Effects of Imidacloprid in the Development of Non-Alcoholic Fatty Liver Disease and the Effects of Exercise Training

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List of abbreviations

4-HNE	4-Hydroxynonenal
ACBP	Acyl-CoA Binding Protein
ACC	Acetyl-CoA carboxylase
ACL	Acyl-CoA Ligase
Akt	Protein Kinase B
ASO	Anti-sense oligonucleotide
CD36	Cluster of differentiation 36
CPT1	Carnitine palmitoyltransferase I
DDT	Dichlorodiphenyltrichloroethane
DGAT	Diacylglycerol acyltransferase
ER	Endoplasmic reticulum
FAS	Fatty acid synthase
FFA	Free fatty acid
GRP78	Glucose-regulated protein 78
HCB	Hexachlorobenzene
HCH	Hexachlorocyclohexane
HFD	High-fat diet
HSL	Hormone sensitive lipase
IRS-1	Insulin-receptor substrate 1
L-FABP	Liver-type fatty acid binding protein
LOEL	Lowest observed effect level
LXR	Liver receptor X
MDA	Malondialdehyde
MTP	Microsomal triglyceride transfer protein
NAFLD	Non-alcoholic fatty liver disease
NASH	Non-alcoholic steatohepatitis
NOAEL	No observed adverse effect level
NOEL	No observed effect level

OXPHOS	Oxidative phosphorylation
PCB	Polychlorinated Biphenyls
PGC-1α	Peroxisome proliferator-activated receptor gamma coactivator 1-alpha
POP	Persistent organic pollutants
PPARα	Peroxisome proliferator-activated receptor
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SCD	Stearoyl-CoA desaturase
SREBP-1c	Sterol regulatory element-binding protein 1c
TNFα	Tumor necrosis factor alpha
UPR	Unfolded protein response
VLDL	Very low density lipoprotein

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Abstract

The non-alcoholic fatty liver disease (NAFLD) is the most common liver pathology in developed countries with an estimated prevalence of 20 to 30% in the American population. A typically benign and asymptomatic pathology, NAFLD is characterized by hepatic steatosis and abnormal levels of hepatic enzymes stemming from an increase in circulating free fatty acids originating from white adipose tissue lipolysis, an increased de novo lipogenesis, reduced fatty acid oxidation and decreased hepatic triglycerides secretion, all within an insulin resistance context. NAFLD has the potential to progress to the non-alcoholic steatohepatitis (NASH), a condition marked by inflammation, advanced oxidative stress and fibrosis. NASH is expected to be the leading cause of liver transplant by 2020 due to its complications (i.e.: cirrhosis, hepatocellular carcinoma and liver failure). Various xenobiotics such as pesticides have been shown to promote the apparition and development of NAFLD. Of interest to this study is the neonicotinoid imidacloprid, more contemporarily known for its suspected role in the colony collapse disorder of various *anthophilae* species. Imidacloprid has been shown to induce hepatic oxidative stress in rats, a significant factor in the development of NAFLD and its progression to NASH. Lifestyle modifications, namely physical exercise, is a current treatment which has been proven beneficial to prevent and treat NAFLD by reducing hepatic steatosis, oxidative stress and improving insulin sensitivity. The role of any neonicotinoid on the development of NAFLD has yet to be examine and few have looked at the role of exercise in the treatment of NAFLD brought about by pesticide contamination.

1. Introduction

1.1 Non-alcoholic fatty liver disease

The non-alcoholic fatty liver disease (NAFLD) is a pathology characterized by abnormal liver function and hepatic steatosis (≥10% of liver total weight) without reported excess alcohol intake [1-3]. NAFLD is viewed as the hepatic manifestation of the metabolic syndrome [1, 2] as it strongly correlates with obesity, hypertension, dyslipidemia (particularly hypertriglyceridemia [4]), insulin resistance and diabetes [1-3]. NAFLD is considered to be the most common liver disorder in developed countries with an estimated prevalence of 20-30% (64 million people) in the United States of America [5]. NAFLD is the result of overnutrition brought about by an energy-dense diet and lack of physical activity [6, 7]. NAFLD is also highly linked to type 2 diabetes as 18 out of 25 million Americans diagnosed with type 2 diabetes are expected to develop it during their lifetime [8].

NAFLD covers a wide histological spectrum of hepatic alterations ranging from simple steatosis to non-alcoholic steatohepatitis (NASH). Whereas NAFLD is typically characterized as asymptomatic, clinically benign and non-progressive, NASH is characterized by mitochondrial dysfunction, hepatocyte injury, inflammation, an immune response and fibrosis which can lead to cirrhosis, liver failure and hepatocellular carcinoma [2, 9-13]. The latter three pathologies account for 14% of liver transplants in the US. NASH is projected to be the leading cause of liver transplant by 2020 [14, 15]

1.2 Imidacloprid

Pesticides have been shown to affect hepatic parameters associated with NAFLD and NASH such as the development of dyslipidemia, oxidative stress and inflammation [16, 17]. Of interest to this study is the pesticide imidacloprid, a neonicotinoid employed for protecting crops by controlling populations of sucking/chewing insects and more contemporarily known for its suspected role in the colony collapse disorder of various bee species [18]. Imidacloprid has been praised since its arrival on the market in 1991 for its high efficiency and specificity in targeting insects-specific receptors, its low persistence in the environment together with its very low bioaccumulative potential as well as low mammalian toxicity [19-21]. Imidacloprid can be applied to crops and structures by means of foliar spray, soil drench or direct soil injection as well as by seed coating [18, 20, 22, 23]. While the foliar spray provides superficial protection, the latter three provide the plant with a systemic protection against insects as imidacloprid is absorbed through its roots from where it distributes itself through the xylem to its leaves, stems, fruits, flowers and pollen [24].

Neonicotinoids enter the human body through dermal contact, inhalation and ingestion [25]. Imidacloprid is thought to be highly absorbed by intestinal cells with a near 100% efficiency by the use of active transporters [26]. No data exists regarding its absorption through dermal contact, the respiratory mucosa or lung alveoli. Imidacloprid penetrates cell membranes through diffusion due to its lipophilicity, yet remains slightly water-soluble (610mg/L at 20°C) [19]. As such, it poorly permeates through the blood brain barrier [27] yet remains capable of traversing it [28, 29]. Imidacloprid is carried by

the plasma to be metabolized in the liver by cytochromes P450 with a strong preference for CYP3A4 (27.4±1.6%) [30-33]. Imidacloprid has a half-life of 3h and was found to be most concentrated in the liver, kidney, lung and skin after 24h in rats, despite being eliminated at 90% [27]. Hepatic peak concentration of the pesticide came 12h after an acute contamination at a dose of 20mg/kg with an estimated total clearance of 37.87ug/ml/h in Wistar rats [28]. A 20mg/kg/day dose was determined to be the lowest observed effect level (LOEL) while a 10mg/kg/day dose to be the no observed adverse effect level (NOAEL) in Wistar rats [34]. In humans, imidacloprid may be excreted at 70-80% through urine and 20-30% in the feces [35].

Recent studies have hinted at the possibility that imidacloprid may play a role in the development of NAFLD and its progression to NASH by affecting insulin pathways, lipid metabolism and generating oxidative stress [21, 23, 36-39]. No direct association has been established to either pathologies. Many studies have been performed to evaluate the role of imidacloprid on the nervous system (reviewed in [29]), yet very little has been done to evaluate the effects of imidacloprid on the liver, the organ responsible for its metabolism.

1.3 Physical activity

From a metabolic standpoint, NAFLD results from alterations between hepatic lipid accumulation and elimination pathways [40]. The accumulation of hepatic triglycerides is well understood and results from the four following factors [40]:

1) An increase in the influx of fatty acids derived from white adipose tissue lipolysis and originating from the dietary intake
2) An increase in *de novo* lipogenesis
3) A decrease in fatty acid oxidation
4) A decrease in triglyceride secretion through VLDL

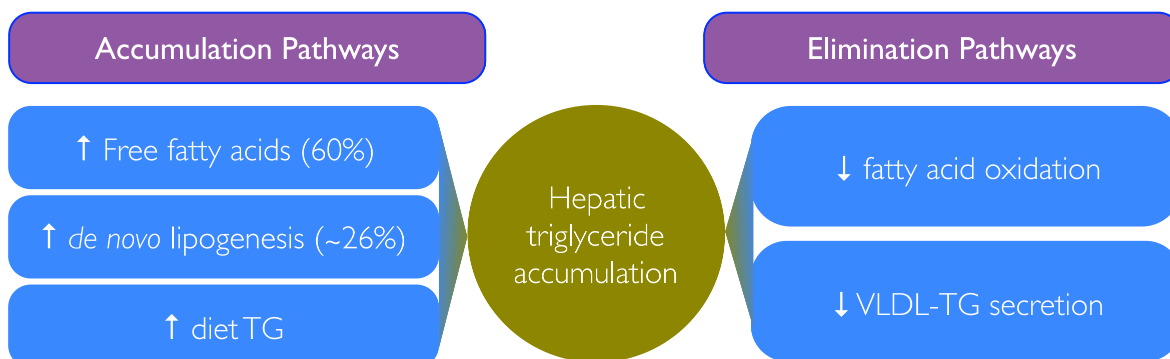

Figure 1: Illustration of hepatic triglyceride accumulation and elimination factors in the development of NAFLD. Fatty acid oxidation relates mainly to β-oxidation although other fatty acid oxidation pathways are present within the liver.

An illustration of the interactions within those metabolic pathways and affiliated proteins of interest to this study is available in Annex 2.

Strategies presently employed to manage NAFLD target extra-hepatic mechanisms to indirectly reduce the ectopic deposition of hepatic triglycerides. No pharmacological treatment has been approved as of 2015 for either NAFLD or NASH [8] and current experimental pharmacological targets (e.g.: insulin sensitizers, antioxidants and lipid lowering drugs) are often accompanied by moderate-to-severe side effects [15, 41]. Lifestyle modifications - namely physical exercise - is the current recommended treat-

ment which has been proven beneficial to prevent and treat NAFLD [6, 41, 42]. Weight loss used to be thought of as the most important factor for NAFLD therapy combined with a negative energy expenditure/intake balance, caloric restriction, dietary macronutrient composition changes as well as physical activity [43]. Recent studies shows physical activity leads to non-significant or benign weight loss while improving insulin sensitivity and hepatic steatosis, with or without diet alterations (reviewed in [44]). Physical activity interferes with the development of hepatic steatosis by stimulating lipid oxidation and inhibiting *de novo* lipogenesis in the liver [45]. Chronic physical exercise can also reduce hepatic oxidative stress by inhibiting pro-inflammatory mediators levels such as TNFα [46, 47]. Patients with NAFLD and NASH report performing significantly less physical activity than their healthy counterparts [48]. Various other studies examining exercise as well as the interactions between exercise*diet have reported net ameliorations in abdominal obesity, visceral fat, waist circumference, glucose metabolism, insulin resistance, steatosis, hepatocyte ballooning, inflammation and fibrosis but conflicting data in serum aminotransferases (reviewed in [6, 15, 48]).

1.4 Hepatic triglyceride accumulation pathways

1.4.1 Influx of free fatty acids

The influx of free fatty acids (FFA) to the liver derives primarily from the lipolysis of white adipose tissue (59.0%±9.9%) and to a lesser extent from absorbed particles through the small intestine (14.9%±7.0%) [40]. The enzyme responsible for white adipose tissue lipolysis is the rate-limiting hormone sensitive lipase (HSL). When activated through phosphorylation, HSL hydrolyzes acylglycerols to form FFA. HSL is strongly in-

hibited by insulin during the postprandial period but becomes hyperactive in an insulin resistance context, thus increasing lipolysis [49, 50]. FFA concentrations in the plasma increase as a result. FFA are highly toxic molecules to biological systems and as such are bound to a transport protein in plasma (albumin) or in the cell (fatty acid binding protein (FABP) and acyl-CoA-binding protein (ACBP) [51, 52]. Cluster of differentiation 36 (CD36) and liver-type fatty acid-binding protein (L-FABP) are two hepatic receptors which enable the entry of plasma FFA into the liver. The microsomal ribonucleic acid (mRNA) expression of both transporters is increased by 2.6-fold and ~3-fold respectively in patients with NAFLD and is correlated with triglyceride accumulation [53, 54]. Upon cellular entry, FFA are converted to fatty acyl-CoA and bound to transport proteins for intracellular transport [51]. From there they are oriented to either the mitochondria for oxidation or the endoplasmic reticulum (ER) to be synthesized into triglycerides and secreted or for immediate oxidation.

1.4.2 *De novo* lipogenesis

1.4.2.1 Sterol regulatory binding protein 1C

The sterol regulatory binding protein 1C (SREBP-1c) is a transcription factor involved in the lipogenic homeostasis of the liver and adipose tissue. SREBP-1c resides in the membranes of the ER as a precursor protein. When activated by a decrease in cholesterol or an increase in insulin, SREBP-1c matures and migrates to the nucleus to regulate the transcription of various lipogenic factors (i.e: acetyl-CoA carboxylase (ACC), acetyl-CoA ligase (ACL), fatty acid synthase (FAS), stearoyl-CoA desaturase (SCD)) and the microsomal triglyceride transport protein (MTP) for triglyceride export

[55-57]. Antisense oligonucleotide (ASO) inhibition of SREBP-1c in mice fed a high-fat diet decreases the expression of ACC, FAS and SCD, reduces hepatic triglyceride accumulation (total plasma triglyceride of 2.82±0.24 mmol/L in HFD mice vs. 0.98±0.12 mmol/L in ASO-HFD mice) and reverses steatosis [58]. SREBP-1c mRNA expression is increased by ~2.5-fold in rats fed a high-fat diet [59]. A ~2- to 5-fold increase was noted for SREBP-1c mRNA in patients with NAFLD and positively correlated to insulin receptor substrate-1 (IRS-1) expression [60, 61]. Enhanced activity of SREBP-1c is also associated with insulin resistance and ER stress [62, 63].

