-1 or p300 mRNA expression in PND180 male offspring, I was only able to measure the indirect action of these enzymes. In future studies, I would like to employ a similar ChIP study to investigate changes in the *in vivo* binding of Sirt1 or p300 to the *FAS* promoter directly.

4.7 Early folic acid intervention in the prevention of permanent nicotine-induced disease outcomes in rat offspring

Since the development of the liver continues during early postnatal life, it is plausible that intervention during perinatal life could help reverse or prevent nicotine-induced hypertriglyceridemia in adulthood (244). For example, short-term injections of Exendin-4™ in neonatal life have been found to prevent oxidative stress, impaired

hepatic glucose production and hepatic insulin resistance normally exhibited in intrauterine growth restricted (IUGR) rat offspring (245).

Interestingly, folic acid supplementation has been demonstrated to reduce long-term hypertriglycidemia in IUGR piglets via increased methylation of the promoter of LXR-target genes (246). Folate, and its synthetic form, folic acid, act as essential cofactors for biochemical reactions, namely the formation of S-adenosylmethionine, the main methyl donor for methylation (247). A clinical study has demonstrated that differences in DNA methylation patterns can be detected in children exposed to smoke *in utero* (248). Futhermore, folic acid has been shown to alleviate ER stress (249, 250). Taken together, it is conceivable that additional supplementation of folic acid may prevent adult onset hypertriglyceridemia in offspring exposed to nicotine through the prevention of aberrant epigenetic modulation and/or reduction of ER stress.

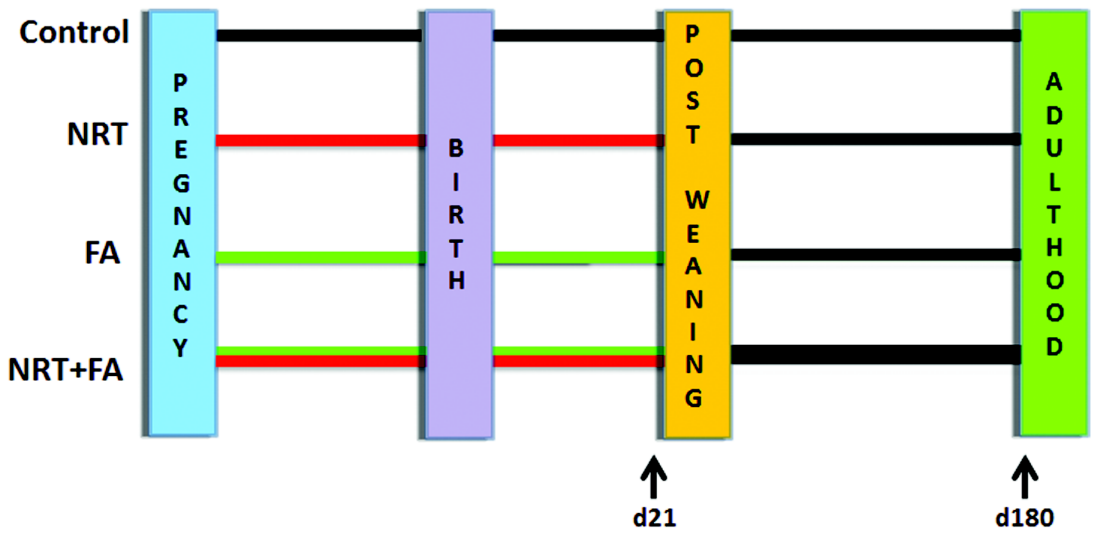

Fig. 4.7 Schematic overview of animal model of MNE-PL and folic acid intervention

Future animal studies will investigate whether dietary folate supplementation can prevent or reduce nicotine-induced hypertriglyceridemia. I will administer nicotine alone, and in combination with folic acid to pregnant rats at clinically relevant doses to female rats during pregnancy and lactation (Fig. 4.7). I will evaluate whether folic acid supplementation prevents hypertriglyceridemia and/or activation of LXRα target genes in adult rat offspring. I will also assess whether folic acid supplementation reduces ER stress in liver of PND180 offspring.

