AHMED AYYASH – PH.D. THESIS – SSRI Fluoxetine and NAFLD

MOLECULAR MECHANISMS UNDERLYING SSRI-INDUCED NON-ALCOHOLIC FATTY LIVER DISEASE

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Lay Abstract

In adults, major depressive disorder (depression) is one of the most common psychiatric illnesses. Recent data suggests that there are more than 4.1 million Canadians who currently suffer from depression. Depression is commonly treated using selective serotonin reuptake inhibitor (SSRI) antidepressants. While these antidepressants do help manage depressive symptoms, they can also cause unwanted side effects including a build-up of fat in the liver, leading to fatty liver disease. The goal of my research is to understand the link between SSRI use and the development of fatty liver disease. This thesis investigates the effects of fluoxetine (Prozac®), a commonly used SSRI antidepressant, on molecular pathways that can lead to the development of fatty liver disease. An understanding of the molecular changes with SSRI treatment may lead to the development of strategies to prevent the harmful effects of SSRI antidepressants on the liver.

Abstract

This thesis aims to investigate fluoxetine, a widely prescribed SSRI antidepressant, for its role in the pathogenesis of NAFLD and uncover novel mechanisms by which it may contribute to drug-induced steatosis. We demonstrated that increased hepatic lipid accumulation was mediated, in part, via elevated serotonin production. The inhibition of hepatic serotonin synthesis prevented lipid accumulation in fluoxetine-treated hepatocytes demonstrating a causal role for serotonin in fluoxetine-induced hepatic steatosis. Interestingly, in several studies, serotonin signaling has been shown to impact prostaglandin biosynthesis. As prostaglandins have been implicated in the development of NAFLD, and fluoxetine has previously been shown to alter the production of prostaglandins I assessed the role of prostaglandins in fluoxetine-induced hepatic lipid accumulation. Fluoxetine treatment increased mRNA expression of prostaglandin biosynthetic enzymes, increased production of prostaglandin 15-deoxy-$\Delta^{12,14}PGJ_2$ (PPARG agonist), and elevated PPARG targets involved in fatty acid uptake. Fluoxetine-induced lipid accumulation, 15-deoxy-$\Delta^{12,14}PGJ_2$ production, and the expression of PPARG lipogenic genes were attenuated with a PTGS1 specific inhibitor. Taken together these findings suggested that fluoxetine-induced lipid accumulation was mediated via PTGS1 and its downstream product 15-deoxy-$\Delta^{12,14}PGJ_2$. Given that *Pparg* was elevated following fluoxetine treatment, and PPARG regulates microRNA involved in hepatic lipid accumulation, my final project focused on PPARG's role in altered miRNA expression. Indeed, fluoxetine treatment increased the miRNA expression of miR-122, an effect that was attenuated when fluoxetine treatment was combined with the PPARG antagonist GW9662, suggesting a fluoxetine-PPARG-*miR122* axis contributing to hepatic steatosis. While these studies have only been performed *in vitro*, an understanding of the molecular changes associated with SSRI treatment may lead to the development of strategies to prevent the increased risk of adverse metabolic outcomes associated with the use of SSRI antidepressants.

Preface & Acknowledgements

This thesis is prepared in the "sandwich" format as outlined in the "Guide for the preparation of Master's and Doctoral Theses" available through the School of Graduate Studies at McMaster University. Chapter 1 is a general introduction to the content of subsequent chapters. The body of this thesis consists of 3 chapters (Chapters 2-3), each one an independent study; Chapters 2 and 3 have been published, and chapter 4 remains a data chapter until being completed and submitted for publication. The author of this thesis, who is also the first author of all included works, wrote all submitted manuscripts included in this thesis. Finally, Chapter 5 includes the discussion of this thesis aimed to summarize the conclusions of the thesis and discuss possible future directions along with reviewing the significance of the work.

Thank you to the members of my committee for providing me with valuable research insight. A special thanks to Dr. Sandeep Raha for being a member of both my master's and Ph.D. committees, as well as a fantastic advisor over the course of my comprehensive examination.

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List of Abbreviations and Symbols

AA Arachidonic Acid
ANOVA Analysis of Variance
BCA Bicinchoninic Acid Assay
C Celsius
cDNA Complementary Deoxyribonucleic Acid
Cd36 Cluster of differentiation 36
CO2 Carbon Dioxide
DNA Deoxyribonucleic Acid
DSM-V Diagnostic and Statistical Manual of Mental Disorder, 5th edition
Epoxyeicosatrienoic acids EETs
ELISA Enzyme-Linked Immunosorbent Assay
Fatp2 Fatty acid transport protein 2
Fatp5 Fatty acid transport protein 5
FLX Fluoxetine
h Hour
Hydroxyeicosatetraenoic acids HETEs
L Liter
µg Micrograms
µL Microliters
µm Microns
µM Micromolar
MDD Major Depressive Disorder
miRNA micro RNA
mRNA Messenger RNA
mL Milliliters
m Minutes
N Sample Size
NAFLD Nonalcoholic fatty liver disease
NASH Nonalcoholic Steatohepatitis
ncRNA non-coding RNA
ng Nanograms
p Level of Significance/probability value
PBS Phosphate-Buffered Saline
PCR Polymerase Chain Reaction
PLA2 Phospholipases A2
Pparg Peroxisome Proliferator Activated Receptor Gamma
Ptgs1 Prostaglandin-Endoperoxide Synthase 1
Ptgs2 Prostaglandin-Endoperoxide Synthase 2
Ptgds Prostaglandin D2 Synthase
SSRI Selective serotonin reuptake inhibitors
SNRI Serotonin and norepinephrine reuptake inhibitors
TCA Tricyclic antidepressants

Tph1 Tryptophan Hydroxylase 1
Tph2 Tryptophan Hydroxylase 2
15d-PGJ$_2$ 15-deoxy-$\Delta^{12,14}$PGJ$_2$
5-HT 5-hydroxytryptamine

Declaration of Academic Achievement

Chapter 2
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Contribution

This study was conceived and designed by AA and ACH. All laboratory experiments Including cell culture, RNA and protein extraction, enzyme-linked immunosorbent assay, real-time polymerase chain reaction, were completed by AA. Data was analyzed by AA and ACH. AA drafted the manuscript while ACH edited the final versions of the manuscript.

Chapter 3
Publication: Ayyash, A., & Holloway, A. C. (2021). Fluoxetine-induced hepatic lipid accumulation is mediated by prostaglandin-endoperoxide synthase 1 and is linked to elevated 15-deoxy-Δ12,14PGJ2. *Journal of Applied Toxicology*, 1–12. https://doi.org/10.1002/jat.4272

Contribution:

This study was conceived and designed by AA and ACH. All laboratory experiments Including cell culture, RNA and protein extraction, enzyme-linked immunosorbent assay, real-time polymerase chain reaction, were completed by AA. Data was analyzed by AA and ACH. AA drafted the manuscript while LJ and ACH edited the final versions of the manuscript.

Chapter 4
Publication: Elevated miR-122 is Mediated by Peroxisome Proliferator-activated Receptor γ and is Among microRNA Altered as a Consequence of Fluoxetine-induced Hepatic Lipid Accumulation.

Contribution:

This study was conceived and designed by AA and ACH. AA, Taylor Donders (TD), Celina Ruan (CR), and Shanza Jamshed (SJ) worked on completing the literature review All laboratory experiments, including cell culture, miRNA extraction, real-time polymerase chain reaction, were completed by AA. Data was analyzed by AA and ACH. AA drafted the manuscript while ACH edited the final versions of the manuscript. This manuscript has not yet been submitted for publication.

1 Chapter 1: Introduction

1.1 Major Depressive Disorder (MDD)

In adults, major depressive disorder (MDD) is the most prevalent psychiatric disorder and one of the leading causes of disease burden (K. Smith, 2014; World Health Organization, 2001). The World Health Organization estimates that MDD affects nearly 350 million individuals worldwide and that by 2030, depression will have become the world's leading cause of disability as measured by disability-adjusted life years (World Health Organization, 2017). MDD is characterized in the Diagnostic and Statistical Manual of Mental Disorders-5 (DSM-V) by symptoms of sadness or anhedonia in addition to at least five other depressive symptoms including altered eating patterns, apathy, restlessness, altered sleeping patterns, fatigue, feelings of worthlessness, excessive or inappropriate guilt, diminished ability to concentrate, indecisiveness, and/or recurrent thoughts of death. These symptoms must exist for a minimum of two weeks and cause significant impairment in functioning (American Psychiatric Association & American Psychiatric Association, 2013). The lifetime prevalence of MDD is approximately 15%–20%, with the prevalence of MDD twice as high for women as it is for men (G. Li et al., 2018). MDD is a significant burden to both the individual and society, resulting in impairments in work productivity, family responsibilities, and education as well as increased rates of morbidity and mortality (Lépine & Briley, 2011; Sheehan et al., 2017; Zuckerman et al., 2018). In Canada, has been reported that 11.3% of people aged 15 and older had symptoms consistent with depression, but this percentage could be much higher since many cases of depression are underdiagnosed by primary health care providers (Government of Canada, 2013; VanItallie, 2005). The annual economic burden of mental illness in Canada was estimated to be upwards of $50 billion CAD (Government of Canada, 2007; K.-L. Lim et al., 2008). Even though MDD has a profound impact on quality of life, after decades of research, the pathophysiology of MDD remains elusive (Das et al., 2019; Zuckerman et al., 2018).

1.2 Pathology of Depression & Etiology: The Various Hypotheses of MDD

Despite the substantial prevalence and impact of MDD, its etiology remains uncertain. The familial contribution to MDD is predicted to range between 30–40% while environmental factors, such as maternal stress, child abuse, neglect, social stress, prenatal infection, traumatic events, and endocrine abnormalities, account for the remaining 60–70% (Fava & Kendler, 2000; Horowitz & Zunszain, 2015; Larrieu & Sandi, 2018; Laugharne et al., 2010; Y.-L. Lin & Wang, 2014; Nemeroff, 2016; Sperner-Unterweger, 2015; Sullivan et al., 2000; Takahashi et al., 2018; Verdolini et al., 2015; Weinstock, 2017). In pursuit of identifying a candidate gene underlying the pathogenesis of MDD, 1500 publications have assessed variants of over 200 gene targets (Kendall et al., 2021). Genes involved in monoamine synthesis, such as serotonin (tryptophan hydroxylase) and dopamine (tyrosine hydroxylase), and monoamine metabolism (catechol-*O*-methyl transferase) were all once regarded as potential candidates, however, most of these studies were largely

underpowered, did not correct for population stratification, and the reported significance levels were not greater than what would be expected by chance (Flint & Kendler, 2014; Tamatam et al., 2012). More recently, genome-wide association studies using large sets of samples, including thousands of patients with different forms of MDD and tens of thousands of patients in meta-analyses, have failed to identify any specific loci responsible for a predisposition for MDD (Border et al., 2019; Shadrina et al., 2018; Thompson et al., 2014; Wray et al., 2018). This led authors to suggest that MDD is a complicated multifactor heterogeneous psychiatric disorder influenced by many genes and their interactions in gene networks and with the environment (Shadrina et al., 2018). Several theories, including hypotheses pertaining to increased inflammation, decreased neurogenesis, hyperactive hypothalamus-pituitary-adrenal (HPA) axis or impaired neurotransmission have been suggested to explain MDD onset (Bao et al., 2008; Cobb et al., 2013; Hodes et al., 2015; Sheline et al., 1996; Swaab et al., 2005).

1.2.1 Elevated Inflammation and MDD

While it is unclear whether inflammation plays a causal role in the development of depression, elevated levels of pro-inflammatory cytokines present in the brain and circulating in the cerebrospinal fluid of depressed individuals have led to speculation that inflammation is involved in the development of MDD (Capuron & Miller, 2011; Felger & Lotrich, 2013; Maes, 1999; Martinez et al., 2012). Pro-inflammatory cytokines produced by innate immune cells including interleukin-1, interleukin-6, tumor necrosis factor-alpha, and acute-phase C-reactive protein (CRP) produced by hepatocytes, have all been reported to be elevated in individuals with MDD, nonetheless at levels much lower than in individuals diagnosed with an infection or autoimmune disease (Dowlati et al., 2010; Howren et al., 2009; Lindqvist et al., 2017). In addition, inflammation can lead to the activation of the kynurenine pathway of tryptophan metabolism, resulting in an accumulation of neurotoxic metabolites such as quinolinic acid which have been hypothesized to contribute to the pathogenesis of MDD (Dantzer et al., 2011; Lindqvist et al., 2017; Öztürk et al., 2021)

1.2.2 Hyperactive Hypothalamus–Pituitary–Adrenal axis and MDD

The hypothalamic-pituitary-adrenal (HPA) axis is directly affected by chronic stress and perturbations in this axis have been thought to be associated with the development of MDD. Dysfunction of the HPA axis has been considered a hallmark of depression, as neuroendocrine studies effectively demonstrated HPA axis overactivity in individuals with MDD (Mello et al., 2003). The HPA axis regulates the secretion of glucocorticoids, such as cortisol; nearly 40-60% of people with MDD have hypercortisolemia (Keller et al., 2017; Murphy, 1991). Hypercortisolemia can be caused by chronic stress and involves a

prolonged excess of serum levels of cortisol as a result of abnormalities in the HPA axis culminating in abnormal glucocorticoid receptor (GR) signaling and glucocorticoid resistance (Dean & Keshavan, 2017; Ignácio et al., 2019; Meijer et al., 2018; Nemeroff, 1996). Furthermore, glucocorticoid neurotoxicity has been implicated in the decreased volume in the hippocampus, driving some of the structural and functional changes observed in depressed individuals (L. Dai et al., 2019; Sapolsky, 2000).