The transcription of SREBP-1c is enhanced by the liver X receptor (LXR), a nuclear hormone ligand-activated receptor highly expressed in the liver, through a response element in its promoter [64-66]. LXR is activated by various oxysterols as well as by insulin [64, 67]. The LXR subfamilies comprise LXRα (expressed exclusively in the liver, adipose tissue, small intestine and macrophages) and LXRβ (expressed ubiquitously). Knockout studies suggest LXRα to hold a more prominent role than LXRβ [67]. Due to its association with SREBP-1c, LXRα activation is associated with triglyceride accumulation, *de novo* lipogenesis and subsequent very low density lipoproteins (VLDL) clearance [66].

1.4.2.2 Acetyl-CoA carboxylase

De novo lipogenesis enables hepatocytes to synthesize fatty acids and triglycerides from the citrate produced by the Krebs cycle. The acetyl-CoA carboxylase (ACC) is a protein responsible for the synthesis of the fatty acid precursor malonyl-CoA and is

tightly regulated by SREBP-1c [60]. Due to the inhibitory role of this precursor, ACC has the capability through its activation/deactivation of selecting for either the β-oxidation or *de novo* lipogenesis pathways. There exist two isoforms of ACC in rodents and humans: ACC1 and ACC2. ACC1 is highly expressed in the cytosol of hepatocytes and adipose tissue whereas ACC2 is predominantly expressed in the mitochondria of heart and muscle tissue, and to a lesser extent in the liver [68, 69]. ACC1 is believed to have a greater role in regulation of fatty acid synthesis and β-oxidation due to its role in the production of malonyl-CoA whereas ACC2 is solely involved in regulating mitochondrial β-oxidation due to its closer proximity to this organelle [69, 70]. The importance of ACC1 is highlighted by its embryonically lethal knockout phenotype, unlike an ACC2 knockout which leads to an 80-90% decrease in hepatocyte triglycerides - possibly due to an increase in β-oxidation [68, 70]. Insulin can activate both ACC through reversible dephosphorylation to enable fatty acid synthesis in the post-prandial state [68]. Inversely, prolonged food withdrawal, high-fat diets or insulin resistance decreases ACC activity through phosphorylation [68, 71]. ACC inhibition is thought to be more metabolically effective by enabling β-oxidation rather than by inhibiting lipogenesis [69] as ACC2-knockout mice are resistant to obesity [68, 70]. Decreasing ACC1 and ACC2 increases fatty acid oxidation in the fed state [69]. Biopsies from patients with NAFLD show an increase of ACC1 mRNA expression of ~2-fold and a 50% decrease in CPT1 mRNA expression. The expression of ACC2 was not affected [60]. Inhibiting ACC1 and ACC2 with a single ASO leads to a reversal of hepatic steatosis and hepatic insulin resistance in high-fat fed rats [69].

1.4.2.3 Diglyceride acyltransferase

The diglyceride acyltransferase (DGAT) is the final enzyme catalyzing triglyceride formation and is present in two isoforms: DGAT1 and DGAT2. DGAT1 is located inside the lumen of the ER and promotes triglyceride formation for VLDL secretion, whereas DGAT2 is located on the surface of the ER, oriented towards the cytosol and promotes intracellular triglyceride synthesis [72, 73]. DGAT1 is mainly expressed in the small intestine [74] while DGAT2 is mainly expressed in the liver. Both are expressed in white adipose tissue [75, 76]. DGAT activity is mainly dictated by the availability of substrate. The synthesis of triglycerides from fatty acids protects cells against fatty acid lipotoxicity in the short-term [52, 77, 78]. Impaired triglyceride synthesis becomes problematic when coupled with peripheral insulin resistance as the liver becomes flooded with FFA stemming from white adipose tissue, oversaturating fatty acid disposal pathways and exacerbating lipoxicity [78]. An important purpose of DGAT2 then becomes the protection of the liver from this lipotoxicity [78]. A DGAT2-knockout produces an embryonically lethal phenotype in mice as DGAT1 is incapable of compensating for its absence [76, 79-81]. ASO treatment in Sprague-Dawley rats for both DGAT confirms their respective potency: an inhibition of hepatic DGAT2 greatly lessens hepatic triglyceride levels (-50%) whereas inhibition of hepatic DGAT1 does not. However, DGAT1-deficient mice fed a high-fat diet displayed an improved insulin sensitivity [82] whereas DGAT2 ASO mice under the same diet did not [76]. Contrary to those findings, another study examining DGAT2 ASO inhibition in Sprague-Dawley rats after a 4 week treatment saw DGAT2 ASO groups improvements in both hepatic and systemic insulin resistance, but not

DGAT1 [80]. DGAT2 ASO reduction in mice and rats fed a high-fat diet have a lower triglyceride content, lower plasma triglyceride levels and improved steatosis. This reduction was also accompanied by an mRNA expression reduction of multiple lipogenic genes for both short- and long-term (i.e: SREBP-1c, SCD1, FAS & ACC) [76, 80]. Therefore DGAT2 is considered more potent than DGAT1 in promoting hepatic steatosis. The overexpression of DGAT in the liver leads to steatosis without an increase in plasma triglyceride or the development of insulin resistance and inflammation. This lack of insulin resistance in particular suggests that insulin resistance induces steatosis rather than steatosis inducing insulin resistance in mice [81]. This dissociation between insulin resistance and steatosis has been reported in humans through a DGAT2 polymorphism where a lifestyle intervention did not always result in a beneficial effect on insulin sensitivity [83].

1.5 Imidacloprid and triglycerides accumulation pathways

There is a dearth of studies pertaining to the effects of imidacloprid and other neonicotinoids on triglyceride accumulation pathways. mRNA expression of phosphorylated hepatic ACC was found to be higher in mice administered imidacloprid at 0.06 mg/kg/day and 6 mg/kg/day under standard and high-fat diets with stronger fold changes in the high-fat group (~1.6-fold vs. ~3-fold, respectively) [39]. Light insulin resistance was found in mice under both diets and may explain the higher phosphorylated state of ACC. These data suggest a possible heightened state of production of triglycerides. S. Bhardwaj et al. (2010) studied the effects of chronic imidacloprid oral administration at various doses (0, 5, 10, 20 mg/kg/day) during 90 days in female Wistar rats. Plasma

triglycerides remained unchanged in rats at 20mg/kg/day which led them to suggest that imidacloprid did not play a role in triglyceride metabolism. However, rats contaminated at the highest dose had decreased weight, altered glucose metabolism with some areas of their livers exhibiting mild focal necrosis [34]. Sun et al. (2016) examined the effects of various imidacloprid doses (0, 0.06, 0.6 and 6 mg/kg/day) on the liver and adipose tissue of C57BL/6J mice fed a high-fat diet during twelve weeks. Contaminated mice fed the high-fat diet all gained weight regardless of the dose of imidacloprid, but noted a gradual decrease in total food intake as the pesticide dose increased. Contaminated mice fed the high-fat diet also had higher blood glucose and higher blood insulin at 6 mg/kg/day than the non-contaminated high-fat diet group. Serum triglycerides but not circulating fatty acids were also shown to be elevated in the 6mg/kg/day group for the high-fat diet group [39]. Although data exists pertaining to hepatic and plasma lipid parameters, there is currently no published data on the effects of neonicotinoids on either DGATs. Albeit limited, the current data hints to a possible development of steatosis and insulin resistance in rats contaminated with imidacloprid.

1.6 Physical activity and fatty acid accumulation pathways

Accumulation pathways in NAFLD stem primarily from an increase in circulating fatty acid influx to the liver and an increased *de novo* lipogenesis. Physical activity aims to attenuate those factors by increasing the clearance of circulating fatty acids and reducing *de novo* lipogenesis. Data pertaining to protein variations in animals and patients performing physical activity is sparse as current studies focus strongly on the clinical treatment (e.g.: weight loss, reduction in steatosis, increased insulin sensitivity) of the

pathology rather than the metabolic pathways underlying it. Reduction of SREBP-1c in Swiss mice trained 50min, 5x/week during 8 weeks and fed a high-fat diet reduced hepatic triglyceride content by 2.2-times as opposed to the sedentary high-fat dieted mice. The same study reported increased hepatic ACC phosphorylation and CPT1 expression in the trained groups versus the mice on a high-fat diet (3.0-fold and 2.9-fold) [84]. Physical activity activates AMPK which inhibits ACC and therefore triglyceride synthesis. This inhibition of ACC also reduces malonyl-CoA concentrations and thus the availability of substrate available for *de novo* lipogenesis [45]. Improving hepatic steatosis reduces insulin resistance, circulating triglycerides and reduces *de novo* lipogenesis [45, 76, 85]. Current studies hereby support the idea that physical activity can reduce the effects of fatty acid accumulation pathways. No studies have examined the influence of physical activity on either hepatic DGAT.

1.7 Hepatic triglyceride elimination pathways

1.7.1 Oxidation

1.7.1.1 Peroxisome proliferator-activated receptor gamma coactivator 1-alpha

The increased flux of FFA emanating from white adipose tissue and increased *de novo* lipogenesis in the presence of hepatic steatosis places a burden on mitochondria which adapt to maintain lipid homeostasis. The peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) is the most important regulator of mitochondrial biogenesis as its activation enhances fatty acid oxidation and it coordinates numerous nuclear transcription factors for related genes (e.g: CPT1). PGC-1α is induced by fatty acids when they become the main energetic substrate such as during periods of

fasting or in a state of hyperglycemia [86, 87]. Paradoxically, the transcription of PGC-1α can be chronically inhibited in an insulin resistance state via IRS-1 and IRS-2 [87]. The effects of this potential reduction has been observed in diabetic patients where OXPHOS-mediated genes by PGC-1α are found to be down-regulated [88]. Accordingly, high plasma insulin levels decrease mitochondrial β-oxidation and aggravate steatosis [43]. Polymorphisms decreasing the function of PGC-1α are associated with lower lipid oxidation and an increased risk of diabetes mellitus [50]. PGC-1α-knockout mice are viable and develop both obesity and hepatic steatosis upon fasting as a result of the combined reduction of mitochondrial respiratory capacity and increased expression of lipogenic genes [89].

1.7.1.2 Peroxisome proliferator-activated receptor alpha

PGC-1α has the ability to co-activate the peroxisome proliferator-activated receptor alpha (PPARα) [90, 91]. PPARα is a nuclear hormone receptor which influences the transcription of genes modulating triglyceride transport, clearance and fatty acid metabolism through three oxidative pathways: mitochondrial, peroxisomal and microsomal [92-96]. PPARα is found in tissues involved in fatty acid metabolism: the liver, kidney, heart and skeletal muscle [94]. Fatty acid accumulation activates transcription of PPARα with unsaturated and very-long-chain fatty acids having the strongest effect [51, 80, 94, 97]. PPARα can also be activated by 4-HNE, a marker of lipid peroxidation commonly found in later stages of NAFLD [98]. PPARα mRNA expression follows PGC-1α by a 5-fold increase in mRNA expression [99]. PPARα is up-regulated by a high-fat diet [59], but unexpectedly reduced in NAFLD - by up to 50% -and in NASH. This decrease has

been associated to the progressive development of insulin resistance and is indicative of impaired β-oxidation. Such a condition may further aggravate steatosis [60, 100]. Hepatic fatty acid oxidation is increased in obese mice and in patients in NASH [50]. PPARα is also essential in the response to prolonged fasting [87, 101]. PPARα-knockout in C57BL/6J mice have severely impaired fatty acids oxidation after a 48-72h fast due to an impairment in the induction of mitochondrial β-oxidation which lead to the apparition of a sudden and severe steatosis [93]. This finding was confirmed by multiple studies (reviewed in [102]). PPARα protects mice from developing NASH when short-term fed a high-fat diet while PPARα-knockouts develop NASH following the enhanced expression of fatty acid metabolizing cytochromes, an increase in TNFα levels and the presence of lipid peroxidation generating oxidative stress as liquid droplets accumulate in hepatocytes [103]. PPARα binds peroxisome proliferators and is thought to mediate the peroxisome proliferation response which can assist lipid homeostasis by increasing sites for fatty acids oxidation outside of the mitochondria [104-106].