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Curriculum Vitae

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EDUCATION

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PUBLICATIONS

Vo T, Revesz A, Ma N and Hardy DB. Maternal Protein Restriction Leads to Disruption of Glucose Homeostasis in Adult Male Rat Offspring via the Liver X Receptor. Journal of Molecular Endocrincology, 2012, Submitted ***(JME-12-0242).***

Ma N and Hardy DB, "The Fetal Origins of the Metabolic Syndrome: Can We Intervene?," Journal of Pregnancy, vol. 2012, Article ID 482690, 11 pages, 2012. doi:10.1155/2012/482690

PRESENTATIONS

Ma N, Wong M, Nicholson CJ, Holloway AC, and Hardy DB. Molecular Mechanisms Underlying Maternal Nicotine Exposure-Induced Elevated Triglycerides in Adult Rat Offspring. **Poster presentation** at the Eastern Canadian Perinatal Investigators Meeting, **Toronto, ON,** 2012**.**

Ma N, Wong, M, Nicholson CJ, Holloway AC, and Hardy DB. Maternal Nicotine Exposure Leads to Elevated Triglycerides in Adult Rat Offspring: The Role of the Liver X Receptor (LXR)**. Poster presentation** at Society for Gynecological Investigation 60th Annual Scientific Meeting, **Orlando, FL**, 2012.

Revesz A, Sohi G, Vo T, Ma N and Hardy DB. Elevated Hepatic miR-29 Expression in Male Growth Restricted Rats is Inversely Correlated with its Target Insulin-like Growth Factor 1 (IGF-1) Long-term. **Poster presentation** at Society for Gynecological Investigation 60th Annual Scientific Meeting, **Orlando, FL,** 2012

Ma N, Wong M, Nicholson CJ, Holloway AC, and Hardy DB. Molecular Mechanisms Underlying Maternal Nicotine Exposure- Induced Elevated Triglycerides in Adult Rat Offspring. **Poster presentation** at the Developmental Origins of Metabolic Disease Symposium 2012, **Ann Arbor, MI**, 2012.

Ma N, Holloway AC, and Hardy DB. The molecular mechanisms underlying maternal nicotine exposure-induced elevated triglycerides in adult rat offspring: role of LXRα. **Oral presentation** at Paul Harding Research Awards Day, **London, ON**, 2012

Ma N, Holloway AC, and Hardy DB. The molecular mechanisms underlying nicotine replacement therapy (NRT)-induced elevated triglycerides in postpartum life. **Poster presentation** at the London Health Research Day, **London, ON** 2012.

Ma N, Revesz A, Vo P and Hardy DB. Maternal protein restriction during pregnancy permanently augments hepatic cyclin D1 associated with premature senescence in rats. **Poster presentation** at Southern Ontario Reproductive Biology Meeting, **London, ON**, 2011.

ACADEMIC AWARDS

2012	**1st Prize Poster Award** at Eastern Canadian Perinatal Investigators Meeting, Toronto, ON
2012	**2nd Prize Poster Award** at Developmental Origins of Metabolic Disease Symposium, Ann Arbor, MI
2012	**1st Prize Oral Presentation** in Graduate Student Category at Paul Harding Research Day, London, ON
2012	**1st Prize Poster Award** in Maternal, Developmental, Fetal, Child & Family Health Category at London Health Research Day, London, ON

FUNDING

2011 – present	Western Graduate Research Scholarship Recipient (Total Award: $4500)
2012	Ontario Graduate Scholarship (Total Award: $15000)
2012 (Total	The Ashley Studentship for Research in Tobacco Control (Total Award: $7200)
2011	Obstetrics & Gynaecology Graduate Scholarship (Total Award: $13700)

WORK EXPERIENCE AND SERVICE TO THE COMMUNITY

Sept 2011 – May 2013 Pharmacology	**Teaching Assistant**, Department of Physiology and University of Western Ontario
Dec 2011 – May 2013	**My Action Plan to Education (M.A.P.) Tutor** The Boys & Girls Club of London, London, ON
Sept 2011 – May 2013 Western	**Let's Talk Science Classroom Leader**, The University of Ontario Chapter, London, ON