1.2.3 Neurodegeneration and MDD

The 'neurotrophin hypothesis' of depression suggests that decreased levels of neurotrophic factors lead to decreased neurogenesis, driving some of the structural and functional changes observed in depressed individuals (L. Dai et al., 2019; Sapolsky, 2000). One of the major neurotrophic factors, brain-derived neurotrophic factor (BDNF) is involved in synaptic plasticity and neurogenesis; both of which are decreased in individuals with MDD (Colucci-D'Amato et al., 2020; Duman, 2002; Emon et al., 2020). Some studies have even suggested that antidepressants alleviate depressive symptoms via their action on BDNF. Indeed there is evidence from animal and clinical studies that have shown that antidepressant exposure can upregulate the expression and activity of BDNF in the hippocampus (de Foubert et al., 2004; B.-H. Lee & Kim, 2010; Russo-Neustadt et al., 1999). Additionally, dysfunctional glutamatergic and GABAergic neurotransmission, as observed in individuals with MDD has also been shown to decrease neuronal plasticity, along with abnormalities in excitatory and/or inhibitory neurotransmission leading to aberrant functional brain connectivity patterns (Barnes et al., 2020; Lener et al., 2017; Sarawagi et al., 2021). Neuroimaging studies have suggested that MDD may have a basis in abnormal structure, connectivity, or function of certain brain regions (S. Ayyash et al., 2021; Y.-K. Kim, 2016; World Health Organization, 2017). However, there remain substantial inconsistencies in the literature regarding the role of structural changes in the pathophysiology of MDD due to the plethora of different neuroimaging techniques, analysis toolboxes, scanning parameters, insufficient sample sizes, and different medications that may influence the results (Bani-Fatemi et al., 2018; G. Li et al., 2018). At this time, the predominant hypothesis of MDD suggests that impaired monoamine neurotransmission plays a central role in the pathophysiology of MDD (Nobis et al., 2020).

1.2.4 Impaired Neurotransmission and MDD and a focus on the 'Monoamine Hypothesis'

The most widely accepted theory for depression is the monoamine hypothesis of MDD. In this paradigm, it is hypothesized that insufficient levels of monoamine neurotransmitters (serotonin, norepinephrine, dopamine) in the central nervous system lead to the development of MDD (Boku et al., 2018; Hasler, 2010). Based on this initial theory of

depression which was postulated in the 1960s, several antidepressants were designed, resulting in the development of tricyclic antidepressants and monoamine reuptake inhibitors (Shadrina et al., 2018). The monoamine hypothesis is the most accepted hypothesis for the etiology of MDD, a view that has dominated the field for several decades (Nagy et al., 2020; Sahli et al., 2016). This hypothesis has been extensively studied, with a specific interest in serotonergic signaling (Artigas, 2013; Nestler et al., 2002; Nestler & Hyman, 2010) In the brain, serotonin is synthesized from tryptophan and is enzymatically converted to 5-hydroxy-L-tryptophan (5-HTP) by the rate-limiting enzyme tryptophan hydroxylase 2 (Tph 2) (Fidalgo et al., 2013). 5-HTP is then converted into serotonin and stored primarily in vesicles (Lubberink & Eriksson, 2020). At the nerve terminals, serotonin stored in synaptic vesicles is released by an exocytotic mechanism into the synaptic cleft upon membrane depolarization; this serotonin subsequently binds to the serotonergic receptors on the post-synaptic neuron (Del-Bel & De-Miguel, 2018; Leon-Pinzon et al., 2014). Serotonin in the synaptic cleft is taken back into the presynaptic neuron via the serotonin transporter (SERT) and oxidized by monoamine oxidase to its metabolite 5-hydroxyindoleacetic acid (Ruddick et al., 2006). Individuals with MDD are believed to have lower levels of monoamines such as serotonin in the post-synaptic cleft. In this regard, antidepressants that target the serotonin transporter (i.e., selective serotonin reuptake inhibitors), are believed to reduce the ability of SERT to remove serotonin from the synaptic cleft which results in sustained effects of serotonin signaling (D. J. David & Gardier, 2016; Torres-Sanchez et al., 2012).

1.3 Antidepressants

Current antidepressant drugs can be divided into five major categories: selective serotonin reuptake inhibitors (SSRIs), tricyclic antidepressants (TCAs), serotonin-norepinephrine reuptake inhibitors (SNRIs), monoamine oxidase inhibitors (MAOIs), and multimodal antidepressants (e.g. bupropion and vortioxetine) (Sangkuhl et al., 2011). These medications remain widely prescribed to treat a variety of illnesses including but not limited to MDD, anxiety, obsessive-compulsive disorder, eating disorders, sleep problems, smoking cessation, and most recently, the antidepressant fluvoxamine has shown efficacy in the treatment of COVID-19 (Agius & Bonnici, 2017; Lenze et al., 2020; Reis et al., 2021; Shefet et al., 2011; Skånland & Cieślar-Pobuda, 2019; Terevnikov et al., 2017). Although these pharmacotherapies all pose potential side effects, this thesis focuses on SSRI antidepressants primarily.

1.3.1 SSRI Pharmacotherapeutic Mechanism of Action

Antidepressants that target the serotonin transporter (i.e., selective serotonin reuptake inhibitors), reduce the ability of the SERT transporter to remove serotonin from the

synaptic cleft (D. J. David & Gardier, 2016). This increases the bioavailability of serotonin at the receptors on the postsynaptic neurons, allowing this neurotransmitter to exert its mood-alleviating effects (Ruddick et al., 2006). Many patients treated with SSRIs experience a lag phase of approximately 6 weeks until clinical efficacy is observed(Machado-Vieira et al., 2010). While the molecular mechanisms of SSRI actions are slowly being unraveled, there still exists a large gap between our understanding of the causes of MDD and the therapeutic effect of SSRIs as this class of antidepressants appears to target other pathways thought to be important in MDD, for instance, their role in promoting BDNF activity and expression (Joshi, 2018; B.-H. Lee & Kim, 2010, p.).

1.3.2 Prevalence of Antidepressant use

Antidepressants are a pharmacological treatment primarily used for the first-line treatment of MDD but have also been a widely prescribed treatment for other psychiatric illnesses and off-label uses (Lochmann & Richardson, 2019; Schneider et al., 2019; St-Amour et al., 2020; Wong et al., 2017). Nearly half of all antidepressant prescriptions were prescribed to treat illnesses other than MDD, with off-label prescriptions representing between 15–30% of all antidepressant prescriptions (Wong et al., 2016, 2017). This increase in the prescription of antidepressants in recent years is a growing trend that is prevalent worldwide (Chien et al., 2007; Ilyas & Moncrieff, 2012; National Center for Health Statistics (US), 2011; A. J. Smith et al., 2008). Antidepressants are among the top five prescription medications used by Canadians, with reports that the prevalence of antidepressant use in Canada ranges from 10 to 17% of adults reported using an antidepressant medication in the past year (Patten et al., 2015; Samuel et al., 2021; St-Amour et al., 2020). The prevalence of prescription of these drugs is also on a rise, as Canadian Primary Care Sentinel Surveillance Network Study found that between 2006 and 2012 the incidence of prescription of antidepressants in primary care did not rise, although the prevalence of antidepressant prescriptions increased from 9.2% to 12.8% (Morkem et al., 2015). Generally, patients with MDD are initially treated with a single antidepressant drug; this first-line treatment often involves an SSRI such as fluoxetine (Papakostas et al., 2018). A cross-sectional analysis of a nationally representative sample of Canadian adults found of the available antidepressants, SSRIs were the most commonly used class of medication (St-Amour et al., 2020). Indeed, SSRIs are the most widely prescribed class of antidepressants and in Canada, as in other countries, SSRIs account for more than half of all prescriptions for MDD (Samuel et al., 2021; A. J. Smith et al., 2008; Trifirò et al., 2013; Wemakor et al., 2014). Furthermore, there has been a growing prevalence of SSRI usage among Canadian youth evidenced by a 39% increase in SSRI prescriptions written by pediatricians between 2005 to 2009, with fluoxetine being the most commonly prescribed and recommended drug of its class, accounting for nearly one-third of all SSRI use in Canadian youth (D. Lam et al., 2013). Approved by the FDA in 1987, fluoxetine (known by the trade name Prozac) rapidly became the most prescribed SSRI and psychotropic drug worldwide (Leo, 1996; Pinna, 2015; A. J. Smith et al., 2008; Trifirò et

al., 2013; Wemakor et al., 2014). However, recently there has been growing concern surrounding the side effects of these antidepressant medications, specifically pertaining to the associated metabolic consequences (Khawam et al., 2006).

1.4 Depression and Metabolic Dysfunction

Metabolic syndrome is characterized by abdominal obesity, elevated triglycerides, blood pressure, fasting glucose, and low high-density lipoprotein (HDL) cholesterol (Wurtman, 1993). There exists a bidirectional relationship between MDD and metabolic syndrome, however, the factors underlying this association remain to be entirely elucidated (Hasan et al., 2015; Kan et al., 2013; Mezuk et al., 2008; A. Pan et al., 2010; A. Pan, Sun, et al., 2012; Renn et al., 2011; Rotella & Mannucci, 2013). A meta-analysis using data from both cross-sectional and cohort studies found that the development of new-onset MDD was highly associated with the presence of the metabolic syndrome (OR= 1.27; 95% CI 1.07-1.51) (A. Pan, Keum, et al., 2012). It has been suggested that factors related to metabolic syndromes, such as obesity-related stigma and increased activation of pro-inflammatory pathways may act as contributors to the development of new-onset MDD (Shea et al., 2021). On the other hand, MDD has been independently associated with diabetes mellitus and obesity, suggesting that MDD may lead to the development of metabolic syndrome (A. Pan, Keum, et al., 2012). A recent systematic review found that affective disorders, including MDD, can double the risk of metabolic syndrome (Ghanei Gheshlagh et al., 2016). In addition, markers of metabolic syndrome increased with increasing severity of depression, an effect which was independent of age, smoking status, socioeconomic factors, and lifestyle (Kinder et al., 2004), supporting the hypothesis that there is a biological link between MDD and development of metabolic syndrome (Skilton et al., 2007). Links between MDD and poor health-related behaviors such as inadequate dietary and exercise habits are among the possible mediators of this association (Bica et al., 2017). Given the relationships between MDD and metabolic syndrome, it is plausible that similar positive associations may exist between MDD and non-alcoholic fatty liver disease (NAFLD), with studies revealing that MDD may lead to the development of NAFLD. NAFLD is considered to be the hepatic manifestation of metabolic syndrome as there is a strong relationship between hepatic fat accumulation and insulin resistance which is thought to be a key driver of metabolic disease (Khan et al., 2019; Kitade et al., 2017; K. Lee et al., 2013; Watt et al., 2019),

1.4.1 Non-Alcoholic Fatty Liver Disease (NAFLD)

NAFLD is classified as a range of diseases varying from simple hepatic steatosis (ie excess accumulation of triglycerides within hepatocytes) to inflammatory non-alcoholic steatohepatitis (NASH) with different levels of fibrosis. These hepatic changes, such as steatosis, inflammation, and fibrosis, occur in the absence of other known etiologies of

hepatic injury such as significant alcohol consumption and viral hepatitis (Chalasani et al., 2012). In accordance with the 'two-hit' hypothesis of NAFLD, the progression from a healthy liver to steatohepatitis occurs in a stepwise fashion beginning with the development of steatosis which leads to hepatic inflammation. As steatosis persists, the increased inflammation may develop into inflammation, fibrosis, and even cirrhosis of the liver (Purohit et al., 2010). Hepatic steatosis, the initiating step of NAFLD, can be a result of one or more mechanisms including diminished fatty acid oxidation; elevated transport of fatty acids from the peripheral organs to the liver; elevated *de novo* fatty acid synthesis, and reduced transport of fatty acids from the liver to the general circulation and peripheral organs (Cimini et al., 2017; Mallat et al., 2011). An estimated 25% of Canadians currently have NAFLD, making it the most common liver disease in Canada (Morris, 2014). With the growing prevalence of obesity, the incidence of NAFLD is also on the rise (Glasgow et al., 1997) yet there is a lack of treatment options for this disease (Younossi et al., 2016). Examining exogenous compounds' contributions to hepatic steatosis ('first hit' of NAFLD) is important as it may prove useful in preventing the subsequent inflammation, fibrosis, and even cirrhosis of the liver associated with severe NAFLD (Cholankeril et al., 2017; Mallat & Lotersztajn, 2008).