1.7.2 Very low density lipoprotein production

1.7.2.1 Microsomal triglyceride transfer protein

The transfer of triglycerides to VLDL requires the rate-limiting enzyme microsomal triglyceride transfer protein (MTP). MTP is an heterodimer located in the ER of hepatocytes critical in enabling the transfer of triglycerides to apoB100 to form VLDL [73, 107]. MTP-knockout in mice leads to an embryonically lethal phenotype [107]. By using Cre/loxP technology, Raabe et al. (1999) discovered MTP-knockout mice suffered a severe decrease in VLDL secretion and developed moderate steatosis even when fed a

low-fat diet [107]. Further research on pharmaceutical agents confirmed those findings [108, 109]. Those observations concluded that reduced MTP alone could induce steatosis and strongly influence circulating triglyceride levels [110]. MTP gene transcription is mediated by high-fat diets, PPARα (activation), SREBPs (inactivation; HepG2 cell cultures), insulin (inactivation) [45, 56, 111] and possibly LXRα (inactivation) [66]. Increased levels of SREBP-1c in ob/ob mice leads to a 45% increase in hepatic MTP mRNA expression, 54% higher MTP activity and 70% higher triglyceride secretion rates as compared to their control counterparts while maintaining similar plasma triglyceride levels with the authors inferring a possible higher systemic clearance of triglycerides for this latter observation [112]. The use of a PPARα agonist in cultured hepatocytes, mice and rats is also associated with increased MTP mRNA (rats: ~0.35 $2^{-\Delta Ct}$ vs ~0.6 $2^{-\Delta Ct}$), protein expression (rats: ~2.5-fold) and protein activity (rats: ~10 pmol/min/mg protein vs ~27 pmol/min/mg protein) [95]. MTP polymorphisms have been shown to have the ability to affect the proper integration of triglycerides into VLDL [113]. It has been hypothesized that the fat-laden liver attempts to increase triglycerides clearance through VLDL to maintain homeostasis. The secretion rate of triglycerides incorporated in VLDL in humans with NAFLD is twice the amount as reported in healthy individuals and reaches a plateau when hepatic steatosis attains ≥10%. VLDL have been reported to be not overproduced, but rather larger in size due to an increase in triglycerides presence [114]. MTP mRNA expression is increased 2-fold in patients with NAFLD but is found to be reduced by ~2-fold in patients with insulin resistance (>2.5 HOMA-IR) and hepatic triglycerides content (>30% triglyceride-laden hepatocytes), demonstrating the inhibiting effects of insulin in an insulin resistance context [54]. Systemic plasma FFA account for

66%±4% and 43%±3% of fatty acids found in VLDL of healthy and NAFLD patients respectively whereas non-systemic FFA account for 34%±4% and 57%±3% [114]. Thus, the increase in VLDL secretion in the NAFLD group is suspected to be caused by an increased contribution of fatty acids derived from the lipolysis of intrahepatic triglycerides, visceral adipose tissue and *de novo* lipogenesis, possibly due to an increase in HSL activity caused by insulin resistance [114].

1.8 Imidacloprid and triglyceride elimination pathways

Very few studies pertaining to triglyceride elimination pathways in regards to imidacloprid and neonicotinoids exist. The reduced activation of Akt following imidacloprid treatments on cell cultures hints at the possibility that imidacloprid may induce insulin resistance [38]. *In vivo* experiments are required to corroborate those findings. Exposure to persistent pesticides (various polychlorinated biphenyls (PCB), dichlorodiphenyltrichloroethane (DDT), hexachlorobenzene (HCB) and hexachlorocyclohexane (HCH) have been shown to reduce PGC-1α [115]. However, the highly lipophilic nature of these pesticides enables their retention by hepatic lipidic macrovacuoles which increases their hepatic concentrations and their toxic potential following their release as triglycerides from those macrovacuoles are metabolized [43]. More pertinent to this review is that imidacloprid can lower PGC-1α (CHOW: ~0.6-fold; HFD: ~0.4-fold) and PPARα expression (CHOW: ~0.6-fold; HFD: ~0.75-fold) in mice chronically administered 0.6 and 6mg/kg/day respectively for 12 weeks under both a standard and high-fat diet [39]. No studies examining the effects of imidacloprid and neonicotinoids have been performed in regards to MTP. The current lack of data pertaining to the effects of imidacloprid and

other neonicotinoids prevents the edification of a coherent hypothesis when predicting its effects on triglyceride elimination pathways.

1.9 Physical activity and elimination pathways

Endurance training has been reported to enhance fatty acid oxidation through the increased release of triglycerides by white adipose tissue, increased FFA clearance through myocyte uptake, increased hepatic lipid oxidation, increased OXPHOS, increased insulin sensitivity and reduced inflammation, all important factors in the prevention and treatment of NAFLD [45, 46, 116]. Hepatocyte triglyceride macro- but not microvesicules were reduced by ~55% in rats with a high-fat diet induced obesity made to run 60min/day, 5x/week for a period of 8 weeks. This reduction was suggested to be mainly the result of 1) a diminished delivery of lipids to the liver, 2) an increased hepatic oxidation of fatty acids and 3) an increased incorporation of triglycerides into VLDL. The researchers also noted a tendency to a decrease in circulating FFA, possibly due to the liver acting as a buffer [117]. Another study in which voluntarily trained (17 weeks) and treadmill-trained rats (60min/day, 5x/week, 8 weeks) fed a high-fat diet confirmed this reduction in hepatic triglycerides and VLDL. This reduction in VLDL was not observed in trained rats fed a standard diet [118]. In individuals living with obesity, 150 to 300 minute of moderate exercise training per week resulted in a 10.3±4.6% decrease in hepatic triglyceride content without a decrease in body weight, percent body fat or hepatic VLDL secretion rate. This decrease has been suggested to be caused by an increase in fatty acid oxidation. Individuals with higher hepatic triglyceride content benefit more from moderate exercise training than healthy individuals [119]. Hepatic CPT1 mRNA expres-

sion is increased in ob/ob mice trained 60min/day, 5x/week for 8 weeks, indicating increased mitochondrial oxidative capacity. Triglyceride secretion in VLDL did not appear to be affected by physical exercise in this same study [120]. A study in patients with NAFLD confirmed VLDL production rate to remain stable but for their clearance to be increased when made to perform aerobic exercise 60min/day, 4-5x/week for 16 weeks at 40-60% VO_{2max}, after which steatosis was reduced by 50% [121]. Exercise training can hence reduce steatosis by 1) reducing the hepatic delivery of FFA produced by the adipose tissue; 2) increasing the hepatic oxidative capacity of hepatocytes and 3) improving clearance of hepatic triglycerides incorporated in VLDL.

1.10 Hepatic markers of cellular stress

1.10.1 Binding immunoglobulin protein GRP78

Misfolded proteins tend to be produced in the ER when oxidative stress is present and aggravate cellular stress by forming toxic protein aggregates. The production and accumulation of misfolded proteins can trigger the unfolded protein response (UPR) in the ER. The UPR has for purpose to restore proper cell function by halting protein translation, enhancing the degradation of misfolded proteins and activating pathways responsible for the production of chaperone proteins. The binding immunoglobulin protein GRP78 is one such chaperone protein occupying the outer membrane and lumen of the ER [122, 123]. When sufficient levels of unfolded proteins accumulate in the endoplasmic reticulum, resident GRP78 sites become occupied and releases other transmembrane ER proteins to the nucleus to induce the expression of UPR-related genes [124, 125]. GRP78 is also sensitive to alterations in calcium concentrations such

as one which can occur in the loss of ER or mitochondrial permeability as observed in NASH [122, 124]. Failure of the UPR to restore normal cell function leads to the activation of transcription factors initiating host defence pathways and apoptosis via caspases [124, 126]. GRP78 has been studied in the context of steatosis in ob/ob mice where overexpression of GRP78 inhibits the cleavage required for the maturation of SREBP-1c thus preventing the transcription of its lipogenic target genes [127, 128]. As expected, its overexpression led to a reduction in hepatic triglycerides and improved insulin sensitivity [127]. However, the context in which GRP78 is activated needs to be taken into account. Upon ER stress induction, SREBP-1c migrates and matures from the endoplasmic reticulum to the nucleus where it transcribes various lipogenic genes involved in *de novo* lipogenesis [129]. Increased palmitate and stearate saturated fatty acid content induces stress in the ER of hepatocytes but not unsaturated (e.g.: oleic & linoleic) fatty acids which inhibited such lipotoxicity in cultured hepatocytes [130]. Another study found that unsaturated (oleic) fatty acids to be able to induce ER stress as determined by higher GRP78 content [123]. Therefore, there appears to be a cycle where ER stress causes a heightened production in fatty acids which in turn generates greater ER stress. Another important factor to consider is the induction of hepatic insulin resistance by ER stress, which has been proposed as a contributor to the development of steatosis and diabetes [123, 131]. ER stress leads to insulin resistance which up-regulates lipogenesis [125]. No pertinent research has been performed in regards to the effects of either imidacloprid or prolonged physical exercise on GRP78 in hepatic cells.

1.10.2 Tumor protein p53

The tumor protein p53 is the first tumour-suppressing gene to have been identified. p53 is localized in low quantities in both the nucleus and cytoplasm and acts as an antioxidant to protect cells by preventing their division while under low levels of stress through the transcription of factors regulating oxidative stress and encouraging DNA repair [132-134]. A sufficient concentration of p53 induces cell death by inhibiting a variety of antiapoptotic genes and encouraging the transcription of proapoptotic genes when oxidative stress overcomes the repair capabilities of the cell [98]. The level of oxidative stress-induced damage dictates whether p53 acts in favour or against cellular integrity [133]. p53 is normally latent only to be activated by oxidative stress, DNA damage, and hypoxia - all factors present in late-stage NAFLD [135]. Oxidative stress markers have the potential to activate, phosphorylate and increase p53 nuclear translocation [136]. p53 has been shown to be present in higher quantities in ob/ob mice and to be implicated in hepatocellular injury [132]. Partial inhibition of the transcriptional activity of p53 diminished hepatic steatosis in mice fed a high-fat diet [137]. A more recent study has shown that p53 inhibition upregulates the PGC1α/PPARα catabolic pathway, leading to a decrease in malonyl-CoA, improved CPT1 activity and improved mitochondrial oxidation of long-chain fatty acids [137]. No studies have examined the role of p53 in imidacloprid metabolism.

1.10.3 Tumor necrosis factor α

In the context of NAFLD, the tumor necrosis factor α (TNFα) is an inflammatory mediator directly secreted by fat-laden hepatocytes, infiltrated macrophages and Kupffer cells or indirectly by fat-engorged adipose tissue to stimulate lipolysis [50, 138, 139].

TNFα is strongly suspected to be a key cytokine in the progression of NAFLD to NASH due to its association to obesity, insulin resistance and fibrosis. TNFα impairs proper mitochondrial function by promoting the formation of reactive oxygen and nitrogen species (ROS and RNS) [73] with ROS proven to contribute in increasing the synthesis of TNFα in patients with NASH [140]. Consequently, TNFα has been shown to be increased in patients with NASH with those levels correlating positively with the severity of inflammation and the progression of fibrosis [140, 141]. TNFα can be induced by a high-fat diet [142, 143]. TNFα can also decrease the mRNA expression of CPT1, LXRα, PGC-1α PPARα and SREBP-1c in Hep3B cells and C57BL/6 mice, affecting proper mitochondrial β-oxidation and fatty acid metabolism in general [144, 145]. Mice lacking TNFα receptors (TNF-R) are protected from steatohepatitis, confirming TNFα to be required to develop NASH [146]. One study in humans however did not find any correlations between TNFα levels between patients with either NAFLD or NASH suggesting that while TNFα levels rise during NAFLD, they may not be required to progress it to NASH [147]. The release of TNFα stimulates triglyceride synthesis in rats within the hepatic tissue exclusively and increases plasma triglyceride concentrations [148]. TNFα, along with FFA, interfere with insulin signalling pathways favouring the development of steatosis and insulin resistance [140, 149]. Various treatments inhibiting TNFα were reported to reduce both NAFLD induced and NASH [150]. Imidacloprid contamination has been observed to increase TNFα mRNA in rats following an acute and chronic exposure [23, 36]. The key role of TNFα in the progression of NAFLD to NASH hence serves as a valid marker as to its development.

1.11 Statement of the problem

The high prevalence of NAFLD coupled with the multiple serious co-morbidities associated with its progression have warranted extensive investigation into the pathology. Multiple xenobiotics including pesticides have been linked with the development of NAFLD. No studies have been performed to evaluate the possible role of imidacloprid in NAFLD development despite it being the most commonly and widely used neonicotinoid pesticide on the North American continent in 2008 [22]. Exercise training has been demonstrated to improve various parameters associated with NAFLD, most notably by reducing hepatic steatosis - one of its hallmark features [45, 46, 116]. The present study aims to bridge this gap by examining how imidacloprid affects both hepatic and plasmatic parameters associated with the development of NAFLD and how exercise training may reduce or prevent altogether the apparition of said pathology.

1.12 Study objectives

The objectives of this study are two-fold. The first objective is to investigate the potential link between the administration of a chronic dose of imidacloprid and NAFLD development as no studies have documented this link despite previous research on xenobiotics (i.e.: persistent organic pollutants) linking them to the development of NAFLD [115, 151]. However, imidacloprid behaves differently than those pollutants as it bioaccumulates poorly and is fairly readily eliminated from the organism. The current hypothesized pathway for imidacloprid to induce NAFLD is through the initiation of hepatic inflammation by the formation of ROS/RNS [23] - a crucial step to the progression of insulin resistance and NAFLD [151] - followed by the development of hepatic insulin

resistance which induces steatosis. On that assumption imidacloprid is believed to be capable of initiating the development of NAFLD. This assumption will be investigated via macromolecules/proteins related to the pathways of accumulation and elimination of hepatic triglycerides. Markers of oxidative stress (i.e.: GRP78, p53, TNFα) will also be examined as oxidative stress is a hallmark characteristic of NASH.

The second objective is to examine how an exercise training program may prevent the development of NAFLD in contaminated rats. Current therapies for NAFLD treatment includes endurance training, which has been proved to reduce steatosis and improve insulin resistance [6, 41, 42]. As such, exercise training is hypothesized to be capable of possibly preventing, alleviating, or reversing NAFLD in imidacloprid-contaminated subjects.

2. Protocol and methods

2.1 Rat husbandry

43-days-old Sprague-Dawley rats (n=40; Charles River, QC, Canada) weighing between 181 and 213 grams were paired and acclimated for a week. Rats were maintained under controlled conditions (12:12h light-dark cycle starting at 0600; constant humidity, room temperature at 20-23°C). Rats had access to a standard diet of pelleted food (Harlan 2018 Rodent Diet, Harlan Teklad Laboratory) and water *ad libitum*. Diet consumption was not monitored. Paired rats were divided randomly into four groups: control sedentary (CTRL-SED; 10 rats), imidacloprid sedentary (IMI-SED; 10 rats), control exercise (CTRL-EX; 10 rats) and imidacloprid exercise (IMI-EX; 10 rats). All procedures were approved by the Animal Care Committee of the University of Ottawa (ACC 2739) and adhered to the guidelines established by the Canadian Council on Animal Care.

2.2 Exercise training program

Swimming was chosen as the preferred method of training due to previous compliance issues with treadmill training in prior experiments by our laboratory. Trained animals begun their training session between 0900 and 1200 in 60 x 90 cm plastic tanks filled with 50cm of water so as to prevent rats from standing on their tail and ensure compliance with the exercise training regimen. Water temperature was maintained between 32-35°C and rats were kept under constant supervision. Rats were familiarized with the exercise training program and apparatus prior to starting the protocol, beginning with a 30min swimming period increased by 5min increments over a period of five

days at the end of which rats became trained 60min/day, 5x/week for a period of six weeks. All rats underwent a total of 30 swimming sessions during this period. Rats were placed in incubators set at 26°C to dry after completing a swimming session. The rats assigned to the untrained group were manipulated daily. Basins were thoroughly washed and decontaminated at the end of each session with 70% ethanol.

2.3 Pesticide administration

Imidacloprid (1-((6-chloro-3-pyridinyl)methyl)-N-nitro-2-imidazolidinimine; Sigma, 37894) was diluted in corn oil (0.14mg/kg, President's Choice 100% pure corn oil). Due to its very low solubility in the medium, the solution was let to vortex in a bain-marie set at 50°C overnight. Each dose was adjusted to accommodate any changes in body weight by adjusting the gavage volume. Administration was concurrent with the exercise training program. The solution was thoroughly vortexed throughout the gavage. Imidacloprid was administered during weekdays at a dose of 10mg/kg/day via gastric probe. Control rats were administered corn oil (0.14 ml/kg; President's Choice 100% pure) at an isotropic volume. Gavage was performed after training in an attempt to increase compliance with the treatment. Rats were given Cheerios (Honey Nut Cheerios, General Mills) after gavage to further increase compliance. No behavioural changes were noted in contaminated rats at any time.

2.4 Tissue sampling

Exercise training and imidacloprid administration were ceased 48-72h prior to the sacrifice to avoid acute exercise effects. Rats were anesthetized using isoflurane. Ocu-

lar, tail and paw reflexes were verified to confirm proper anesthetization prior to tissue collection. An abdominal medial incision was performed to expose the abdominal cavity. Blood was first collected from the inferior *vena cava,* transferred to an EDTA collection tube and centrifuged at 4000rpm for 7min (Thermo Scientific, Cat#75004381) to isolate the plasma. The plasma was placed in eppendorfs and flash frozen in liquid nitrogen. The liver was then excised and its median lobe isolated. Solid tissues were placed in marked aluminium foil and flash frozen in liquid nitrogen at -196°C. All samples were placed on dry ice for transport and stored at -80°C upon arrival until further analysis.

2.5 Western Blotting

200mg of each liver was collected and placed in 1ml of lysis buffer (Annex 1) and homogenized by a TissueLyser II (Qiagen, 85300) at 30Hz for 3min. Samples were then centrifuged at 14000 RPM for 20min at 4°C. The infranatant was collected and stored at -80°C. Protein dosage was completed spectrophotometrically (Biotek Synergy HT, Biotek) by Bradford with DC™ Protein Assay (DC™ Protein Assay Reagent S, Cat#500-0115; DC™ Protein Assay Reagent A, Cat#500-0113; DC™ Protein Assay Reagent B, Cat#500-0114) with bovine serum albumin (BSA) (Sigma, A7906-100G) as the standard. All samples were run in duplicates. Protein concentration was set at 40μg of protein per sample. Laemmli buffer 4x was added to samples (Annex 1), vortexed 10s, heated for 5min at 95°C on a dry heater (Thermo Scientific, Cat#2001), vortexed 10s again and cooled on ice for 5min.

SDS-PAGE electrophoresis was employed for protein separation (Biorad, Cat#1645052 and Cat#1658001) using a trisaminomethane-based buffer. The stacking gel was set at 4% for all gels while the resolving segment varied according to the protein of interest (7 to 15%). Migration was done at 85V until reaching the resolving segment when it was increased to 125V until completion. Gels were soaked in transfer buffer 1x (Annex 1) during 15min prior to their placement in the transfer apparatus (Biorad, Cat#1703935EDU). Proteins were transferred onto 0.45μm PVDF membranes (Millipore, Cat#IPVH00010). Transfer parameters were 100V for 90min. A cooling unit, cold transfer buffer (4°C) and the placement of the transfer apparatuses on ice were used to maintain a low buffer temperature during the transfer. PVDF membranes were left to air dry after completion for protein transfer to be confirmed visually. PVDF membranes were briefly rehydrated in methanol and rinsed in TBS-T 0.1% 5min before blocking in a 5% solution of milk (Carnation, Fat Free Instant Skim Milk Powder) in TBS-T 0.1% for 45min at room temperature on an agitating plate (Thermo Scientific, Cat#2314). Membranes were washed thrice 5min in TBS-T 0.1% before incubation with primary antibodies. Primary antibodies (ACCα, NB100-92011; DGAT1, NB110-41487; DGAT2, NBP1-71701H; GRP78, NB300-520; LXR, NB400-157; MTP, NBP1-62489; p53, NB200-103H; PGC-1α, NBP1-04676H and; SREBP-1c, NBP100-2215; Novus Biological, Littleton, CO) were added at the dilution specified by the manufacturer to a solution of BSA 5% in TBS-T 0.1%. A housekeeping gene of a different molecular mass than the antibody of interest was added to the solution to establish a ratio between bands. Housekeeping proteins included α-tubulin (Novus Biological, NB100-690H), β-actin (Sigma, A3854-200UL) and GAPDH (Novus Biological NB300-221). Membranes were

incubated at 4°C overnight, isolated from light and on an agitating plate. Upon incubation, membranes were washed thrice five minutes in TBS-T 0.1% before the addition of the proper secondary antibody (Novus Biological: Goat Anti-Rabbit IgG NB7160; Cell Signalling: Anti-mouse IgG, #7076S) in powdered milk 5% in TBS-T 0.1% for 45min at room temperature on an agitating plate. Membranes were washed thrice in TBS-T 0.1%, once ten minutes in TBS 1x and incubated in Clarity™ Western ECL Substrate (Bio-Rad, #1705060) for 10min. Revelation was performed in a dark chamber (UVP, Chemi-DocIt^2 810 Imager). Bands were isolated for analysis by VisionWorks®LS Analysis Software (v.8.15.16057.9028) and the data exported to excel for further analysis.

2.6 Triglycerides quantification

Triglycerides were extracted from the liver and plasma individually from each rat. 50mg of liver was added to 500μl of KOH 0.5M (Fisher, P2501) in a 95% ethanol solution and homogenized with a TissueLyser II (Qiagen, 85300). Eppendorfs were transferred onto a dry heater (Thermo Scientific, Cat#2001) at 70°C for 10min, vortexed, and placed another 10min onto the dry heater. 1ml of $MgSO_4$ 0.15M (Fischer, Cat#10034-99-8) was added and samples were centrifuged at 5000RPM for 7min. The superior liquid phase was recuperated. Hepatic triglycerides were quantified using a free glycerol reagent kit according to the manufacturer's protocol (Sigma, F6428). Plasma samples were assayed directly without any prior manipulations using a serum triglycerides determination kit (Sigma, TR0100) and the accompanying protocol of the manufacturer. A glycerol solution was used as the standard (Sigma, G7793) in both hepatic and plasma triglycerides quantification. All manipulations were performed in 96-

well clear flat bottom plates (Sigma, P7366). Spectrometric data was exported to excel for further analysis. Three essays were performed for hepatic triglycerides quantification. The first essay was discarded from the dataset as all liver samples were treated simultaneously, exposing earlier prepared samples longer to room temperature while on ice. To our surprise, a second test where liver samples were treated five samples at a time yielded similar results. Results were confirmed in a third test. An average of the two latter tests was employed for the final analysis.

2.7 Free fatty acid quantification

The colorimetric Free Fatty Acid Quantification Kit (Sigma, MAK044) was employed according to the protocol provided by the manufacturer to quantify plasma FFA. A 96-well clear flat bottom plate (Sigma, P7366) was employed. A plasma volume of 25μl was added per well. Absorbance data were exported to Excel after completion.

2.8 Tumor necrosis factor α quantification

The inflammatory marker TNFα was quantified in the serum (plasma) using a colorimetric ELISA kit (Novus Biological, NBP1-92681) as per the manufacturer's instructions. Plates were washed between steps using a microplate strip washer (BioTek, ELx50). Absorbance data were exported to Excel after completion.

2.9 Statistical analysis

Data is presented as means ± SD. Statistical analyzes were performed using SPSS 23.0.0.0 64-bit edition for Mac (IBM Corp, NY, 2015). Outliers below Q_1-1.5*IQR

and above Q_3+1.5*IQR were screened for once and eliminated from datasets with the exception of rat weights. Proteins violating the Levene's test (DGAT1, DGAT2 and GR-P78) were only screened for extreme outliers (Q_1-3.0*IQR and above Q_3+3.0*IQR). The second and third test for hepatic triglycerides quantification were each individually screened for outliers before being averaged and analyzed. All data were analyzed using a two-way analysis of variance and tested for Levene's test for homogeneity of variance and subsequent violations were mentioned. Independent t-tests were performed to identify significant differences between individual groups. A Bonferroni correction was performed to adjust the alpha value when required. Analyzes violating the Levene's test for equality of variance had their significance examined using the Welch's t-test for both contamination and exercise independently to examine the variation in significance for each factor. Differences among the groups were considered significant at $p \lesssim 0.05$.

3. Results

3.1 Hepatic triglyceride accumulation pathways

Steatosis and obesity were absent from rats. Rat weights were found to be significantly reduced in contaminated groups ($F(1,36)=6.301$, $p=0.017$) by 7.8% (Figure 1a) but not in trained animals (Table 1). Further analysis reveals this reduction to lie between the CTRL-SED and the IMI-SED groups ($t(18)=2.888$, $p=0.01$). Calculation of the net body gain (final weight minus initial weight) confirms IMI-SED rats to have only gained 84.30±9.18g. Group analysis confirms the IMI-SED ($M=84.30$, $SD=10.08$) but not IMI-EX to have a lower average weight delta than the CTRL-SED ($M=106.6$, $SD=10.32$) group ($t(18)=3.475$, $p=0.003$).

Weight Component	CTRL-SED	IMI-SED	CTRL-EX	IMI-EX
Pre-test (g)	195.50 ± 8.32	194.20 ± 5.92	193.70.± 6.40	195.10 ± 4.93
Post-test (g)	302.10 ± 20.78	278.50 ± 15.37*	295.40 ± 23.60	286.40 ± 21.49
Net body weight gain (g/ 100g bw)	106.6 ± 10.32	84.30 ± 9.18*	101.70 ± 10.08	91.30 ± 9.56

Table 1: Pre-test, post-test and net body weight gain in control-sedentary (CTRL-SED), imidacloprid-sedentary (IMI-SED), control-exercise (CTRL-EX) and imidacloprid-exercise (IMI-EX) rats. n=40; * = p≤0.05 vs. CTRL-SED)

3.1.1 Influx of free fatty acids

Hepatic triglycerides (Figure 1b) and plasma FFA (Figure 2a) content were not significantly affected by either contamination or training. Plasma triglycerides (Figure

2b) were markedly reduced in trained ($F(1,32)=5.543$, $p=0.025$) but not in contaminated rats, with no significant differences noted between individual groups.

3.1.2 *De novo* lipogenesis

Key proteins associated with *de novo* lipogenesis were quantified. The protein levels of the lipogenic markers LXR and SREBP-1c (Figure 3) remained unaltered for both treatment conditions. DGAT1 (Figure 4), the protein responsible for promoting triglycerides formation for VLDL secretion [72, 73], was increased in contaminated rats ($F(1,36)=35.818$, $p<0.001$). DGAT2, responsible for intracellular triglycerides synthesis [72, 73], was decreased in trained rats ($F(1,36)=5.818$, $p=0.028$) with no differences between individual groups (Figure 4).

3.2 Hepatic triglycerides elimination pathways

3.2.1 Oxidation

PGC-1α is the most important regulator of mitochondrial biogenesis [86, 87]. PGC-1α protein level was found to be significantly higher in contaminated rats ($F(1,28)=3.974$, $p=0.056$) but not trained ($p=0.965$; Figure 5a). Further analysis reveals the difference to lie exclusively between the CTRL-SED ($M=0.0784$, $SD=0.1041$) and IMI-SED ($M=0.0104$, $SD=0.0202$) groups ($t(18)=-3.320$, $p=0.004$).