1.4.2 Bidirectional Relationship Between MDD and NAFLD?

Although there appears to be a consistent association between MDD and metabolic syndrome, the nature of an association between NAFLD and MDD is less clear. Some studies suggest that there is a strong positive association between NAFLD and MDD whereas other studies report no association at all (K. Lee et al., 2013; Q. Ma et al., 2021; Tomeno et al., 2015; Weinstein et al., 2011). Some studies suggest NAFLD may be a major contributor to MDD (Labenz et al., 2020; Weinstein et al., 2011; J. Xiao et al., 2021; Youssef et al., 2013). For example, Weinstein and colleagues utilized clinical and self-reported data from patients with chronic liver disease and reported that patients with NAFLD had a 27.2% prevalence of MDD, a percentage that eclipses the prevalence of MDD in the control population (2%−5%) (Weinstein et al., 2011). Using a database of 567 patients with biopsy-confirmed NAFLD, Youssef *et al*. examined this association further and discovered a dose-dependent relationship between the severity of depressive symptoms and the degree of histological severity of NAFLD, after adjustment for potential confounding factors (Youssef et al., 2013). This finding was further supported by a recent study involving a Korean population, which found a positive relationship between the severity of NAFLD and depression, supporting the notion that advanced stages of NAFLD potentially had a stronger association with depression, even after adjusting for confounding factors(Jung et al., 2019). A 10-year retrospective follow-up study by Labenz et al. assessed the incidence of depression in 19,871 NAFLD patients and discovered a significant association between MDD and NAFLD (HR: 1.21, 95% CI: 1.14–1.26, $p < 0.001$)(Labenz et al., 2020). The 10-year incidence of MDD after controlling for variables including diabetes and obesity was 18.2% in individuals without NAFLD, but 21.2% in patients with NAFLD ($p < 0.001$), suggesting an elevated incidence of MDD in patients

with NAFLD independent of these comorbidities (Labenz et al., 2020). Additionally, a meta-analysis and systematic review aimed at assessing this association found a 15% prevalence of depression in NAFLD patients with a significantly heightened risk of development of MDD in patients with NAFLD (OR: 1.29, 95% CI: 1.02–1.64, $p = 0.03$) (J. Xiao et al., 2021). Alternatively, population-based analysis of 10,231 adult patients with chronic liver disease, data obtained from the National Health and Nutrition Examination Survey (NHANES 2005–2010), found that MDD was not associated with NAFLD after controlling for confounders including components metabolic syndrome, which is in contrast to the notion of NAFLD being a mediator of MDD (K. Lee et al., 2013). This led authors to suggest that other studies that have found this association to have potentially been influenced by the difficulty of carefully controlling for other components of metabolic syndrome such as type 2 diabetes or obesity (Carta et al., 2007; K. Lee et al., 2013).

While MDD is prevalent in patients with NAFLD, some studies have shown that MDD can mediate the onset and development of NAFLD (Shao et al., 2021). Compared with non-depressed patients, patients with subclinical MDD were 2.1 times more likely to display excessive hepatocyte lipid accumulation, while patients with clinical MDD were 3.6 times more likely to display excessive hepatocyte lipid accumulation (Youssef et al., 2013). This finding was supported by a more recent study conducted on data from U.S. adults between 2007 to 2016, which found that MDD patients were 1.6 to 2.2 times more likely to have NAFLD, relative to those without MDD, further supporting the possibility of MDD mediating the progression of NAFLD (D. Kim et al., 2019). Although numerous clinical and epidemiological studies have reported a potential association between MDD and NAFLD, the psychiatric medications used to treat MDD may also pose an independent risk factor for the development of MDD, thus adding a layer of complexity to the MDD-NAFLD paradigm (Shea et al., 2021).

1.4.3 Are Antidepressants the missing link between MDD and metabolic disease

It has been well documented in the literature that SSRI use can contribute to the development of adverse metabolic outcomes including weight gain, type 2 diabetes, and NAFLD, effectively limiting patient adherence to therapy (Deuschle, 2013; Joshi, 2018; A. Pan, Sun, et al., 2012; Rubin et al., 2008). While weight gain or loss in MDD can be the result of disordered eating, significant weight gain during the acute phase of treatment or weight gain that continues despite achieving full remission of depressive symptoms is to be expected to be a side effect of antidepressant treatment (Fava & Kendler, 2000). Approximately 2% of fatty liver disease cases are estimated to be drug-induced, mostly associated with prolonged intake of medications (Farrell, 2002; Pavlik et al., 2019; Rabinowich & Shibolet, 2015; Satapathy et al., 2015). Although the relationship is complex, there is a substantial body of evidence suggesting some classes of antidepressants may increase weight in a significant proportion of patients, with some suggesting that increased antidepressant usage may be a driving force for the obesity pandemic (S. H. Lee et al., 2016; Serretti & Mandelli, 2010). While SSRI use has been associated with weight

loss during acute treatment, several studies have indicated that long-term use, extending beyond six months of treatment, was associated with weight gain (S. H. Lee et al., 2016). For example, the Hordaland Health Study analysis demonstrated a link between the use of SSRIs and abdominal obesity (OR = 1.40, 95% CI = 1.08 to 1.81) in 461 subjects (Raeder et al., 2006; Skilton et al., 2007). Further, nearly half of all patients taking SSRI antidepressants experience significant weight gain (Olfson & Marcus, 2010). Additionally, studies examining mean weight gains in participants after 6 to 12 months of SSRI therapy have shown an average increase of 15 lbs (6.75 kg), 24 lbs (10.80 kg), and 21 lbs (9.45 kg) for people taking sertraline, paroxetine and for fluoxetine respectively (Ferguson, 2001; Raeder et al., 2006; Sussman & Ginsberg, 1998). In addition to weight gain, SSRI use has been associated with an increased risk of new-onset diabetes. A systematic review and meta-analysis found that adults with any use of antidepressants, including SSRIs and TCAs, were more likely to develop new-onset diabetes compared with those without any use of antidepressants (OR = 1.5, 95% CI 1.08-2.10; HR = 1.19, 95% CI 1.08-1.32) (Bhattacharjee et al., 2013; Derijks et al., 2008; Khoza & Barner, 2011; Yoon et al., 2013). Taken together these studies provide a strong basis to support the hypothesis that SSRI use may play a role in the development of hallmarks of metabolic syndrome (Deuschle, 2013; Labenz et al., 2020; A. Pan, Sun, et al., 2012; Rubin et al., 2008).

Similarly, there is increasing evidence to suggest that SSRI antidepressant use is associated with an increased risk of NAFLD. A recent population-based study utilizing The Health Improvement Network database, the largest medical database in the United Kingdom, was used to identify incident NAFLD (n = 19,053) in patients between 1986 and 2017 (Shaheen et al., 2021). After adjusting for age, sex, socio-economic status, and comorbidities, Shaheen and colleagues found that antidepressant usage was more common in patients with NAFLD (40.8%), relative to the general population (11%) (Abbing-Karahagopian et al., 2014; Heald et al., 2020; Shaheen et al., 2021). Although there is a paucity of evidence in clinical literature, animal and cell culture studies suggest that SSRIs can cause increased hepatic steatosis (X.-M. Feng et al., 2012; Xiong et al., 2014). SERT knockout animal models are similar to those exposed to SSRIs in that in these models there is attenuated serotonin uptake from the synaptic cleft (D. J. David & Gardier, 2016) which is similar to the mechanism of action of SSRI antidepressants. Chen et al. demonstrated that SERT knockout mice had hepatic steatosis independent of food intake, with pronounced hepatic steatosis in 6-month-old SERT knockout mice fed a normal diet (X. Chen et al., 2012). Studies have also reported that *in vivo* administration of SSRIs leads to excess lipid accumulation in the liver (Carvalho et al., 2016; Wilde et al., 1993). Similarly, treatment of hepatic cell lines with fluoxetine has resulted in an increase in hepatic lipid accumulation through the promotion of lipogenesis and reduction of lipolysis (X.-M. Feng et al., 2012; Xiong et al., 2014). These findings, among others, prompted further research into the mechanism by which SSRIs may be contributing to the pathogenesis of NAFLD (Shaheen et al., 2021; Youssef et al., 2013).

1.5 Rationale

MDD is a prevalent disorder that is predicted to be the second leading cause of disease burden worldwide by the year 2030 (World Health Organization, 2017). Pharmacotherapy continues to be the leading treatment for MDD, with SSRIs being the most prescribed (Samuel et al., 2021; A. J. Smith et al., 2008; Trifirò et al., 2013; Wemakor et al., 2014). In fact, SSRI use has been associated with metabolic dysfunction and NAFLD, an association that requires further exploration (Deuschle, 2013; Labenz et al., 2020; A. Pan, Sun, et al., 2012; Rubin et al., 2008; Shaheen et al., 2021). NAFLD progression occurs in a stepwise fashion beginning with the development of steatosis, an excess accumulation of triglycerides within hepatocytes. As steatosis persists, it can lead to increased inflammation which in its later stages leads to fibrosis, and even cirrhosis of the liver (Purohit et al., 2010). Current estimates suggest that nearly 25% of Canadians currently suffer from NAFLD, making it the most common liver disease in Canada (Morris, 2014). This thesis aims to investigate fluoxetine, a widely prescribed SSRI antidepressant, for its role in the pathogenesis of NAFLD and uncover novel mechanisms by which it may contribute to drug-induced steatosis. Of particular interest was the effects of fluoxetine on the regulation of peripheral serotonin signaling pathways. Interestingly, serotonin (5-HT) produced in peripheral tissues is an important regulator of metabolism and energy storage by promoting insulin secretion and hepatic *de novo* lipogenesis as well as by decreasing lipolysis in adipose tissue (Yabut et al., 2019). It has been suggested that SSRIs can inhibit SERT function in metabolically active tissues, thus leading to prolonged serotonin effects including increased liver gluconeogenesis, decreased hepatic glucose uptake, increased hepatocyte lipid storage, and progression of NAFLD (Yabut et al., 2019). Furthermore, exposure to SSRIs have previously been shown to disrupt the central serotonergic system resulting in elevated neuronal serotonin content *in vitro*, as well as elevated serotonin biosynthetic enzyme activity and protein expression *in vivo* (S. W. Kim, 2002). Also of importance, activation of serotonin receptor signaling pathways may also contribute to NAFLD. The 5-HT_2 receptors are a receptor family comprised of three subtypes (5-HT_{2A}, 5-HT_{2B}, 5-HT_{2C} subtypes), all of which are structurally distinct but functionally conserved (Julius et al., 1990; Locker et al., 2006). It has recently been shown that peripheral serotonin acts via the 2A serotonin receptor (5-HT_{2A}) to up-regulate the expression of lipogenic proteins resulting in hepatic steatosis (Choi et al., 2018; Niture et al., 2018; L. Wang, Fan, et al., 2020). Among the subtypes of 5-HT receptors, liver-specific 5-HT_{2A} knockout mice showed a reduction in liver size, weight, and lipid accumulation, as indicated by histological data, NAFLD activity score, and hepatic triglyceride concentrations, without affecting systemic energy homeostasis (Choi et al., 2018) These 5-HT_{2A} receptors are G protein-coupled receptors and are recognized for being coupled to the phospholipase A2 (PLA2) signaling pathway, stimulating the release of the second messenger, arachidonic acid (AA) from membrane phospholipids (Felder et al., 1990; Qu et al., 2003). Arachidonic acid serves as a precursor to several biologically active acid lipids, including prostaglandins, leukotrienes, and thromboxanes (Calder, 2020). The prostaglandins are especially important as increased levels of certain prostaglandins are associated with

NAFLD (Chung et al., 2014; Kumar et al., 2020; Maciejewska et al., 2020; Qin et al., 2015). Some of these prostaglandins, such as 15d-PGJ2 are endogenous PPARG ligands, and PPARG acts as a master regulator of lipid homeostasis in hepatocytes. We aimed to further explore the influence of fluoxetine on PPARG with a focus on epigenetic mechanisms linking PPARG activation to changes in miRNA signatures and mRNA expression of lipogenic gene targets. Interestingly, the PPARG pathway has been shown to influence several miRNAs, many of which have been shown to be altered by fluoxetine treatment and NAFLD (Baudry et al., 2010a; Craig et al., 2014; Launay et al., 2011; Miao et al., 2018). An understanding of the molecular changes associated with SSRI treatment may lead to the development of strategies to prevent the harmful effects of SSRI antidepressants with regard to NAFLD.

1.6 Hypothesis

The overall hypothesis of this thesis is that the SSRI fluoxetine induces hepatic lipid accumulation.

1.7 Objectives

1.7.1 Objective 1

The first objective of this Ph.D. thesis was to determine whether a model SSRI, fluoxetine, could affect hepatic lipid accumulation *in vitro* via changes in peripheral serotonin production. At the time of undertaking this project, there were no studies to our knowledge addressing the effects of fluoxetine on hepatic serotonin production. Based on emerging evidence suggesting that peripheral serotonin production may be instrumental to the pathophysiology of NAFLD, and the use of SSRI antidepressants has been linked to hepatic lipid accumulation and NAFLD, we aimed to investigate if the SSRI fluoxetine was linked to altered serotonin signaling which may in part, contribute to hepatic lipid accumulation. I hypothesized that SSRIs could alter serotonin production and induce changes in hepatic lipid accumulation in hepatocytes.

1.7.2 Objective 2

In objective 1, I identified that in hepatic cells exposed to the SSRI antidepressant, fluoxetine resulted in increased hepatic lipid accumulation, which may in part be linked to elevated serotonin production. Since peripheral serotonin acts via the 5-HT_{2A} receptor, an effect has been shown to result in an up-regulation of the expression of lipogenic proteins resulting in hepatic steatosis. Activation of the 5-HT_{2A} receptor has been shown to activate phospholipase A2 (PLA2) to release the second messenger, arachidonic acid which is a

primary substrate for prostaglandin synthesis (Basselin et al., 2005; Choi et al., 2018; Felder et al., 1990; Niture et al., 2018; Qu et al., 2003; L. Wang, Fan, et al., 2020). Although arachidonic acid serves as a precursor to several biologically active acid lipids, including prostaglandins, leukotrienes, and thromboxanes, I explored the prostaglandin pathway further due to the literature that implicates prostaglandins in the progression of NAFLD (Calder, 2020; Chung et al., 2014; Kumar et al., 2020; Maciejewska et al., 2020; Qin et al., 2015). '15-deoxy-Δ12,14-prostaglandin J2' (15d-PGJ2) acts as a potent endogenous ligand for peroxisome proliferator-activated receptor γ (PPARG) and may play a crucial role in the fluoxetine-induced lipid accumulation (Álvarez-Almazán et al., 2017; Fujitani et al., 2010; J. Li et al., 2019). PPARG serves as a ligand-dependent transcription factor that plays a pivotal role in the regulation of lipid utilization and storage, which are likely to contribute to the development of NAFLD (Yamazaki et al., 2011). In this chapter, I investigated the role of altered prostaglandin signaling in SSRI-induced hepatic lipid accumulation. I hypothesized that SSRIs could alter serotonin production and induce changes in hepatic lipid accumulation in hepatocytes.