3.2.2 Very low density lipoprotein production

MTP is the key regulatory protein associated with triglycerides export from the liver. Protein levels for the key hepatic triglycerides export MTP was significantly lower

(F(1,31)=6.444, p=0.016) in contaminated but not trained rats (Figure 5b). No differences were found within groups.

3.3 Cellular stress

The stress marker GRP78 is activated following the accumulation of unfolded protein to trigger the UPR and restore proper cell function. GRP78 was found to be significantly reduced in trained rats ($F(1,36)=16.123$, $p<0.001$) but not contaminated rats (Figure 6a).

p53 initially attempts to protect cells under low oxidative stress but induces cell death when said stress overcomes the repair capabilities of the cell [98]. p53 protein levels were not affected by contamination but were increased by 2-fold in trained rats ($F(1,35)=25.741$, $p<0.001$; Figure 6b). No significant differences were found within any of the individual four groups.

The inflammatory marker TNFα (serum) has been reported to increase as NAFLD progresses towards an inflammatory stage. TNFα was found to be reduced in both contaminated ($F(1,34)=14.225$, $p<0.005$) and trained rats ($F(1,34)=56.755$, $p<0.001$; Figure 7).

Absolute values for the upcoming figures are available in a table in Annex 3.

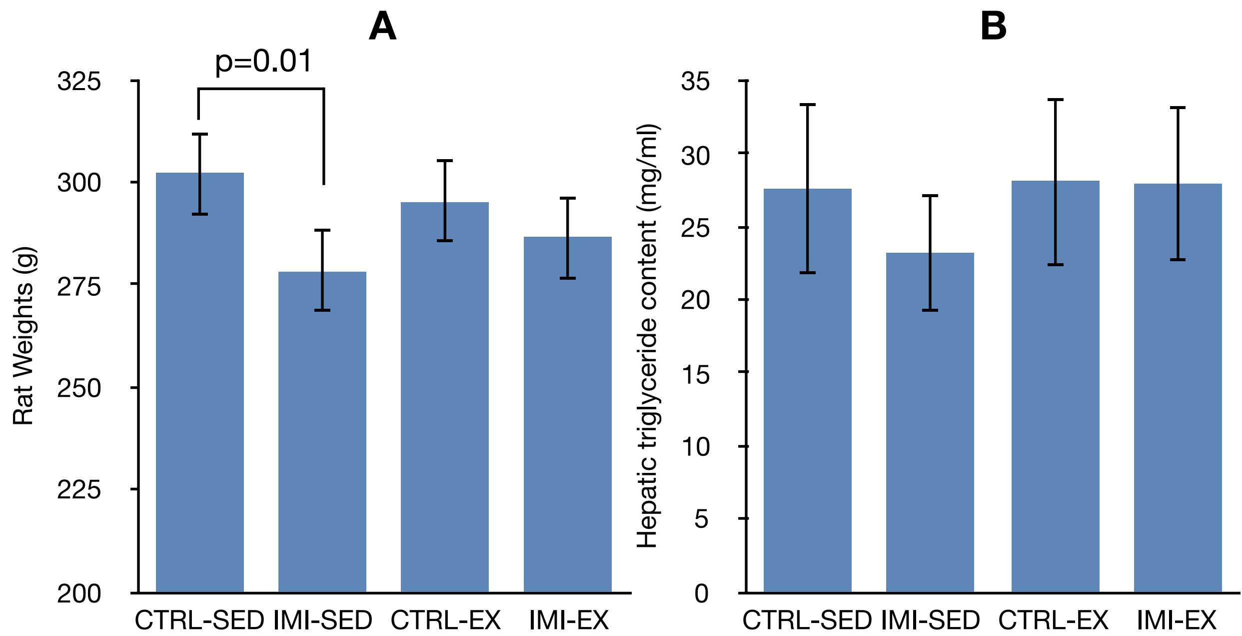

Figure 1: Average **A)** rat weights of control-sedentary (CTRL-SED, n=10), contaminated-sedentary (IMI-SED, n=10), control-trained (CTRL-EX, n=10) and contaminated-trained (IMI-EX; n=10) rats; **B)** hepatic triglycerides content of control-sedentary (CTRL-SED; n=10), contaminated-sedentary (IMI-SED, n=10), control-trained (CTRL-EX, n=10) and contaminated-trained (IMI-EX, n=10) rats after 30 days of exposure to imidacloprid and/or exercise training. A significant decrease in weight was observed in **A** for the IMI-SED group but not the CTRL-EX or IMI-EX group. The data for **B** did not show any significant differences. Values are means ± SD).

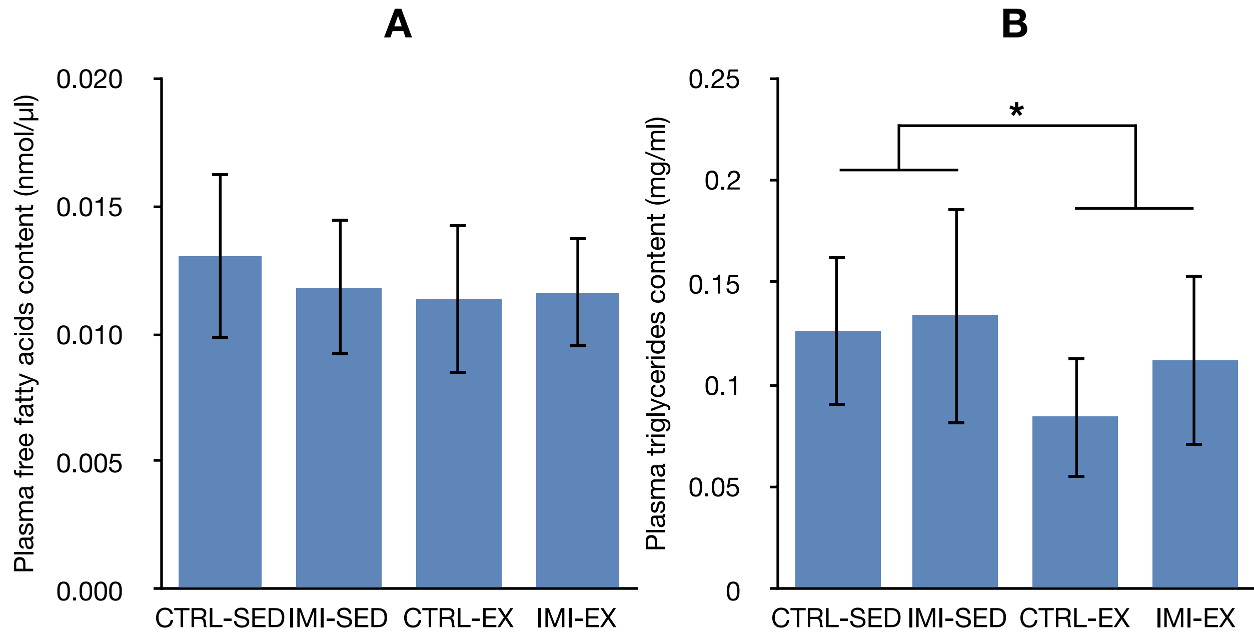

Figure 2: Average **A)** plasma free fatty acids content of control-sedentary (CTRL-SED; n=10), contaminated-sedentary (IMI-SED, n=10), control-trained (CTRL-EX, n=7) and contaminated-trained (IMI-EX, n=10) rats **B)** plasma triglycerides content of control-sedentary (CTRL-SED, n=8), contaminated-sedentary (IMI-SED; n=9), control-trained (CTRL-EX, n=9) and contaminated-trained (IMI-EX, n=10) rats by group after 30 days of exposure to imidacloprid and/or exercise training. No statistical differences between groups were noted in **A**, but a significant decrease in plasma triglycerides was observed for trained rats in **B** ($p<0.05$).

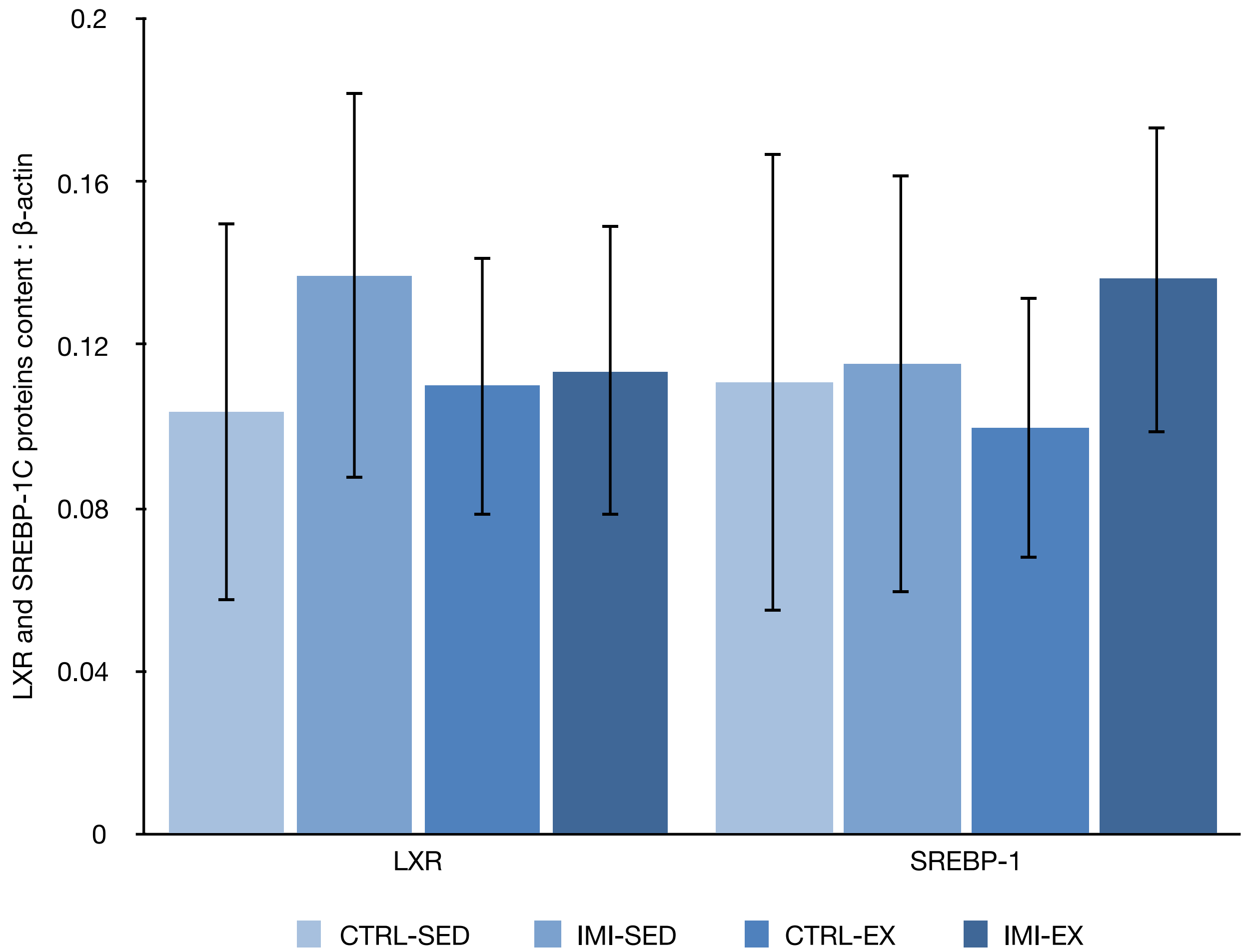

Figure 3: Average LXR and SREBP-1c proteins content of control-sedentary (CTRL-SED, n=10), contaminated-sedentary (IMI-SED, n=10), control-trained (CTRL-EX, n=10) and contaminated-trained (IMI-EX, n=10) rats by group after 30 days of exposure to imidacloprid and/or exercise training. Western blotting analysis shows no significance was reached in any groups. β-actin was employed as the housekeeping reference gene. All data were relativized against their corresponding β-actin band value. Values are means ± SD; * Different from the control group by $p \leq 0.05$.