1.7.3 Objective 3

In objective 2, I demonstrated that SSRI-induced lipid accumulation was mediated by prostaglandin-endoperoxide synthase 1 (Ptgs1), which was linked to elevated 15-deoxy-$\Delta^{12,14}PGJ_2$, and resulted in increased expression of *Pparg* and its downstream targets. My next objective explored the influence of fluoxetine exposure on lipid accumulation via a miRNA-PPARG pathway. Of particular interest were miRNAs that are altered by fluoxetine exposure but are also central to the progression of NAFLD. Fluoxetine has been shown to alter miRNA signatures and has recently been implicated in its role in the progression of liver steatosis, inflammation, fibrosis, and cirrhosis (Soto-Angona et al., 2020) We performed a literature review and selected the primary miRNA candidates to investigate in fluoxetine treated hepatocytes. Interestingly, miR-122 has been recently shown to be regulated by the transcription factor PPARG, which has been shown in our earlier chapter to be altered by fluoxetine exposure (A. Ayyash & Holloway, 2021b). In this chapter, I further examined the effects of fluoxetine treatment on the expression of miRNAs linked to NAFLD. I hypothesized that the ability of fluoxetine to cause lipid accumulation in hepatocytes is mediated, in part, via changes in the expression of miRNA which regulate lipogenic genes.

2 Chapter 2

Fluoxetine-induced Hepatic Lipid Accumulation is Linked to Elevated Serotonin Production.

Title: Fluoxetine-induced Hepatic Lipid Accumulation is Linked to Elevated Serotonin Production

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2.1 Abstract

Fluoxetine, a commonly prescribed selective serotonin reuptake inhibitor antidepressant, has been shown to increase hepatic lipid accumulation, a key factor in the development of non-alcoholic fatty liver disease. Interestingly, fluoxetine has also been reported to increase peripheral serotonin synthesis. As emerging evidence suggests that serotonin may be involved in the development of non-alcoholic fatty liver disease we sought to determine if fluoxetine-induced hepatic lipid accumulation is mediated via altered serotonin production. Fluoxetine treatment increased lipid accumulation in association with increased mRNA expression of tryptophan hydroxylase 1 (*Tph1*, serotonin biosynthetic enzyme) and intracellular serotonin content. Serotonin alone had a similar effect to increase lipid accumulation. Moreover, blocking serotonin synthesis reversed the fluoxetine-induced increases in lipid accumulation. Collectively, these data suggest that fluoxetine induced lipid accumulation can be mediated, in part, by elevated serotonin production. These results suggest a potential therapeutic target to ameliorate the adverse metabolic effects of fluoxetine exposure.

Keywords: Selective Serotonin Reuptake Inhibitor (SSRI), Fluoxetine, Steatosis, Non-Alcoholic Fatty Liver Disease (NAFLD), *de novo* Lipogenesis, Serotonin, Metabolic Disorder

2.2 Introduction:

In Canada, it has been reported that 11.3 % of people aged 15 and older had symptoms consistent with depression, with approximately one-third of individuals reported taking antidepressants to manage symptoms (Patten et al., 2015). Pharmacotherapy is the leading option for treatment and management of moderate to severe depression, with a marked increase of nearly 500% in the percentage of adults taking antidepressants between 1988 and 2008 (National Center for Health Statistics (US), 2011). In particular, selective serotonin reuptake inhibitors (SSRIs) have been the leading class of antidepressants drugs prescribed in Canada (A. J. Smith et al., 2008). While SSRIs are effective at managing depression in many patients, they have also been implicated in the development of adverse metabolic outcomes including weight gain, type 2 diabetes and non-alcoholic fatty liver disease (NAFLD) (De Long, Stepita, et al., 2015).

NAFLD is characterized by excess accumulation of lipid in the liver in the absence of excessive alcohol intake (Chalasani et al., 2012). An estimated 25% of Canadians currently suffer from NAFLD, making it the most common liver disease in Canada (Morris, 2014). NAFLD has the potential to progress into more serious illnesses such as nonalcoholic steatohepatitis (NASH), cirrhosis and fibrosis of the liver, and ultimately, hepatocellular carcinoma. This poses a significant clinical and economic burden as the rising rate of NAFLD is compounded with the lack of treatment (Younossi et al., 2016). There is now considerable evidence that long-term SSRIs use may lead to an increased prevalence of metabolic disturbances including the hepatic lipid accumulation which is characteristic of NAFLD. Indeed, animal experiments and cell culture studies have demonstrated that exposure to the commonly prescribed SSRI antidepressant fluoxetine significantly increases hepatic lipid accumulation (De Long et al., 2017; X.-M. Feng et al., 2012; Lu et al., 2020; S. Pan et al., 2018a; Xiong et al., 2014). The mechanisms by which fluoxetine can induce hepatic lipid accumulation have not been fully elucidated but may involve its ability to modulate peripheral serotonin signaling. Emerging evidence suggests that serotonin production in the periphery may be instrumental to the pathophysiology of NAFLD (Choi et al., 2018; Crane et al., 2015; Yabut et al., 2019); peripheral serotonin has been reported to act via the 2A serotonin receptor (HTR2A) to up-regulate the expression of lipogenic proteins and increase hepatic steatosis (Choi et al., 2018; Niture et al., 2018; L. Wang, Fan, et al., 2020). SSRIs including fluoxetine have been shown to increase serotonin synthesis in association with an increase in the expression of tryptophan hydroxylase 1 (Tph1), the rate-limiting enzyme in the synthesis of peripheral serotonin (Abumaria et al., 2007; Baik et al., 2005; S. W. Kim et al., 2002). However, no study has ascribed fluoxetine-induced changes in hepatic lipid accumulation to altered serotonin production. Therefore, the goal of this study was to test the hypothesis that fluoxetine exposure contributes to excess hepatic lipid accumulation via increased serotonin production.

2.3 Materials and Methods:

2.3.1 Cell Culture Maintenance and Treatment

H4-II-E-C3 hepatoma cells were grown in Corning™ Dulbecco's modified Eagle's medium (DMEM) (Corning, NY) supplemented with 10 % heat-inactivated fetal bovine serum (Hyclone, South Logan, UT), 2% L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin (Gibco, Long Island, NY) at 37 °C in a humidified atmosphere of 95 % O_2 and 5 % CO_2.

All cell treatments were made in supplemented DMEM as described above. When H4-II-E-C3 cells reached 80% confluence, they were treated for 6 and 24 h with 10 µM fluoxetine hydrochloride (Toronto Research Chemicals, North York, ON) to investigate its effect on lipid accumulation and serotonin synthesis (N = 5 independent experiments). Significant lipid accumulation has previously been shown in mouse liver at 6 and 24 h following a single dose of fluoxetine (10 mg/kg or 25 mg/kg) (Feng et al., 2011). Elevated lipid accumulation was also observed in primary mouse hepatocytes following a 24 h exposure to 10µM fluoxetine (X.-M. Feng et al., 2012; Xiong et al., 2014). Based on these results we selected 6 h of treatment to determine any early changes in gene expression and 24 h as a timepoint where we expected to see lipid accumulation based on prior *in vitro* studies. (X.-M. Feng et al., 2011, 2012; Xiong et al., 2014). Next, cells were treated for 24h with 500µM of serotonin hydrochloride (MilliporeSigma, Burlington, MA), a dose previously shown to promote steatosis in other liver cancer cell lines, to investigate the effects of serotonin on lipid accumulation (Niture et al., 2018). To examine the contribution of serotonin production in fluoxetine-induced lipid accumulation, cells were treated for 24 h with 10µM fluoxetine ± 50 µM *para*-chlorophenylalanine (4-Chloro-DL-phenylalanine; PCPA) (Sigma-Aldrich), an inhibitor of tryptophan hydroxylase, the rate-limiting enzyme for serotonin synthesis (Koe & Weissman, 1966) to assess changes in lipid accumulation and intracellular serotonin synthesis.

2.3.2 Quantitative Real-Time PCR

To determine changes in gene expression in each experiment, we assessed steady-state mRNA expression of fatty acid synthase [*Fasn;* Forward 5′-GAG-TCC-GAG-TCT-GTC-TCC-CGC-TTG-A, Reverse GCC-GTG-AGG-TTG-CTG-TTG-TCT-GTA-G,], and tryptophan hydroxylase 1 [*Tph1;* Forward 5′-TGG-CTA-TCG-GGA-AGA-CAA-CG-3', Reverse 5′-GGA-CGG-CTG-GAA-AAC-CCT-GT-3'] as markers of lipid accumulation and serotonin synthesis respectively using real-time quantitative PCR. After treatment, cells were washed with phosphate-buffered saline (PBS), and total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA). RNA concentrations were determined using the NanoDrop One™ Microvolume UV-Vis Spectrophotometer (Thermo Scientific, Waltham, MA). Complementary DNA (cDNA) was made from 2 µg of mRNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) as per the manufacturer's instructions. The resulting cDNA was a template for qPCR, which was carried out using PerfeCTa® SYBR® Green FastMix® (Quanta Biosciences,

Gaithersburg, MD) on the CFX384 Touch™ Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA). The PCR cycling settings included polymerase activation (95 °C for 10 m), followed by 40 cycles of denaturing (95 °C for 15 s), and annealing/elongation (60 °C for 1 m). Levels of gene expression were generated using the ΔΔCt method (Livak & Schmittgen, 2001) and normalized using the geometric means of three reference genes Glyceraldehyde 3-phosphate dehydrogenase [*GAPDH;* Forward 5′-TGG-AGT-CTA-CTG-GCG-TCT TCA-C-3', Reverse 5′-GGC-ATG-GAC-TGT-GGT-CAT-GA-3'], Beta-actin [*ACTB*; Forward 5′-CAC-AGC-TGA-GAG-GGA-AAT-3', Reverse 5′-TCA-GCA-ATG-CCT-GGG-TAC-3'] and [*RPS18*; Forward 5′-GCG-ATG-CGG-CGG-CGT-TAT-3', Reverse 5′-AGA-CTT-TGG-TTT-CCC-GGA-AGC-3'].

2.3.3 Lipid Staining and Quantification

To assess the effects of our cell treatments on lipid accumulation, cells were fixed with 10% formaldehyde followed by staining with Oil Red O (Sigma-Aldrich) solution for 20 m and then washed with water as previously described (Ramírez-Zacarías, Castro-Muñozledo, and Kuri-Harcuch, 1992). The samples' absorbance was measured at 510 nm in a plate reader (Synergy™ H4 Hybrid Microplate Reader, BioTek Instruments, Winooski, VT, USA).

2.3.4 Intracellular serotonin content

To determine effects of fluoxetine ± PCPA on intracellular serotonin content, at the end of the treatment period cells were pelleted by centrifugation (2000 rpm for 5 m), re-suspended in RIPA lysis buffer (15mM Tris–HCl, 1%(v/v)Triton X 100, 0.1% (w/v) SDS, 167 nM NaCl, 0.5% (w/v) sodium deoxycholic acid), with Complete Mini EDTA-free protease inhibitor cocktail tablets (Roche Applied Science) followed by sonication (Misonix 2000, Qsonica LLC., Newtown, CT) at 7Hz for 15s. Intracellular serotonin concentrations were determined using a commercially available ELISA kit according to the manufacturer's instructions (Cat# BA E-8900, Serotonin: LDN, Nordhorn, Germany). Absorbance was measured at 450 nm (Synergy™ H4 Hybrid Microplate Reader, BioTek Instruments, Winooski, VT).

2.3.5 Statistical Analysis

All statistical analyses were conducted using SigmaPlot (v.11.2, Systat Software, San Jose, CA). Data were tested for outliers (Grubbs' test), normality, and equal variance. Comparisons among the two groups were analyzed using Student's t-tests. Comparisons among multiple groups were analyzed using One-Way Analysis of Variance (ANOVA) followed by the Bonferroni multiple comparisons test. When normality or equal variance failed, the Mann-Whitney Rank Sum Test or Student-Newman-Keuls One-Way ANOVA on Ranks were used to determine significance. All data are presented as mean ± SEM and were considered significant when $P \leq 0.05$.

2.4 Results:

After 24 h, cells exposed to 10μM fluoxetine had significantly increased lipid accumulation in association with increased mRNA expression of fatty acid synthase, a key marker of *de novo* lipogenesis (Figure 1A & 1B). Treatment with exogenous serotonin for 24 h significantly increased both lipid accumulation (Figure 1C) and mRNA expression of *Fasn* (Figure 1D). At this same dose, fluoxetine significantly increased expression of *Tph1* at both 6 and 24 h (i.e. prior to measurable lipid accumulation) and intracellular serotonin synthesis was significantly increased at 24 h (Figure 2) suggesting fluoxetine-induced lipid accumulation may be mediated, in part, via increased serotonin production. To confirm that increased serotonin production mediates fluoxetine's effects on lipid accumulation, cells were treated with fluoxetine ± PCPA. Fluoxetine significantly increased intracellular serotonin content and lipid accumulation whereas co-treatment with PCPA attenuated fluoxetine-induced increases in serotonin and lipid accumulation (Figure 3).

2.5 Discussion:

Depressive disorders are frequently managed with long-term use of SSRI antidepressant medications, including the commonly prescribed SSRI fluoxetine. However, there is increasing evidence from clinical studies, animal experiments, and cell culture studies that SSRIs can cause perturbations in lipid metabolism which may lead to metabolic disease with long term use (X.-M. Feng et al., 2012; Fjukstad et al., 2016; S. Pan et al., 2018a; Xiong et al., 2014). Cell-based studies have shown that exposure to fluoxetine increased lipid accumulation in association with increased expression of key components of *de novo* lipogenesis in primary hepatocytes (X.-M. Feng et al., 2012; Xiong et al., 2014). Similarly in this study, we observed an increase in lipid accumulation and steady-state mRNA expression of fatty acid synthase, a key enzyme in *de novo* lipogenesis (Maier et al., 2006; Rendina & Cheng, 2005), following exposure to fluoxetine. These results are consistent with an observed increase in hepatic triglyceride content and fatty acid synthase protein expression in mice treated for four weeks with fluoxetine (Pan et al., 2018) however the mechanism(s) remain to be fully elucidated.

It is well established that exposure to SSRIs can disrupt the central serotonergic system resulting in alterations in brain serotonin content (Kroeze et al., 2012). However, more recently exposure to SSRIs has also been shown to alter peripheral serotonin levels (S. W. Kim et al., 2002). Peripheral serotonin, which accounts for 95% of circulating serotonin, is primarily synthesized in the gastrointestinal tract, but all of the key components of the serotonergic pathway including the plasma membrane serotonin transporter, serotonin receptors and tryptophan hydroxylase (Tph1; the rate-limiting enzyme for peripheral serotonin production) have been identified in the liver (Choi et al., 2020; Kyritsi et al., 2020; Y. Nagao et al., 2011). Importantly, serotonin has been shown to increase lipid accumulation and the expression of lipogenic enzymes, including fatty synthase, in hepatic cells (Niture et al., 2018). In rats with experimentally induced fatty liver, serum serotonin levels were positively correlated with a NAFLD activity score; a similar correlation was observed in humans with NAFLD (L. Wang, Fan, et al., 2020). Moreover, pharmacological inhibition or genetic deletion of *Tph1 in vivo* has been reported to reduce hepatic lipid accumulation (Crane et al., 2015; Namkung et al., 2018). Therefore, we hypothesized that the ability of fluoxetine to increase hepatic lipid accumulation may be mediated in part via increased hepatic serotonin production. We observed a significant increase in the expression of *Tph1* following 6 and 24 h of treatment. The increase in *Tph1* expression at 6 h preceded any measurable changes in lipid accumulation, however by 24 h there was a significant increase in both lipid accumulation and intracellular serotonin content.

We hypothesized that an increase in serotonin could lead to increased lipid accumulation. Indeed, consistent with what has been reported in Hep-G2 and SK-Hep-1 liver cells (Niture et al., 2018), treatment of H4-II-E-C3 hepatoma cells with serotonin in this study resulted in a significant increase in lipid accumulation and expression of fatty acid synthase. To confirm that increased serotonin can be causally related to fluoxetine-induced lipid accumulation, we treated cells with PCPA, an inhibitor of serotonin synthesis. PCPA

treatment abolished the fluoxetine-induced increase in serotonin synthesis and lipid accumulation suggesting that hepatic serotonin production is mechanistically linked to the lipid perturbations reported following fluoxetine exposure. Interestingly, the tricyclic antidepressant amitriptyline has also been reported to increase hepatic steatosis (Kampa et al., 2020) and serotonin release (Nagayasu et al., 2013); these data suggest that perturbations in serotonin synthesis or signaling may play a role in the metabolic deficits associated with the use of tricyclic antidepressants (Dortland et al., 2010). While our study suggests that hepatic serotonin production is important for fluoxetine-induced hepatic lipid accumulation, Choi et al. (2018) reported that *Tph1* was not expressed in murine liver. Conversely, Wang et al. (2020) reported *Tph1* mRNA expression in the liver but did not find any increase in hepatic *Tph1* expression in association with increased serum serotonin concentrations and elevated hepatic triglycerides in a mouse model of NAFLD. However, as Wang et al. (2020) only measured *Tph1* mRNA expression after 50 days on a steatogenic diet it is possible that changes in hepatic serotonin synthesis are an earlier event in the development of steatosis. Alternatively, it is possible that the relationship between increased serotonin production and fluoxetine-induced lipid accumulation is unique to this class of medications and does not extend to diet-induced models of NAFLD. In the present study, we demonstrated that hepatic lipid accumulation following fluoxetine treatment is mediated, in part, via increased serotonin production. Although it has been shown that serotonin can act through its receptor, HTR2A, to increase hepatic steatosis, there is evidence in other cell lines that fluoxetine decreases HTR2A expression (Koura et al., 2019). Moreover, fluoxetine has also been reported to inhibit the activity of other monoamine transporters, including the dopamine (*SLC6A3*) and norepinephrine (*SLC6A2*) transporters (Owens et al., 2001; Zwartsen et al., 2017). Dopamine agonists have been shown to cause dysregulated hepatic lipid accumulation and both dopamine and epinephrine induce an inflammatory response in liver cells (Aninat et al., 2008), a key component of NAFLD (Chalasani et al., 2012). Therefore, although our results suggest a key role for increased serotonin production underlying fluoxetine-induced hepatic lipid accumulation, it may also involve changes in expression of serotonin receptors or signaling by other monoamines. Further studies are warranted to identify targets of intervention for the treatment or prevention of NAFLD in people using antidepressants and reduce the adverse metabolic consequences associated with this class of medications.

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2.8 Figures:

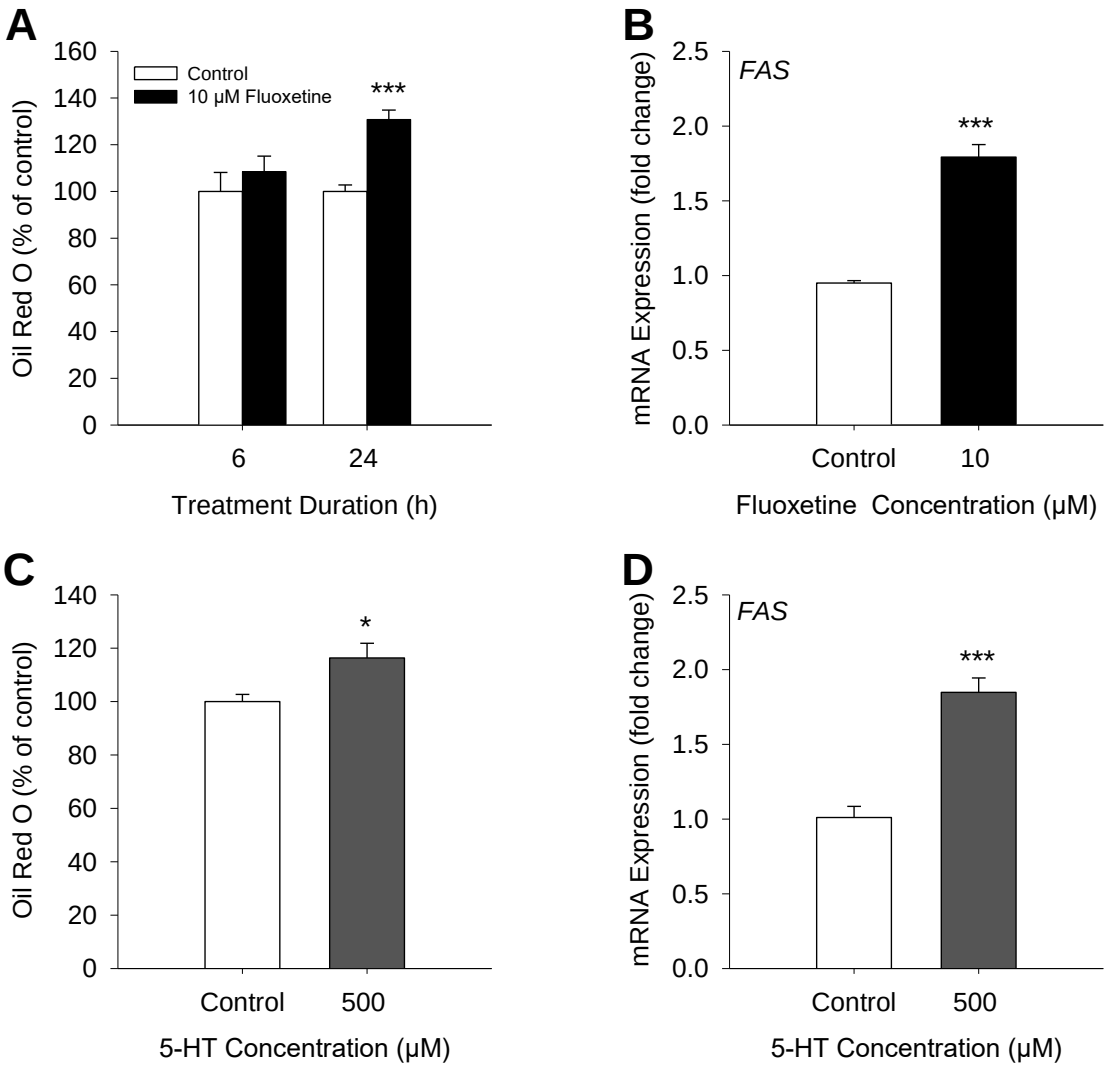

Figure 1. (**A**) Lipid accumulation was determined by Oil Red O staining following treatment with fluoxetine, for 6 and 24 h. (**B**) Relative mRNA expression of enzyme *Fas* following 24 h treatment with 10μM fluoxetine. ***, $P \leq 0.001$ vs. control group calculated via Student's T-test. **(C**) Lipid accumulation was determined by Oil Red O staining following 24 h of treatment with Serotonin (5-HT). *, $P \leq 0.05$ vs. control group calculated via Student's T-test. (**D**) Relative mRNA expression of key hepatic lipogenic enzyme *Fas* following 24 h of treatment with 5-HT. ***, $P \leq 0.001$ vs. control group calculated via Student's T-test. All data presented as mean ± SEM, (n = 4-5 independent experiments).

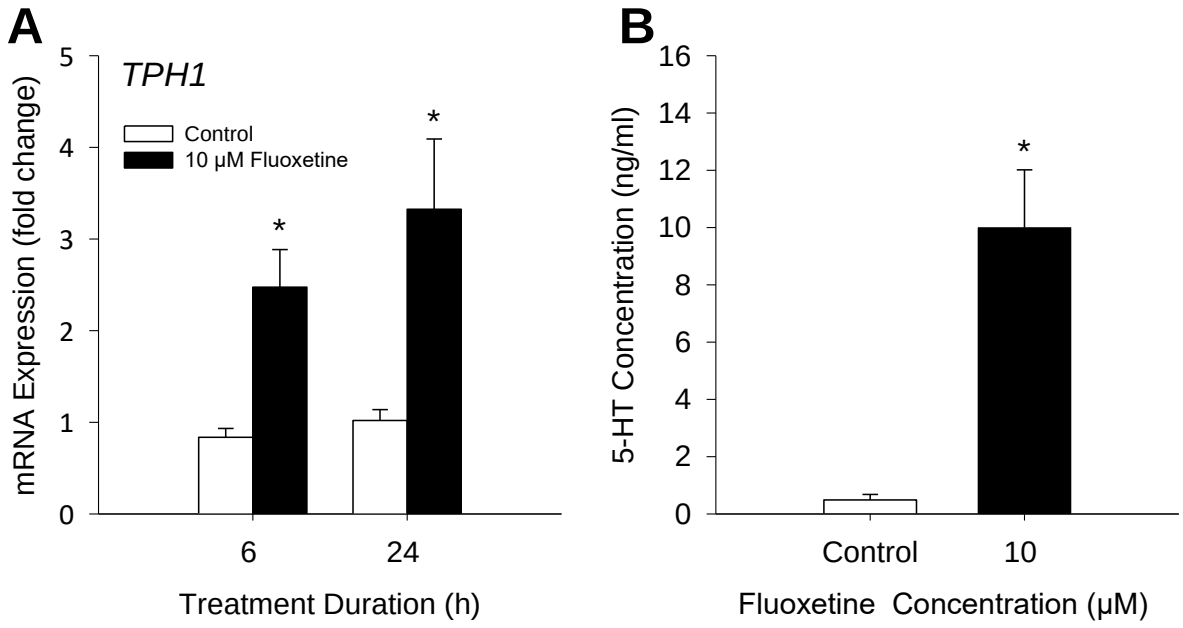

Figure 2. (**A**) Measurement of rate-limiting enzyme *Tph1* relative mRNA expression in H4-II-E-C3 treated with 10μM fluoxetine, for 6 and 24 h. *, $P \leq 0.05$ vs. control group calculated via Student's T-test. (**B)** Intracellular serotonin was quantified at these same doses 24 h post-treatment using commercially available serotonin ELISA as per the manufacturer's instructions (LDN, Nordhorn, Germany). $P \leq 0.05$ vs. control group calculated via Student's T-test. All data are represented as mean ± SEM, (n = 4-5 independent experiments).