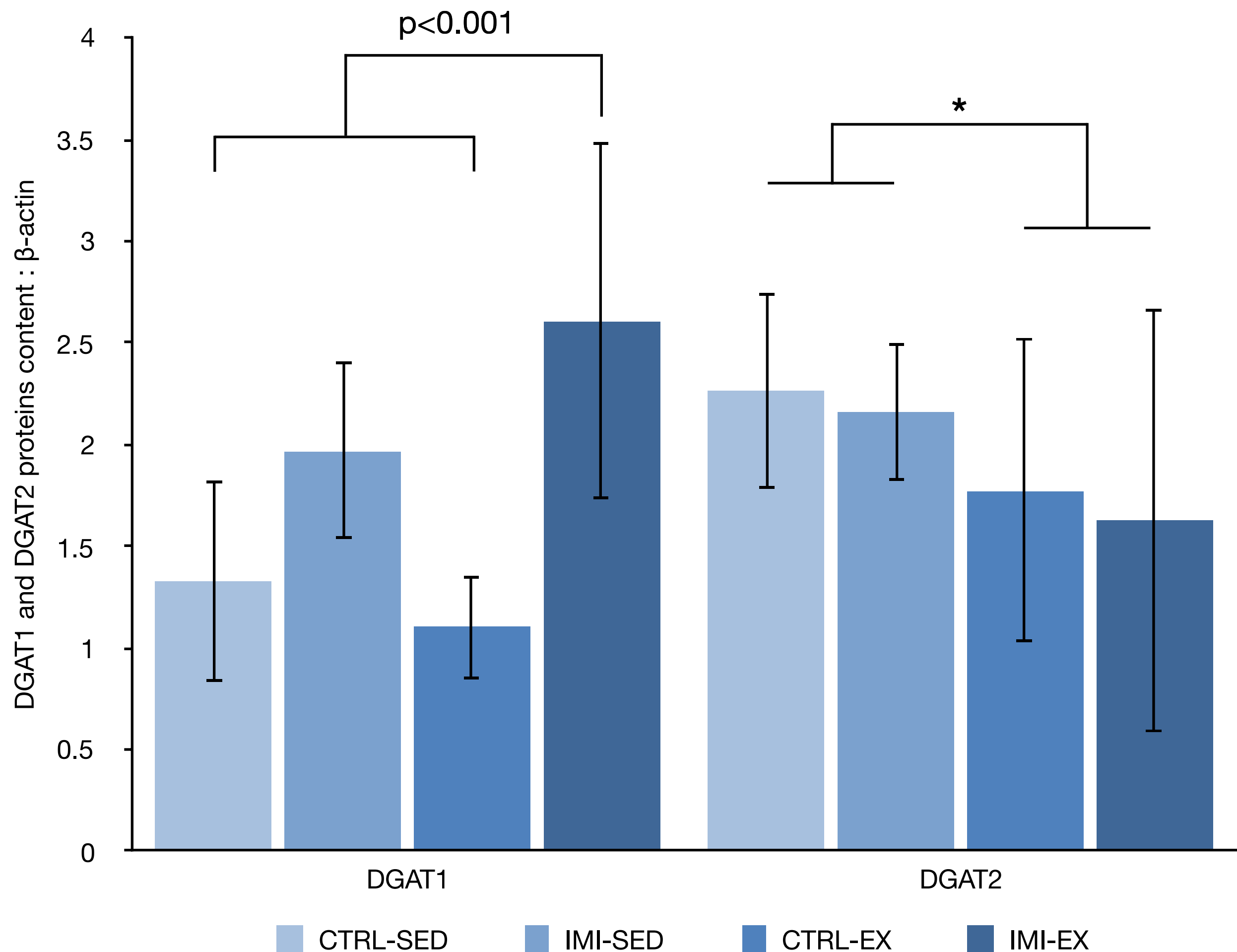

Figure 4: Average DGAT1 and DGAT2 proteins content of control-sedentary (CTRL-SED, n=10), contaminated-sedentary (IMI-SED, n=10), control-trained (CTRL-EX, n=10) and contaminated-trained (IMI-EX; n(DGAT1)=10, n(DGAT2)=9) rats by group after 30 days of exposure to imidacloprid and/or exercise training. Western blotting analysis shows a significant increase in DGAT1 levels in IMI rats with the exception of CTRL-SED and IMI-SED ($p<0.001$). A significant decrease in DGAT2 levels in trained rats overall ($p<0.05$). β-actin was employed as the housekeeping reference gene. All data were relativized against their corresponding β-actin band value. Data for DGAT1 and DGAT2 violated the Levene's test. Values are means ± SD; * Different from the control group by $p \leq 0.05$.

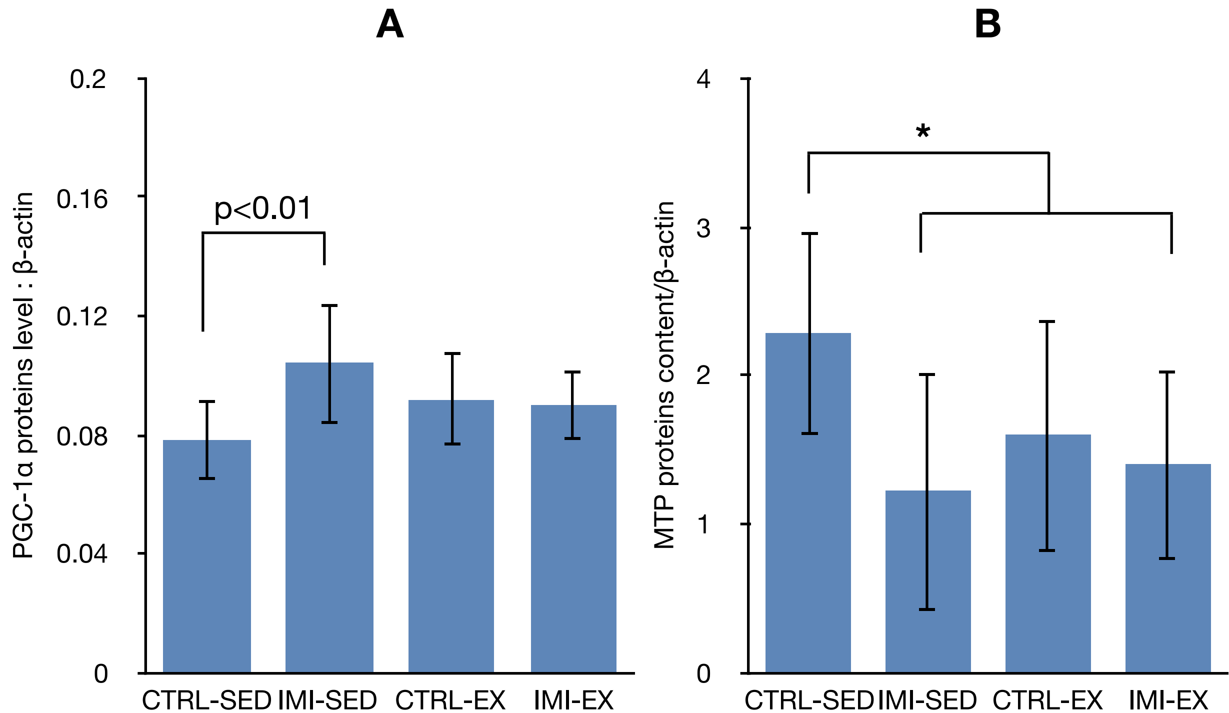

Figure 5: Average **A)** PGC-1α proteins content of control-sedentary (CTRL-SED; n=10), contaminated-sedentary (IMI-SED; n=10), control-trained (CTRL-EX; n=6) and contaminated-trained (IMI-EX; n=6) and **B)** MTP proteins content of control-sedentary (CTRL-SED; n=7), contaminated-sedentary (IMI-SED; n=8), control-trained (CTRL-EX; n=10) and contaminated-trained (IMI-EX; n=10) rats by group after 30 days of exposure to imidacloprid and/or exercise training. Western blotting analysis shows a significant decrease in MTP in contaminated but not in trained rats. A significant difference was identified for PGC-1α proteins level ($p=0.056$ for contamination) with this increase confirmed to be present between the CTRL-SED and IMI-SED. β-actin was employed as the housekeeping reference gene. All data were relativized against their corresponding β-actin band value. Values are means ± SD; *Different from the control group by $p \leq 0.05$.

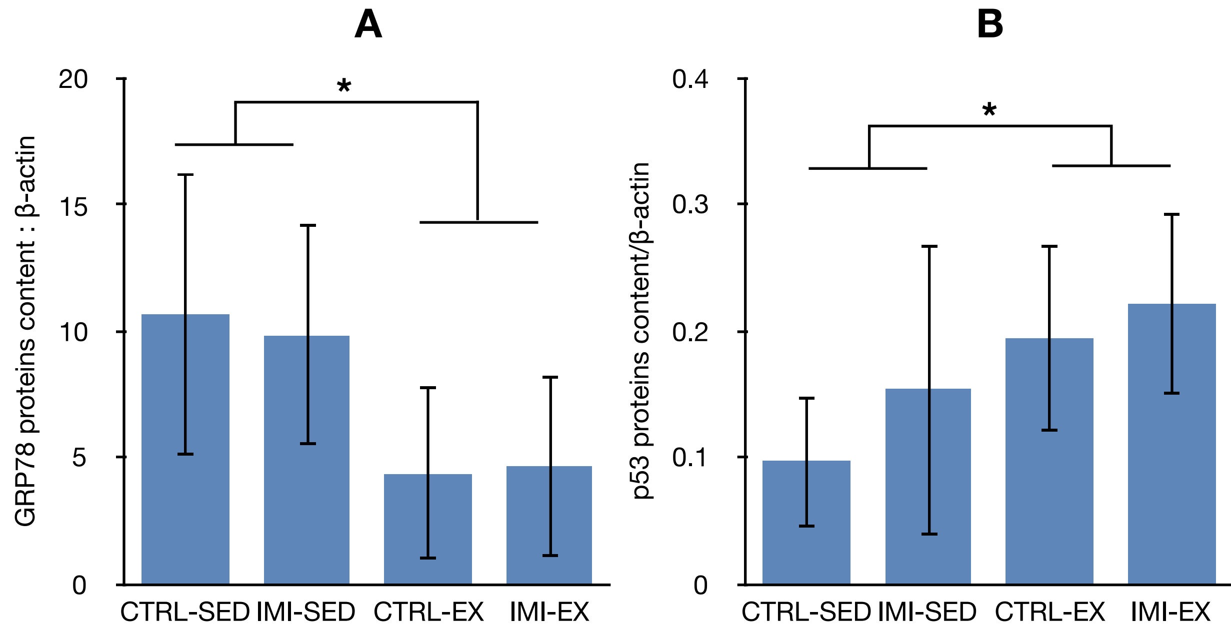

Figure 6: Average A) GRP78 proteins content for control-sedentary (CTRL-SED; n=10), contaminated-sedentary (IMI-SED; n=10), control-trained (CTRL-EX; n=10) and contaminated-trained (IMI-EX; n=10) and **B)** MTP proteins content of control-sedentary (CTRL-SED; n=10), contaminated-sedentary (IMI-SED; n=9), control-trained (CTRL-EX; n=10) and contaminated-trained (IMI-EX; n=10) rats by group after 30 days of exposure to imidacloprid and/or exercise training. Western blotting analysis show a significant decrease in GRP78 levels in trained rats as opposed to sedentary rats ($p<0.001$). p53 was significantly increased in EX groups but unaffected by contamination. β-actin was employed as the housekeeping reference gene. All data were relativized against their corresponding β-actin band value. Data for GRP78 violated the Levene's test. Values are means ± SD; *Different from the control group by $p \leq 0.05$.

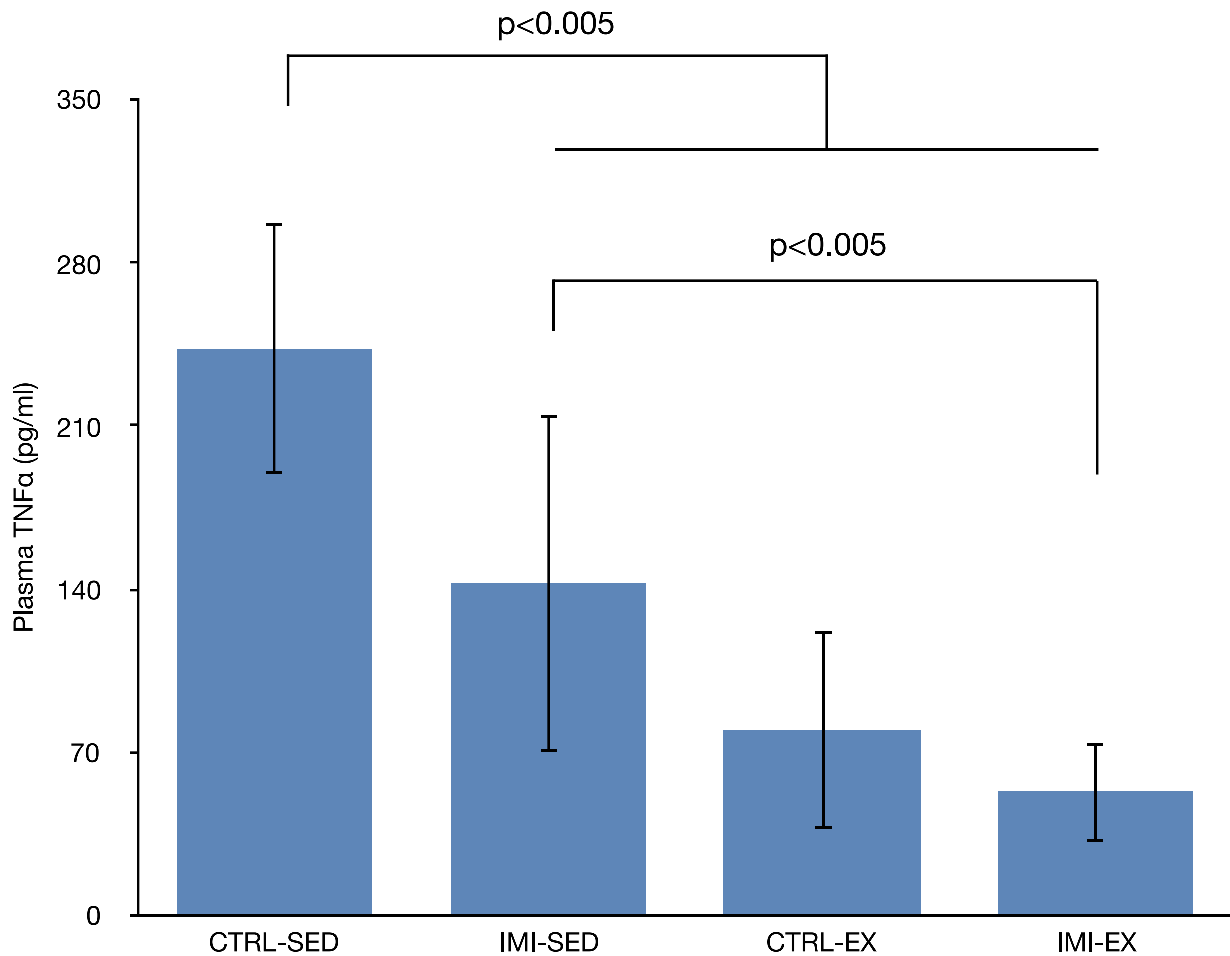

Figure 7: Average TNFα levels of control-sedentary (CTRL-SED; n=10), contaminated-sedentary (IMI-SED; n=8), control-trained (CTRL-EX; n=9) and contaminated-trained (IMI-EX; n=9) rats by group after 30 days of exposure to imidacloprid and/or exercise training. Spectrophotometric analysis shows a significant decrease in TNFα levels in all groups as opposed to CTRL-SED rats. IMI-EX rats also have lower average TNFα levels as opposed to their IMI-SED counterparts. Samples were standardized using a water blank. Data for TNFα violated the Levene's test. Values are means ± SD; *Different from the control group by $p \leq 0.05$.