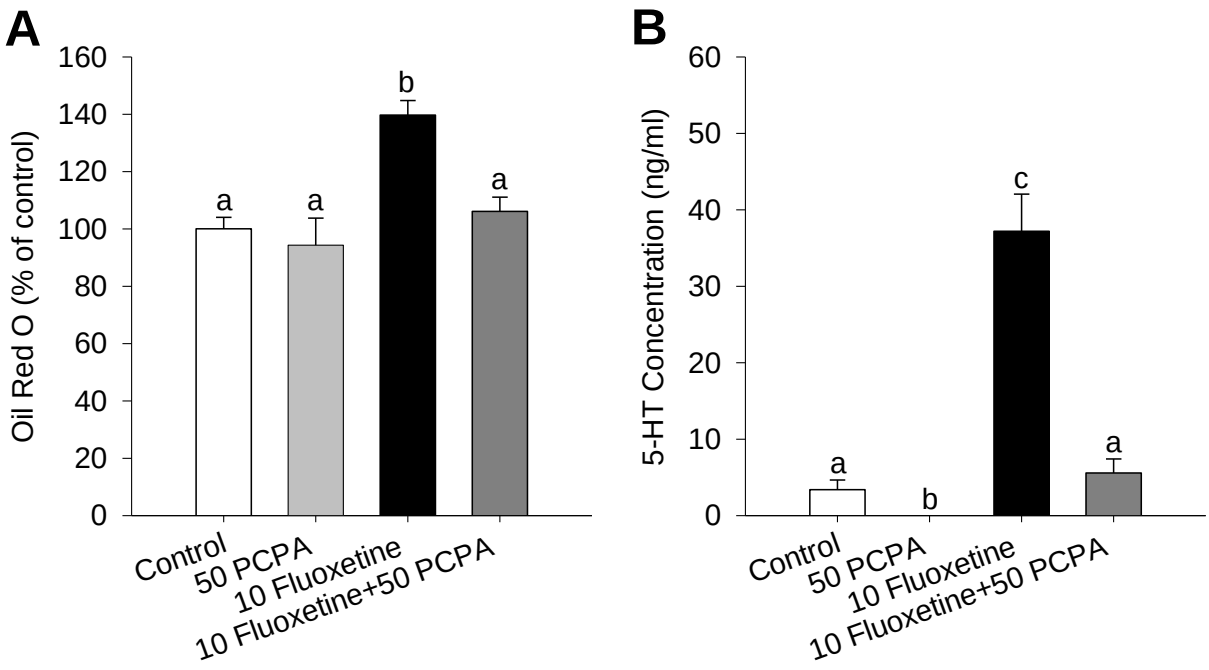

Figure 3. (**A**) Lipid accumulation was quantified in H4-II-E-C3 cells treated for 24 h with 50 μM PCPA, 10 μM Fluoxetine, and co-treated with 50 μM PCPA and 10 μM Fluoxetine. Different superscript letters indicate a significant difference $P \leq 0.05$ compared to other treatment groups. Significance was calculated by a one-way ANOVA, followed by the Bonferroni multiple comparisons test. (**B**) Intracellular serotonin was determined using commercially available serotonin ELISA as per the manufacturer's instructions (LDN, Nordhorn, Germany) following 24 h treatment above. Bars with the same superscript are not statistically different, whereas different superscript letters indicate a significant difference compared to other treatment groups $P \leq 0.05$. Statistical significance assessed by a Student-Newman-Keuls One-Way ANOVA on Ranks. All data represented as mean ± SEM, (n = 4-5 independent experiments).

3 Chapter 3

Fluoxetine-induced Hepatic Lipid Accumulation is Mediated by Prostaglandin Endoperoxide Synthase 1 and is linked to elevated 15-deoxy-$\Delta^{12,14}PGJ_2$

Full title: Fluoxetine-induced Hepatic Lipid Accumulation is Mediated by Prostaglandin Endoperoxide Synthase 1 and is linked to elevated 15-deoxy-$\Delta^{12,14}PGJ_2$

Short title:

Fluoxetine induced Steatosis and Prostaglandin Production

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Keywords: Selective Serotonin Reuptake Inhibitor (SSRI), Fluoxetine, Steatosis, Non-Alcoholic Fatty Liver Disease (NAFLD), Fatty Acid Uptake, 15-deoxy-$\Delta^{12,14}$PGJ2 (15d-PGJ_2), peroxisome proliferator-activated receptor gamma (PPARG), prostaglandin-endoperoxide synthase 1 (PTGS1), prostaglandin-endoperoxide synthase 2 (PTGS2), prostaglandin

3.1 Abstract:

Major depressive disorder and other neuropsychiatric disorders are often managed with long-term use of antidepressant medication. Fluoxetine, an SSRI antidepressant, is widely used as a first-line treatment for neuropsychiatric disorders. However, fluoxetine has also been shown to increase the risk of metabolic diseases such as non-alcoholic fatty liver disease. Fluoxetine has been shown to increase hepatic lipid accumulation *in vivo* and *in vitro*. In addition, fluoxetine has been shown to alter the production of prostaglandins which have also been implicated in the development of non-alcoholic fatty liver disease. The goal of this study was to assess the effect of fluoxetine exposure on the prostaglandin biosynthetic pathway and lipid accumulation in a hepatic cell line (H4-II-E-C3 cells). Fluoxetine treatment increased mRNA expression of prostaglandin biosynthetic enzymes (*Ptgs1*, *Ptgs2*, *Ptgds*), PPAR gamma (*Pparg),* and PPAR gamma downstream targets involved in fatty acid uptake (*Cd36*, *Fatp2,* and *Fatp5*) as well as production of 15-deoxy-$\Delta^{12,14}PGJ_2$ a PPAR gamma ligand. The effects of fluoxetine to induce lipid accumulation was attenuated with a PTGS1 specific inhibitor (SC-560), whereas inhibition of PTGS2 had no effect. Moreover, SC-560 attenuated 15-deoxy-$\Delta^{12,14}PGJ_2$ production and expression of PPAR gamma downstream target genes. Taken together these results suggest that fluoxetine-induced lipid abnormalities appear to be mediated via PTGS1 and its downstream product 15d-PGJ_2 and suggest a novel therapeutic target to prevent some of the adverse effects of fluoxetine treatment.

3.2 Short abstract:

The use of SSRI antidepressants has been linked to adverse metabolic outcomes, including non-alcoholic fatty liver disease. This study aimed to further investigate the mechanism by which the SSRI fluoxetine contributes to elevated hepatic lipid accumulation. Fluoxetine treatment caused an increase in 15-deoxy-$\Delta^{12,14}PGJ_2$ and mRNA expression of key components of the prostaglandin biosynthetic pathway. Fluoxetine-induced lipid accumulation was blocked with the selective Ptgs1 inhibitor, SC-560. Taken together these results suggest the altered prostaglandin production is a key mediator of fluoxetine-induced lipid accumulation.

3.3 Introduction:

Major Depressive Disorder (MDD) is a prevalent and often recurrent illness affecting nearly 350 million individuals worldwide and is predicted to be the leading cause of disability by 2030 (Longfei et al., 2015; World Health Organization, 2017). A global burden of disease study saw a 49.86% percent increase in incident cases of depression worldwide from 1990 to 2017 (Q. Liu, He, et al., 2020), with the financial burden of MDD in 2010 exceeding USD 210.5 billion in the United States alone (Greenberg et al., 2015; R. W. Lam et al., 2016). MDD can be attributed to biochemical, structural, and functional abnormalities in the brain (S. Ayyash et al., 2021; Y.-K. Kim, 2016; World Health Organization, 2017). In particular, it has been speculated that MDD results from the dysregulation of monoaminergic transmission, with an emphasis on the serotonergic systems, a view that has dominated the field of MDD research for the past 60 years (Dell'Osso et al., 2016; Sahli et al., 2016) Thus, antidepressants that aim to modulate serotonin signaling, which includes selective serotonin reuptake inhibitors (SSRIs) have become front-line pharmacotherapies in the treatment of MDD (Joshi, 2018).

While SSRIs such as fluoxetine are efficacious at managing MDD, they have also been implicated in the development of adverse metabolic outcomes including weight gain, type 2 diabetes, and non-alcoholic fatty liver disease (NAFLD) (De Long, Stepita, et al., 2015; Deuschle, 2013; A. Pan, Sun, et al., 2012; Rubin et al., 2008). NAFLD is classified as a range of diseases varying from simple hepatic steatosis to inflammatory non-alcoholic steatohepatitis (NASH) with different levels of fibrosis (Chalasani et al., 2012). These illnesses are observed in the absence of other known etiologies of hepatic injury such as significant alcohol consumption and viral hepatitis (Chalasani et al., 2012). Steatosis can be identified by excess accumulation of triglycerides within hepatocytes. Hepatic steatosis can be a result of one or more mechanisms including elevated *de novo* fatty acid synthesis; diminished fatty acid oxidation; elevated transport of fatty acids from the peripheral organs to the liver; and reduced transport of fatty acids from the liver to the general circulation and peripheral organs (Cimini et al., 2017; Mallat et al., 2011). Animal experiments have shown that fluoxetine treatment resulted in a significant increase in hepatic lipid accumulation; a hallmark of NAFLD (X.-M. Feng et al., 2012; Lu et al., 2020; Xiong et al., 2014). The accumulation of hepatic lipid is often associated with an inflammatory response which includes increased expression of the 2-series prostaglandins, a subgroup of eicosanoids, which includes PGD_2, PGE_2, PGF_{2A}, and PGI_2 (Weixuan Wang, Zhong, & Guo, 2021).

Prostaglandins are converted from arachidonic acid catalyzed by the rate-limiting enzyme prostaglandin-endoperoxide synthase 1 (PTGS1) and prostaglandin-endoperoxide synthase 2 (PTGS2), also known as cyclooxygenase 1 (COX-1) and cyclooxygenase 2 (COX-2) respectively. Interestingly, in a rat model of diet-induced obesity, a PTGS2 selective inhibitor suppressed the development of NAFLD (Hsieh et al., 2009; Martín-Sanz et al., 2017). In contrast, Wang et al. suggested that PTGS1 but not PTGS2 mediates NAFLD progression (W. Wang et al., 2018). One of the cyclooxygenase-derived prostaglandins of

interest is prostaglandin D_2 (PGD_2). The PGD_2 metabolite 15-deoxy-$\Delta^{12,14}PGJ_2$ (15d-PGJ_2) functions as a potent endogenous ligand and agonist for peroxisome proliferator-activated receptor gamma (PPARG) (Álvarez-Almazán et al., 2017; Forman et al., 1995). In hepatocytes, PPARG is a central regulator of lipid metabolism, targeting genes involved in *de novo* lipogenesis and free fatty acid uptake (Skat-Rørdam et al., 2019); PPARG expression was found to be significantly elevated in the liver of obese rats and involved in the development of NAFLD (Edvardsson et al., 2006; Gavrilova et al., 2003; Matsusue et al., 2008; Pettinelli & Videla, 2011; Schadinger et al., 2005). Importantly the inhibition of cyclooxygenase suppressed hepatic steatosis by suppressing the production of 15d-PGJ_2 and the subsequent expression of PPARG (Tsujimoto et al., 2016). While there is a paucity of literature examining fluoxetine's effect on the expression or activity of cyclooxygenase enzymes, other SSRIs have been shown to alter the expression and activity of these rate-limiting enzymes. For example, the SSRI vortioxetine has been shown to inhibit the activity of both PTGS1 and PTGS2, while fluvoxamine significantly decreased the expression of PTGS2 (Marčec & Likić, 2021; Naji Esfahani et al., 2019; Talmon et al., 2020). Notably, The SSRI fluoxetine has been shown to increase the protein expression of PTGS1 and PTGS2, elevate the cerebrospinal fluid concentration of 15d-PGJ_2 and increase secretion of 15d-PGJ_2 from raphe serotonergic neurons (Launay et al., 2011a; Salem Sokar et al., 2016; Simplicio et al., 2015). Therefore, the goal of this study was to test the hypothesis that increases in hepatic prostaglandin production underlie increased fluoxetine-induced lipid accumulation *in vitro*.

3.4 Materials and Methods:

3.4.1 Cell Culture Maintenance and Treatment

H4-II-E-C3 hepatoma cells (ATCC CRL-1600, Manassas, VA) were grown in Corning™ Dulbecco's modified Eagle's medium (DMEM; Corning, NY) supplemented with 10 % fetal bovine serum (Hyclone, South Logan, UT), 2% L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin (Gibco, Long Island, NY) at 37 °C in a humidified atmosphere of 95 % O2 and 5 % CO2. All cell treatments were made in supplemented DMEM as described above. When H4-II-E-C3 cells reached 80% confluence, they were treated with 10 µM fluoxetine hydrochloride (Toronto Research Chemicals, North York, ON; cat. F597100; N = 5 independent experiments) to assess lipid accumulation and involvement of the prostaglandin biosynthetic pathway as described below. The 10 µM concentration of fluoxetine and 24 h time point used has been shown to result in increased lipid accumulation in the H4-II-E-C3 (A. Ayyash & Holloway, 2021a) and primary rat hepatocytes (X.-M. Feng et al., 2012; Xiong et al., 2014).