4. Discussion

4.1 Hepatic triglycerides accumulation pathways - Contamination

Steatosis, the hallmark feature of NAFLD, is characterized by the accumulation of hepatic triglycerides (≧10% of liver weight) as well as the increase in both hepatic and plasma FFA contents [8] whereas the increase in plasma triglycerides tends to fluctuates to the degree of insulin resistance [114]. None of those features were observed in contaminated groups: hepatic triglycerides (Figure 1b), plasma FFA (Figure 2a) as well as plasma triglycerides (Figure 2b) were found to remain similar to their control counterparts. NAFLD is hence not believed to have developed in contaminated rats. Despite the absence of steatosis, proteins associated with accumulation and elimination pathways were nonetheless quantified as the dosage at which rats had been contaminated (10mg/kg/day) may have been sufficient to affect some parameters of interest without inducing NAFLD features.

4.2 Hepatic triglycerides accumulation pathways - Exercise training

Hepatic triglycerides and plasma FFA were unaffected by exercise training, but a decrease was noted in plasma triglyceride content (Figure 2b). This finding is consistent with existing literature which reports lower plasma triglycerides due to a greater lipid oxidation in skeletal muscle of trained Sprague-Dawley rats [152] as well as lower hepatic triglycerides secretion in trained Zucker rats, a rat model of genetic obesity [153]. Human studies also observed an increase in triglycerides intake by exercised muscles resulting in a decrease in plasma triglycerides levels [154, 155]. Exercising Sprague-Dawley rats for a period of 60min, 5x/week for six weeks can hence lower plasma trigly-

cerides, although the mechanism through which they are lowered has not been investigated in the present study.

4.3 *De novo* lipogenesis and VLDL production - Contamination

The lack of an increase in plasma FFA alludes to the absence of an increased *de novo* lipogenesis. Indeed, the principal actors in lipogenesis LXR and SREBP-1c remained unaffected by both contamination and exercise (Figure 3). The rate limiting enzyme for triglyceride formation DGAT1 was found increased in IMI rats with no differences within them (Figure 4). Its homologue DGAT2 was not affected by contamination (Figure 4). An increase in DGAT1 would be expected to be followed by a subsequent increase in plasma triglycerides and hepatic MTP as DGAT1 assists in the synthesis of triglycerides meant for VLDL export. None of those increases were observed: plasma triglyceride levels remained stable across contaminated groups (Figure 2b) and MTP protein levels were significantly lower in both IMI groups (Figure 5b). It is worth noting that IMI-SED rats had a lower (M=23.18, SD=4.00) albeit non-significant ($t_{(1,18)}$=1.643, p=0.065) hepatic triglyceride content as compared to CTRL-SED rats (M=27.56, SD=5.82). However, the initial ANOVA did not find any significance between contaminated groups ($F_{(1,36)}$=1.855, p=0.182). A lower level of hepatic MTP protein levels may reflect a lower hepatic triglycerides content, but this claim remains unsubstantiated by current literature. An important factor opposing this hypothesis is the lower MTP protein content present in the IMI-EX group while its hepatic triglycerides content remains at the level of CTRL-SED rats rather than be increased. An earlier study reported a lower hepatic triglycerides secretion in trained Zucker rats [153] but did not report whether exer-

cise training affected MTP as the role of this protein had yet to be discovered at the time of its publication. The lower MTP protein content could have been the result of alterations to insulin signalling pathways as MTP mRNA has been shown to be decrease by ~2-fold in patients with insulin resistance. While imidacloprid has been shown to have the potential of negatively affect insulin signalling pathways in mice [39] and rats [34], the present study does not support such an observation as parameters related to insulin resistance (local and systemic) were not altered: LXR & SREBP-1c protein levels (Figure 3), hepatic triglycerides and plasma FFA (Figure 1b; Figure 2a) all remained at levels similar to their control counterparts when examining contamination. A final hypothesis would be that although statistically significant, the observed decrease in MTP proteins content was simply not sufficient enough to increase hepatic triglycerides despite it playing a pivotal role in the development of steatosis [110].

4.4 *De novo* lipogenesis and VLDL production - Exercise

The only notable change in protein levels was a decrease in DGAT2 protein content in trained rats (Figure 4). This decrease concords with at least one other study which also observed a concomitant increase in DGAT1 protein levels [156]. Such an increase in DGAT1 was not observed in this present study. Despite the significant decrease in DGAT2, hepatic triglycerides were not found to be decreased (Figure 1b). The quantification of hepatic fatty acids may have provided some additional insight as to whether this decrease would have been capable of inducing their accumulation considering that DGAT2 catalyzes the formation of hepatic triglycerides from a pool of fatty acids. MTP was not affected by exercise training.

4.5 Oxidation - Contamination

An increase in the flux of FFA common to NAFLD places a burden on the mitochondria which adapts to maintain lipid homeostasis. Fatty acids stimulate the transcription of PGC-1α [86]. As such, PGC-1α impairment can be associated with NAFLD as fatty acids accumulate in hepatocytes [157]. PGC-1α protein levels were found to be significantly higher only in the IMI-SED group (Figure 5a). PGC-1α has been found to be activated by fasting in order to accommodate the increased influx of fatty acids stemming from an increased lipolysis [157]. No increase in plasma FFA was noted (Figure 2a), but the fact remains that IMI-SED rats gained significantly less weight than their counterparts (Figure 1a). Although the increase in PGC-1α appears to substantiate the noted weight loss in IMI-SED rats, the lack of an increase in plasma FFA undermines the hypothesis that fasting may have contributed to increasing PGC-1α.

4.6 Cellular stress - Contamination

Oxidative stress brought about by pesticides has been proposed to initiate the development of NAFLD [115, 151]. Markers for oxidative stress associated with the development of NAFLD (i.e.: GRP78, p53 and TNFα) were examined to establish if imidacloprid could induce oxidative stress. The synthesis of the protein chaperone GRP78 has been found to be increased in the hours following hepatic toxic injury and to remain high afterwards as misfolded proteins accumulate within the ER [158, 159]. This accumulation of misfolded proteins has been shown to cause ER stress which can lead to insulin resistance and steatosis [123]. Inversely, the the overexpression of GRP78 leads

to a decrease in hepatic triglycerides and improved insulin sensitivity [127]. No variations in GRP78 were detected in contaminated rats (Figure 6a). Results for p53, known to be induced by various pesticides through the generation of oxidative stress [16, 133, 135, 160, 161], follows a similar trend where its levels in the contaminated groups remain similar to those of their control counterparts (Figure 6b). No current research has examined the effects of imidacloprid on either GRP78 or p53. TNFα, a key inflammatory marker in the progression of NAFLD to NASH [139], was found in a lesser concentration in all groups relative to the CTRL-SED with a significant decrease in the IMI-EX group compared to the IMI-SED (Figure 7). A previous study observed a 2-fold increase in hepatic TNFα mRNA of Sprague-Dawley rats contaminated with imidacloprid at a dose of 20mg/kg/day [23], but no mention was made of its protein levels. The noted decrease in plasma TNFα in this present study suggests the *statu quo* to be maintained: TNFα directly inhibits insulin signalling, thus increasing insulin resistance [149]. The decrease in TNFα observed in IMI rats suggests insulin resistance stemming from pesticide contamination to be an unlikely outcome, at least from the perspective of this pathway. As such, the data obtained for the proteins GRP78, p53 and TNFα suggests imidacloprid administered orally at a dose of 10mg/kg/day does not generate oxidative stress in quantifiable levels under the present parameters of this study.

4.7 Cellular stress - Exercise

Aerobic exercise has been found to decrease hepatic oxidative stress by modulating ROS/RNS and enhancing the activity of various antioxidant enzymes to attenuate the naturally occurring oxidative stress stemming from exercise itself [47, 162, 163].

Oxidative stress markers examined in this study were found to be affected by exercise training to various degrees. GRP78 was decreased in trained rats, which corroborates a study performed on obese patients where exercise was found to alleviate ER stress through a reduction in the expression of circulating GRP78 [164]. p53 was instead found to be increased in CTRL-EX and IMI-EX groups (vs. CTRL-SED and IMI-SED respectively) with no significant differences within both (Figure 6b). A single similar study found p53 to remain at control values in trained Sprague-Dawley rats made to swim 5x/week for 12 weeks, contradicting our findings [165]. TNFα was decreased for both trained groups (CTRL-EX and IMI-EX) as opposed to the control groups. This observation is supported by a study where moderate swimming exercise in middle-aged Wistar rats (20min/day, 4 weeks w/ load 3% of bw) lead to a decrease of ~20% of plasma TNFα in trained rats as well as other various inflammatory markers [163]. This same study did not observe any variations in reactive species associated with oxidative stress within the liver and only noted an increase in one hepatic antioxidant (GSH) in trained rats. In humans, exercise (light jog, 3x/week for 12 weeks) without dietary restriction was shown to decrease plasma TNFα levels by 35% [46].

4.8 Addendum to cellular stress

While oxidative stress markers would have been expected to yield conclusive results should NAFLD had developed, the fact that the condition did not leaves those results open to many interpretations as the quantified oxidative stress markers yield ambiguous results. This combined with the paucity of data pertaining to oxidative stress markers (particularly for GRP78 and p53) as well as the absence of antioxidant activity

status renders the formulation of assumptions futile as the extent to which hepatocytes were capable of buffering oxidative stress in either contaminated or trained rats is unknown. Limitations pertaining to the evaluation of oxidative stress will be discussed further below.

4.9 Weight variations

A novel observation by this study is the relative lack of weight gain of IMI-SED rats as opposed to the CTRL-SED rats. Another observation in a similar vein was the preservation in weight of IMI-EX rats, who maintained a weight similar to both control groups (Table 1; Figure 1b). Previous studies have reported weight loss in rats contaminated with imidacloprid but did not specify the origin of said loss. Repeated exposure to imidacloprid from postnatal day 21 to 42 at a dose of 10 mg/kg/day has been shown to reduce the weight of Wistar pups [166]. Adult female Wistar rats contaminated at a dose of 20 mg/kg/day of imidacloprid during 90 days gained only 53% of their initial weight as opposed to 64% for their control counterparts. Weight gain in rats exposed to 5mg/kg/day and 10mg/kg/day in this same study was not observed [34]. One study on female albino rats reports a reduction in net body weight gain (25.35g/100g bw) in animals contaminated with 9 mg/kg/day of imidacloprid for a period of four weeks, although their food intake was not significantly reduced [167]. This present study corroborates this latter finding in Sprague-Dawley rats, being that a dose as low as 10 mg/kg/day of imidacloprid chronically administered (six weeks, omitting weekends) appears to be sufficient to reduce weight gain by up to 7.8% in IMI-SED rats as opposed to CTRL-SED. Another interesting observation is the mitigation of overall weight loss in IMI-EX rats whose

weight remains at levels similar to the CTRL-SED and CTRL-EX groups (Table 1, Figure 1a). We are not aware of known mechanisms which may be responsible for this observation, although it may be partially explained by an increase in food consumption following exercise sessions or a gain in global muscle mass due to the exercise training - neither of which were monitored in this study. Our observations contradict the currently established NOEL of 10mg/kg/day of imidacloprid as a threshold dose as weight alterations are observed at this level of contamination and confirm the findings of Toor et al. (2013) [167] on an unspecified breed of female albino rats.

5. Limitations of this study and further directions

The first limitation of this study relates to the protocol and sacrifice of the rats. The unforeseen paucity of imidacloprid forced us to cease pesticide gavages and exercise training on a Thursday in an attempt to acquire more in the upcoming days. Our laboratory was advised on Monday by our supplying company that the pesticide had been backordered for multiple weeks. This incited the immediate sacrifice of all 20 IMI rats on a Tuesday while CTRL rats were sacrificed the following day. Due to the clearance of the pesticide, rats may have had enough time (~144h) to properly metabolize and eliminate the pesticide from their organism as well as potentially recover from any pesticide-induced damage. A very good reference as to the repartition and elimination of imidacloprid can be found in [28]. It is likely that rats would have been able to recover from contamination to a certain degree, leading to results closer in value to their CTRL counterparts than expected had they been sacrificed 48h following the last physical training session. The level of this probable recovery is unknown.

Another time-related concern would be the duration of the contamination and training program which may have been too short in length to properly allow tendencies to appear in both groups. Three other studies examining imidacloprid contamination in rats reported various significant physiological changes at typically higher doses (9, 20, 40 and 80mg/kg/day) for a duration of 30 days [23, 168] whereas exercise training of at least eight weeks appears to be the minimal period required for it to have a definitive effect on rats [117, 118, 120, 156]. It is hereby conceivable that results for contaminated but mostly trained rats did not have sufficient time to acclimate to their training program.

A severe limitation was the absence of food intake monitoring and body composition. This makes it impossible to confirm whether the lesser weight gain in IMI-SED rats as opposed to the CTRL-SED or IMI-EX was caused by a reduced food consumption, an increase in muscle mass, by some unforeseen metabolic effects of imidacloprid or a combination of those factors. A single study examining imidacloprid contamination (9mg/kg/day, 4 weeks) found no differences in food intake despite a lower weight gain compared to the control group [167]. It is also impossible to confirm whether exercise improved weight gain in contaminated rats by improving their appetite or by some unsuspected metabolic process (e.g.: improved antioxidative capabilities or higher pesticide clearance).

The absence of plasma insulin quantification and/or insulin sensitivity test limits the interpretation of some of our results. SREBP-1c and MTP are two proteins affected by insulin: the former is stimulated and matures by its presence to enable *de novo* lipogenesis [66] while the latter is reduced at a transcriptional level to limit triglyceride export by VLDL [111]. Under conditions of insulin resistance, SREBP-1c becomes more expressed and MTP less expressed, both contributing to steatosis. While SREBP-1c levels remained unaffected regardless of the group (Figure 3), MTP was markedly reduced in contaminated rats (Figure 5b) which led to some questioning as to whether MTP protein levels had decreased due to a lower yet not significant hepatic triglycerides content as seen in the IMI-SED group (Figure 1b) or if imidacloprid itself could have affected MTP as can be seen in the IMI-EX group, where its levels remain similar to the

CTRL-SED group (Figure 5b). Imidacloprid was found to have the potential to negatively affect the insulin signalling pathway via Akt2 in cell cultures [38]. Research in mice contaminated with a dose of 6mg/kg/day of imidacloprid showed a higher AUC for insulin and glucose tolerance tests in mice fed a high-fat diet while only a higher AUC to be present for the glucose tolerance test in mice fed a low-fat diet [39]. However, PGC-1α has been shown to be inhibited at a transcriptional level by Akt2/PKB pathway in primary hepatocytes. Should imidacloprid have affected insulin signalling in this pathway, PGC-1α levels would be expected to be lower [169] whereas they were higher in the IMI-SED group (Figure 5a). The quantification of circulating insulin and/or glucose/insulin sensitivity tests may have provided some insight as to the insulinemic state of rats and offered another perspective on the metabolic status of the liver, namely in regards to the observed decrease in MTP in the contaminated groups.

Some pesticides have been shown to elicit an increase in antioxidant activity *in vitro* as a compensatory mechanism against oxidative stress [160]. Antioxidants and the antioxidative pathways were not investigated in this study. It is possible that an overall reduction in oxidative stress and its markers may have been brought about by an increase in antioxidants activity. Imidacloprid has been shown to generate oxidative stress at doses of 26mg/kg (acute, IP) [23] and 20mg/kg/day (90 days, oral) but not at 10mg/kg/day days (30 days, oral) [34, 37]. Mice contaminated by a single oral dose of 14,976mg/kg of imidacloprid saw an overall increase hepatic antioxidant enzymes activity (i.e.: CAT, SOD, GPX and GST) but an overall decrease in their activity when treated with an antioxidant (*in hoc*: vitamin C) 30min prior to contamination (i.e.: GSH, CAT,

SOD, GPX and GST). MDA levels were also increased by contamination and decreased by vitamin C administration [21]. *Per contra*, chronic administration of imidacloprid at 20mg/kg/day for 90 days was found to decrease various antioxidants (i.e.: CAT, SOD and GPX) but not at 10mg/kg/day [37]. Although imidacloprid has not been reported to generate oxidative stress at the doses employed in this study, having quantified antioxidant activity would have enabled us to ascertain and clarify the nature of the oxidative stress (or absence hereof) as to its origin (imidacloprid, exercise or both) as well as the hepatic reaction to said stress.

Perhaps not a limitation *per se* but worth addressing is the relatively low dosage of pesticide employed. Imidacloprid doses administered orally at 10mg/kg/day are considered to be the NOEL) and 20mg/kg/day the minimum dose required to observe morphological and histological changes in rat livers [34, 37]. The reason for employing a lower dose was to examine whether the chronic oral administration of a dose at the current established threshold would be able to induce pathological effects.

A point worth mentioning in regards to the increase in DGAT1 in IMI-EX groups is the weak presence of the protein within the liver. DGAT1 is mainly located in the intestines to promote triglycerides synthesis from dietary FFA and is seldom found in the liver [74]. Thus, even small variations on Western blots could potentially yield a statistically significant result.

A final point to address is the use of Sprague-Dawley rats as models from which to extrapolate human contamination. The general lack of availability of human livers and the ethical considerations regarding the use of live test subjects incites to the use of an animal model. Extensive studies have been made using the Sprague-Dawley rat in the context of xenobiotics as well as NAFLD, and results appear to corroborate findings in humans, with some notable exceptions [105].

6. Conclusion

Parameters associated with fatty acid intake, *de novo* lipogenesis, fatty acid oxidation and excretion suggest imidacloprid contamination under the present conditions did not induce the development of NAFLD in rats. Two reasons for the absence of its development may be the use of an imidacloprid dose at the NOEL (10mg/kg/day), a limited (six week) exposition to imidacloprid, a late sacrifice enabling rats to recuperate as well as the limited weight gain stemming from a probable lower food intake by IMI-SED rats could explain why NAFLD failed to develop. The absence in development of NAFLD renders inconsequential the examination of exercise as a means to prevent it. Further studies will want to improve upon the limitations of this study by a) quantifying food intake and body composition so as to determine the cause behind the weight loss in IMI-SED and weight gain in IMI-EX rats; b) examining the possible onset of insulin resistance as imidacloprid has been shown to affect the insulin pathway *in vitro* and *in vivo*, which could affect protein expression through various pre- and post-transcriptional mechanisms; c) examine ROS/RNS, antioxidants and antioxidative pathways to investigate the potential development of oxidative stress within the liver (e.g.: MDA, 4-HNE,

CAT, GSH, GSX & SOD); d) employ multiple doses (e.g.: 5, 10 and 20mg/kg/day per rat) along a timeline (e.g.: 4 and 8 weeks) so to establish the effects of various dosages on hepatic alterations observed in this present study and e) examine extra analytes pertaining to hepatic lipid metabolism so to bolster the validity of observations and establish better links between the four factors involved in the development of NAFLD if need be (e.g.: ATGL, CD36, FATP & SCD-1)

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Annex 1

Lysis buffer

2.3g SDS (Sigma, L3771)

200μl EDTA 0.5M pH 8.0 (1mM) (Sigma ED2P)

38.04mg EGTA (1mM) (Sigma E4378)

83.98mg NaF (20mM) (Sigma S7820)

Completed at 100ml with PBS 1x, pH 7.2

Added immediately prior to use:

500μl Na_3VO_4 200mM (1mM) (Sigma S6508)

Protease inhibitor (according to manufacturer; Sigma 8340)

Laemmli buffer 4x (for 5ml)

2.0ml Glycerol

1.2ml Tris HCl 1M pH 6.8

1.0ml β-mercaptoethanol

0.4 g SDS (Sigma, L3771)

3-4 grains of Bromophenol Blue (Biorad, #1610404)

0.8ml dH_2O

Electrophoresis buffer (10x)

30g Tris base (Sigma, T1503)

144g Glycine (Fisher, BP3811)

10.0 g of SDS (Sigma, L3771)

Complete volume to 1L of dH_2O

Transfer buffer 10x

30.3g Tris base (Sigma, T1503)

144g Glycine (Fisher, BP3811)

Dissolve in 500ml of dH_2O then complete volume to 1L of dH_2O

Transfer buffer 1x

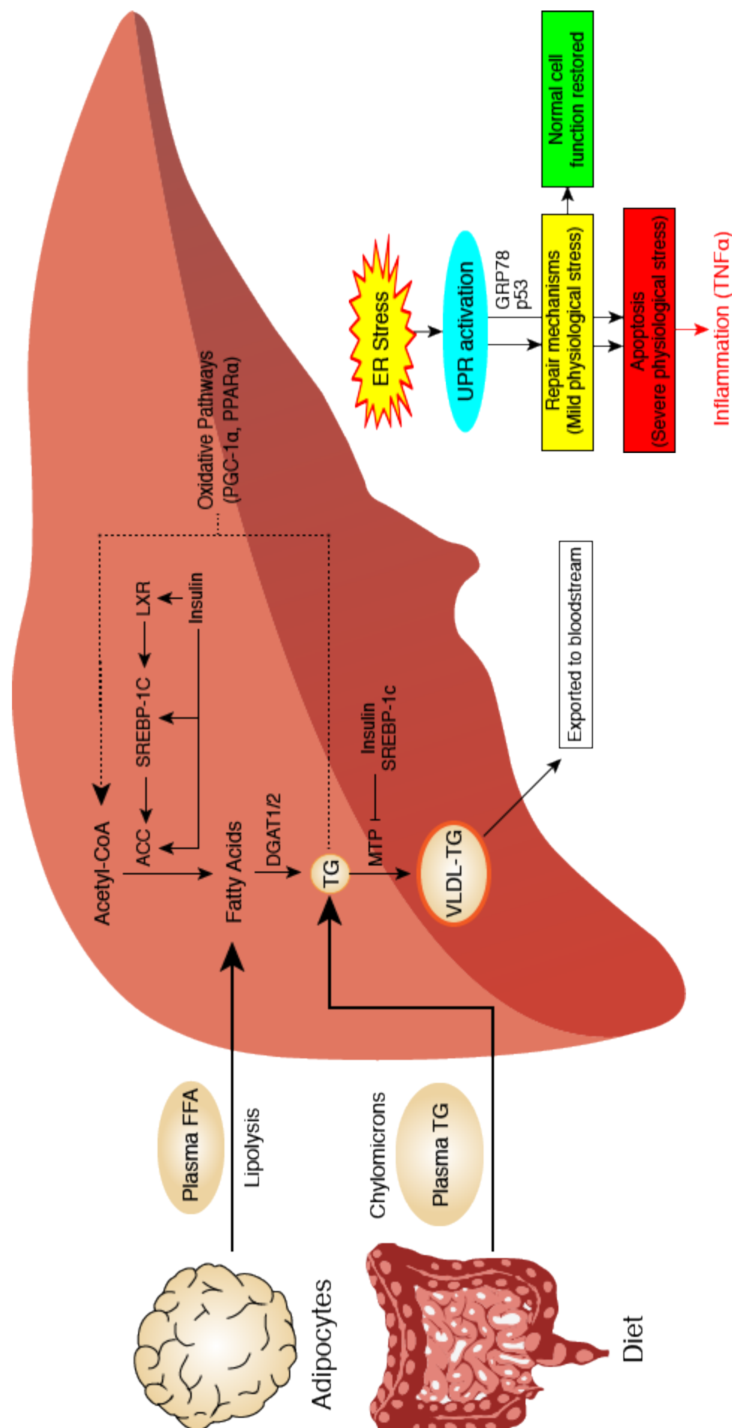
100ml of transfer buffer 10x

200ml methanol (Fisher, A4124)

700ml of dH_2O

Annex 2

For reference. Summary of metabolic pathways associated with the influx of fatty acids and triglycerides, *de novo* lipogenesis, triglyceride oxidative pathways, VLDL-TG export as well as the inflammatory pathway originating from the ER stress found in early stages of NASH.



Annex 3: Hepatic/plasma triglycerides content and proteins ratio parameters by group

Measurement	CTRL-SED	IMI-SED	CTRL-EX	IMI-EX
Hepatic triglycerides content (mg/g)	27.55 ± 5.82	23.18 ± 4.00	28.10 ± 5.77	27.92 ± 5.35
Plasma triglycerides content (mg/ml)	0.127 ± 0.037	0.134 ± 0.053	0.082 ± 0.029*	0.111 ± 0.047
Plasma free fatty acids (nmol/µl)	1.306E-02 ± 3.225E-03	1.182E-02 ± 2.649E-03	1.139E-02 ± 2.948E-03	1.161E-02 ± 1.535E-03
DGAT1	1.328 ± 0.500	1.968 ± 0.438*	1.102 ± 0.252	2.608 ± 0.883*
DGAT2	2.263 ± 0.482	2.163 ± 0.342	1.776 ± 0.743	1.632 ± 1.040
GRP78	10.62 ± 5.58	9.85 ± 4.35	4.35 ± 3.43*	4.94 ± 3.97*
LXR	0.103 ± 0.046	0.137 ± 0.050	0.110 ± 0.032	0.114 ± 0.036*
MTP	2.28 ± 0.68	1.22 ± 0.80*	1.59 ± 0.78	1.40 ± 0.64*
p53	0.096 ± 0.051	0.153 ± 0.115	0.194 ± 0.074*	0.222 ± 0.072*
PGC-1α	0.078 ± 0.014	0.092 ± 0.016*	0.104 ± 0.020	0.090 ± 0.012
SREBP-1c	0.111 ± 0.046	0.010 ± 0.032	0.116 ± 0.056	0.136 ± 0.038
TNFα (pg/ml)	243.0 ± 54.1	142.0 ± 42.6	79.3 ± 42.0	52.6 ± 20.8