3.4.2 Lipid accumulation

Following 24 h of fluoxetine treatment cells were fixed with 10% formaldehyde followed by staining with Oil Red O (Sigma-Aldrich, St. Louis, MO) solution for 20 m and then washed with water as previously described (A. Ayyash & Holloway, 2021a; Ramírez-Zacarías et al., 1992a). Absorbance was measured at 510 nm in a plate reader (Synergy™ H4 Hybrid Microplate Reader, BioTek Instruments, Winooski, VT).

3.4.3 Prostaglandin synthesis and PPAR activation

Cells were treated for 24 h with 10 µM fluoxetine hydrochloride as described above. We assessed changes in the relative mRNA expression of *Ptgs1* (encodes prostaglandin-endoperoxide synthase 1; cyclooxygenase-1; COX1), *Ptgs2* (encodes prostaglandin-endoperoxide synthase 2; cyclooxygenase-2; COX2), *Ptgds* (encodes prostaglandin D2 synthase), *Pparg*, and the downstream PPARG targets involved in fatty acid uptake [cluster of differentiation 36 (*Cd36*), fatty acid transport protein 2 (*Fatp2*), fatty acid transport protein 5 (*Fatp5*)] using quantitative real-time PCR (Gao et al., 2020; Pettinelli & Videla, 2011; Videla & Pettinelli, 2012; Yamazaki et al., 2011). After treatment, cells were washed with phosphate-buffered saline (PBS), and total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA). RNA concentrations were determined using the NanoDrop One™ Microvolume UV-Vis Spectrophotometer 10 (Thermo Scientific, Waltham, MA). Complementary DNA (cDNA) was made from 2 µg of mRNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) as per the manufacturer's instructions. The resulting cDNA was a template for qPCR, which was carried out using PerfeCTa® SYBR® Green FastMix® (Quanta Biosciences, Gaithersburg, MD) on the CFX384 Touch™ Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA). The PCR cycling settings included polymerase activation (95 °C for 10 m), followed by 40 cycles of denaturing (95 °C for 15 s), and annealing/elongation (60 °C for 1 m). Levels of gene expression were generated using the double delta-Ct method (Livak & Schmittgen, 2001) and normalized using the geometric

means of two reference genes glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*) and beta-actin (*Actb*).

3.4.4 Fatty acid uptake

To determine the effect of fluoxetine on fatty acid uptake, H4-II-E-C3 cells were grown to 80% confluence, treated for 1 h with 10 μM fluoxetine hydrochloride (Toronto Research Chemicals, cat. F597100) and 0.15 μM insulin (positive control; Sigma-Aldrich, cat. I0516). Fatty acid uptake was determined using a commercially available fatty acid uptake assay kit according to the manufacturer's instructions (Biovision, cat. K408-100). (N = 10 independent experiments).

3.4.5 Does blocking prostaglandin output prevent fluoxetine-induced lipid accumulation?

To determine whether blocking prostaglandin production would influence SSRI-induced lipid accumulation, cells were treated with fluoxetine (10 μM) ± indomethacin, a non-selective inhibitor of both COX isoforms (Raji et al., 2017). Cells were treated for 24 h with the following: 1) Vehicle, 2) indomethacin (50 μM; Sigma-Aldrich, cat. I7378), 3) indomethacin +fluoxetine, and 4) fluoxetine. Cells were cultured as described above, and indomethacin was added to the treatment wells for 1 h prior to the addition of fluoxetine. After 24 h, cells were stained for lipid accumulation using Oil Red O or collected for quantitative real-time PCR. To further elucidate whether changes in hepatic lipid accumulation were mediated by PTGS1 or PTGS2, cells were treated with either the selective PTGS1 inhibitor, SC-560 (Cayman Chemical, Ann Arbor, MI; cat. 70340) or the selective PTGS2 inhibitor NS-398 (Cayman Chemical, cat. 70590). Cells were treated for 24 h with either 1) Vehicle, 2) SC-560 (1 μM), 3) SC-560 +fluoxetine and 4) fluoxetine or 1) Vehicle, 2) NS-398 (10 μM), 3) NS-398+fluoxetine and 4) fluoxetine. For all treatments with an inhibitor, the inhibitor was added to the respective wells for 1 h prior to the addition of fluoxetine. After 24 h, cells were stained for lipid accumulation using Oil Red O as described above.

3.4.6 Mechanisms of PTGS1 inhibition on fluoxetine-induced lipid accumulation

Because the PTGS1 but not PTGS2 inhibitor was able to prevent fluoxetine-induced lipid accumulation, we treated cells with fluoxetine ± SC-560 for 24 h using the same protocol described above. After 24 h, media was saved for 15-deoxy-Δ12,14-PGJ_2 quantification (as described below) and cells were collected for quantitative real-time PCR of genes involved in prostaglandin production [cyclooxygenase-1 (*Ptgs1*), cyclooxygenase-2 (*Ptgs2*), prostaglandin D2 synthase (*Ptgds*)], regulation of hepatic lipid metabolism [peroxisome proliferator-activated receptor gamma (*Pparg*)], and fatty acid uptake [cluster of differentiation 36 (*Cd36*), fatty acid transport protein 2 (*Fatp2*), fatty acid transport protein 5 (*Fatp5*)]. Primer sequences are supplied in Table 1.

3.4.7 15-deoxy-Δ12,14-PGJ2 ELISA

The concentration of prostaglandin 15-deoxy-Δ12,14-PGJ_2 was quantified from media collected 24 h post-treatment with fluoxetine ± SC-560 using a commercial ELISA per the manufacturer's protocol (Enzo Life Sciences, Switzerland; cat. ADI-900-023).

3.4.8 Statistical Analysis

All statistical analyses were conducted using SigmaPlot (v.11.2, Systat Software, San Jose, CA). Data were tested for outliers (Grubbs' test), normality, and equal variance. Comparisons among the two groups were analyzed using Student's t-tests. Comparisons among multiple groups were analyzed using One-Way Analysis of Variance (ANOVA) followed by the Bonferroni multiple comparisons test. When normality or equal variance failed, the Mann-Whitney Rank Sum Test or Student-Newman-Keuls One-Way ANOVA on Ranks were used to determine significance. All data are presented as mean ± SEM and were considered significant when $P \leq 0.05$

3.5 Results:

3.5.1 Lipid accumulation, prostaglandin biosynthetic pathway, and fatty acid uptake

After 24 h, cells exposed to 10 µM fluoxetine had significantly increased lipid accumulation (Figure 1). We also observed a significant increase in expression of *Ptgs1*, *Ptgs2*, *Ptgds*, and *Pparg* (Figure 3). Treatment with 10 µM fluoxetine resulted in a significant increase in fatty acid uptake following 1 h of treatment (Figure 2).

3.5.2 Role of prostaglandin synthesis on fluoxetine-induced lipid accumulation

Given the elevated mRNA expression of *Ptgs1* and *Ptgs2* observed (Figure 3), we aimed to elucidate whether changes in hepatic lipid accumulation were related to the elevated prostaglandin synthesis. Cells were treated with three different inhibitors for PTGS1 and/or PTGS2. We first used a non-selective PTGS1 and PTGS2 inhibitor, indomethacin, and found that indomethacin treatment attenuated hepatocyte lipid accumulation (Figure 4A). Similarly, the selective PTGS1 inhibitor, SC-560 also attenuated fluoxetine-induced lipid accumulation (Figure 4C) whereas the selective PTGS2 inhibitor, NS-398 did not reduce fluoxetine-induced increases in lipid accumulation (Figure 4B). These findings suggested that PTGS1 inhibition was central to attenuating fluoxetine-induced lipid accumulation in these cells. Next, we treated cells with the Selective PTGS1 inhibitor, SC-560 with and without fluoxetine as described above to assess 15d-PGJ_2 output and PPARG downstream target genes. Fluoxetine treatment caused a significant increase in 15d-PGJ_2 output; an effect which was attenuated by the addition of SC-560 (Figure 5 A). The expression of *PTGDS*, *PPARG*, and downstream PPARG targets involved in fatty acid uptake was also attenuated with the combined treatment with SC-560 and fluoxetine (Figure 5 B-F).

3.6 Discussion:

MDD affects nearly 16% of the global population, and the prevalence continues to rise (Kessler et al., 2003; Q. Liu, He, et al., 2020). SSRI antidepressants are commonly used for the treatment of MDD in children, adolescents, and adults (DeFilippis & Wagner, 2014; Joshi, 2018). In particular, fluoxetine remains one of the most commonly prescribed and recommended drugs in its class (Bachmann et al., 2016; D. Lam et al., 2013; Rossi et al., 2004). However, there is now considerable evidence that long-term treatment with SSRIs leads to an increased prevalence of metabolic disturbances (Skilton et al., 2007). Long-term SSRI use is associated with increased weight gain, dyslipidemia, and new-onset type 2 diabetes (Ferguson, 2001; Jerrell, 2010; Raeder et al., 2006; Sussman & Ginsberg, 1998; Yoon et al., 2013). Moreover, animal and cell culture experiments have shown that fluoxetine exposure increases hepatic triglyceride content and lipid accumulation; hallmarks of NAFLD (A. Ayyash & Holloway, 2021a; De Long, Barry, et al., 2015; X.-M. Feng et al., 2012; Lu et al., 2020; S. Pan et al., 2018b; Xiong et al., 2014). While the mechanisms by which fluoxetine leads to hepatic steatosis are not fully elucidated, there is evidence that altered prostaglandin production may be a key mediator of fluoxetine-induced lipid accumulation.

Both prostaglandin biosynthetic enzymes (i.e. PTGS1 and PTGS2) have been identified as having an important role in the development of NAFLD, as increased prostaglandin production contributes to the dysregulation of lipid metabolism (Feingold et al., 1992; Forman et al., 1995; Hsieh et al., 2009; W. Wang et al., 2018; Yokota et al., 2002). Indeed, the 2-series prostaglandins, PGD_2, PGE_2, and PGF_{2A}, have been shown to play an important role in hepatic lipid accumulation and inflammatory processes involved in NAFLD (Björnsson et al., 1992; Henkel et al., 2012; Nassir et al., 2013; Pérez et al., 2006; W. Wang et al., 2021). These prostaglandins act to substantially diminish the secretion of very-low-density lipoprotein apolipoprotein B (VLDL-apoB), effectively promoting steatosis in primary hepatocytes (Björnsson et al., 1992; Nassir et al., 2013; Pérez et al., 2006). Diminished VLDL-apoB leads to impaired cellular triglyceride recycling and decreased transport of triacylglycerol and cholesterol to the circulation, contributing to hepatic steatosis (Pérez et al., 2006; M. Yang et al., 2020). Furthermore, PGE_2 acts synergistically with insulin to increase the incorporation of glucose into triglycerides in hepatocytes (Najjar & Perdomo, 2019; Pérez et al., 2006). In this study, we have shown that fluoxetine induced hepatic lipid accumulation in association with upregulated mRNA expression of *Ptgs1* and *Ptgs2*. These results are consistent with a previous study which reported that fluoxetine markedly enhanced the expression of PTGS1 and PTGS2 in gastric tissue (Salem Sokar et al., 2016). PTGS1 and PTGS2 are involved in the conversion of arachidonic acid to Prostaglandin H2 (PGH2), an important precursor of other downstream prostaglandins (M. Nagao et al., 2013). While PTGS1 is expressed abundantly in many tissues, the expression of PTGS2 is often elevated in response to a stimulus such as inflammation (Martín-Sanz et al., 2010). The PTGS1 enzyme is constitutively expressed and prefers coupling and co-localization at perinuclear membrane or ER, with thromboxane synthase, prostaglandin F synthase, and two other prostaglandin D synthases (PTGDS) isozymes,

generating thromboxane A2, PGF_{2A}, and PGD_2, respectively (Hanna & Hafez, 2018). PTGS2, the inducible isoform, prefers coupling with prostaglandin I synthase and prostaglandin E synthase, producing PGI_2 and PGE_2, respectively (Hanna & Hafez, 2018). Based on our inhibitor studies it appears that PTGS1 and not PTGS2 is involved in fluoxetine-induced lipid accumulation.

In the current study, the PTGS1/PTGS2 inhibitor indomethacin and the PTGS1 specific inhibitor SC-560 attenuated fluoxetine-induced lipid accumulation, whereas the PTGS2 specific inhibitor NS-398 showed no effect (Figure 4). Interestingly, fluoxetine treatment has previously been shown to increase the protein expression of PTGS1, but not PTGS2, in the rat aorta (Simplicio et al., 2015). In a variety of other tissues, fluoxetine has been shown to display anti-inflammatory activity, reducing the activity and expression of PTGS2, and inhibiting the production of the downstream prostaglandin PGE_2 (Branco-de-Almeida et al., 2012; Kannen et al., 2011; C.-M. Lim et al., 2009; D. Liu et al., 2011). Since the biosynthesis of PGE_2 is primarily regulated by PTGS2, and inhibition of PTGS2 via NS-398 did not reduce fluoxetine-induced lipid accumulation (Figure 4 B), it suggests that PGE_2 is not critical for fluoxetine-induced lipid accumulation (Branco-de-Almeida et al., 2012). However, the observed upregulation of PTGS2 may play a vital role in the progression of hepatic fibrogenesis or hepatocarcinogenesis, neither outcome which was assessed in this study (Martín-Sanz et al., 2010; Motiño et al., 2016; H. Yang et al., 2020). Consistent with our results, there is some evidence from rodent models of NAFLD which suggests that PTGS1 plays a central role in the development of hepatic steatosis (Sztolsztener et al., 2020; W. Wang et al., 2018). In a recent study, rodents fed a high-fat diet to induce NAFLD saw a marked increase in hepatic expression of PTGS1 protein, with no changes in PTGS2 expression (Sztolsztener et al., 2020). Similarly, Wenzhe et al. (2018) reported that in rodents with high-fat-diet-induced NAFLD, PTGS1 protein expression was dramatically upregulated in fatty liver tissues, while PTGS2, was only slightly elevated (W. Wang et al., 2018). At low doses, the inhibitors aspirin and genistein reduced hepatic lipid accumulation, an effect the authors proposed as a result of PTGS1 inhibition (W. Wang et al., 2018).

Arachidonic acid is converted to PGH2 by PTGS1 and PTGS2, followed by conversion by specific prostaglandin synthases (e.g., PTGDS, PGES, PGFS, and PGIS) into the 2-series prostaglandins, PGD_2, PGE_2, PGF_{2A}, and PGI_2 respectively (Álvarez-Almazán et al., 2017; Fujitani et al., 2010). Current evidence suggests that PGD_2 plays a central role in NAFLD by promoting hepatic lipid accumulation (Tsujimoto et al., 2016; W. Wang et al., 2021). Indeed, the PGD_2 metabolite, 15d- PGJ_2 has been reported to be elevated in rodent models of NAFLD (Tsujimoto et al., 2016; W. Wang et al., 2021). Consistent with PTGS1 activation in SSRI-induced lipid accumulation, we observed an increase in *Ptgds* and the PGD_2 metabolite, 15d-PGJ_2, with fluoxetine treatment; an effect which was attenuated by treatment with the PTGS1 selective inhibitor SC-560. Similarly, fluoxetine has been reported to increase the cerebrospinal fluid concentration of 15d-PGJ_2 and secretion of 15d-PGJ_2 from raphe serotonergic neurons (Launay et al., 2011a). 15d-PGJ_2 is a potent endogenous ligand of the nuclear receptor PPARG (Álvarez-Almazán et al., 2017; Fujitani

et al., 2010; Harris & Phipps, 2002; Kumar et al., 2020; Launay et al., 2011a) which may explain, in part, the increased lipid accumulation following fluoxetine treatment.

PPARG serves as a ligand-dependent transcription factor that plays a pivotal role in the regulation of lipid synthesis and uptake, which are likely to contribute to the development of NAFLD (Yamazaki et al., 2011). PPARG activation in hepatic stellate cells and macrophages seems advantageous in protecting against NAFLD and NASH promotion, whereas PPARG has proven to be steatogenic in hepatocytes, promoting the deposition of intracellular lipids (Larter et al., 2008; Matsusue et al., 2003; Skat-Rørdam et al., 2019; Y.-L. Zhang et al., 2006). In addition, hepatocyte-specific knockout of PPARG attenuated hepatic steatosis in high-fat diet-fed mice (Morán-Salvador et al., 2011). 15d-PGJ_2 is an endogenous PPARG ligand and has been shown to increase both PPARG activity and expression in hepatocytes (J. Li et al., 2019; Maggiora et al., 2010). In our study, fluoxetine increased the expression of *Pparg* and its downstream targets *Cd36*, *Fatp2*, and *Fatp5* (Figure 5 C - F). The uptake of fatty acid by the liver is facilitated by CD36, FATP2, and FATP5; these transporters, therefore, play an important role in maintaining intracellular fatty acid homeostasis (Wilson et al., 2016). In humans, NAFLD patients tend to have elevated expression of CD36 and fatty acid transport proteins (FATP2 & FATP5) (Fabbrini et al., 2009; Greco et al., 2008; Nassir et al., 2013). In animal models, CD36 expression was 5-fold higher in livers with steatosis than in healthy livers (Nassir et al., 2013). In our study, the upregulation of the PPARG target genes occurred in association with increased fatty acid uptake suggesting that fluoxetine-activated PPARG signaling pathways influence hepatic lipid accumulation. Importantly the fluoxetine-induced increase in 15d-PGJ_2, *Pparg*, and its downstream targets (*Cd36*, *Fatp2*, *Fatp5*) was attenuated by the PTGS1 selective inhibitor SC-560.

Results from this study have shown that the SSRI fluoxetine led to elevated lipid accumulation in association with increased expression of key components of prostaglandin synthesis. The effects of fluoxetine to induce lipid abnormalities appear to be mediated via PTGS1 and its downstream product 15d-PGJ_2; a PPARG ligand. Future studies should aim to investigate how other arachidonic acid metabolites might also be involved in fluoxetine-induced lipid accumulation and inflammatory processes involved in NAFLD (Q. Li et al., 2020; Maciejewska et al., 2015; Marchix et al., 2020; W. Wang et al., 2021). An assessment of targets of intervention for the treatment and prevention of SSRI-induced hepatic lipid accumulation is also warranted, as it may prove useful in the prevention of subsequent inflammation, fibrosis, and even cirrhosis of the liver associated with severe NAFLD (Cholankeril et al., 2017). Given the high prevalence of MDD and the increasing use of SSRIs to treat a variety of conditions ranging from anxiety to obsessive-compulsive disorder, these results may have significant implications to improve the adverse metabolic associated with SSRI use (Lochmann & Richardson, 2019).

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3.8 Tables:

Table 1. Real-time PCR *Rattus norvegicus* primers used in the study

Gene	Forward primer	Reverse Primer	Genebank accession #
Gapdh	TGGAGTCTACTGGCGTCTTCAC	GGCATGGACTGTGGTCATGA	NM_017008.4
Actb	CACAGCTGAGAGGGAAAT	TCAGCAATGCCTGGGTAC	NM_031144.3
Ptgs1	TACCCACCTTCCGTAGAACAG	CAGATCGTGGAGAAGAGCATCA	NM_017043.4
Ptgs2	TTCCAAACCAGCAGGCTCAT	AAAAGCAGCTCTGGGTCGAA	NM_017232.3
Ptgds	GCTTCCACTCCCTCTCAGTG	GGAACGCGTACTCATCGTAGT	NM_013015.2
Pparg	ACCTCAGGCAGATTGTCACAG	GCAGAGGGTGAAGGCTCATA	NM_013124.3
Cd36	GTACTCTCTCCTCGGATGGC	TGCATGAACAGCAGTATCTGAG	NM_031561.2
Fatp2	TTGAAACCTTCGCCACAGGA	TCCACGTTTGCTTCTCTGCT	NM_031736.2
Fatp5	TCGAATGCTGACTCCCCTTG	TGTTTGTCCCTCACAGGCTC	NM_024143.2

3.9 Figures:

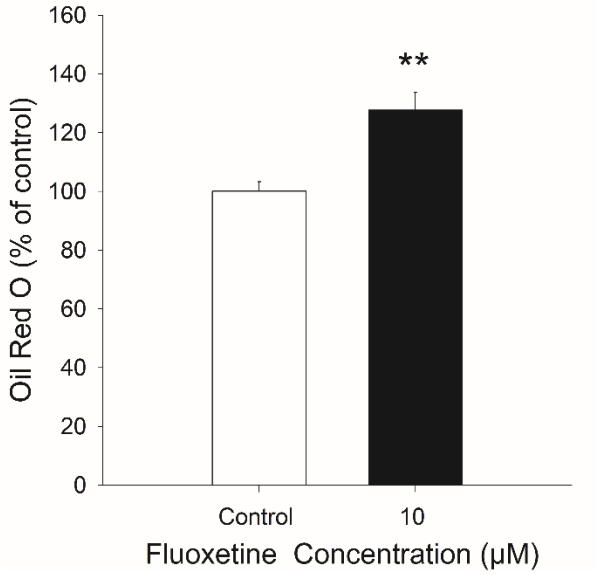

Figure 1. Lipid accumulation was determined by Oil Red O staining following treatment with fluoxetine for 24 h. **, $P \leq 0.01$ vs. control group calculated via Student's T-test. All data represented as mean ± SEM, (n = 5 independent experiments).

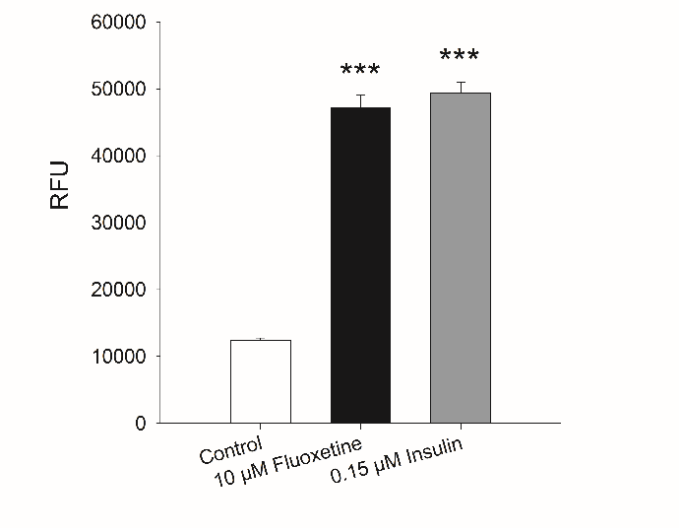

Figure 2. Fatty acid uptake in H4-II-E-C3 treated with 10 µM fluoxetine and 0.15 µM insulin ***, $P \leq 0.001$ vs. control group calculated via one-way ANOVA, followed by the Bonferroni multiple comparisons test. All data represented as mean ± SEM, (n = 10 independent experiments).

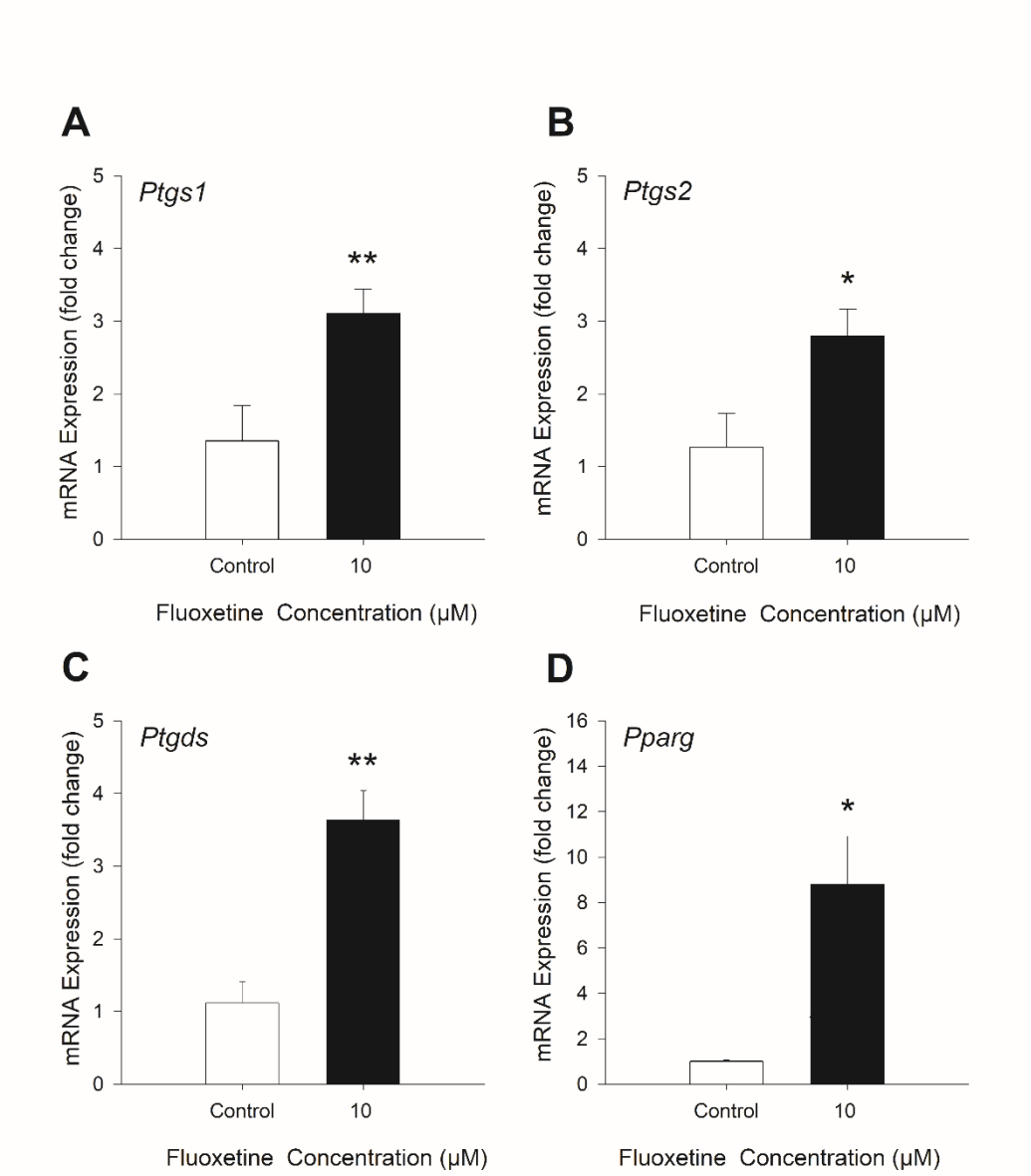

Figure 3. Relative mRNA expression of (**A**) *Ptgs1*, (**B**) *Ptgs2*, (**C**) *Ptgds* and (**D**) *Pparg* in H4-II-E-C3 treated with 10 μM fluoxetine, for 24 h. *, $P \leq 0.05$ vs. control group calculated via Student's T-test.**, $P \leq 0.01$ vs. control group. All data represented as mean ± SEM, (n = 4-5 independent experiments).

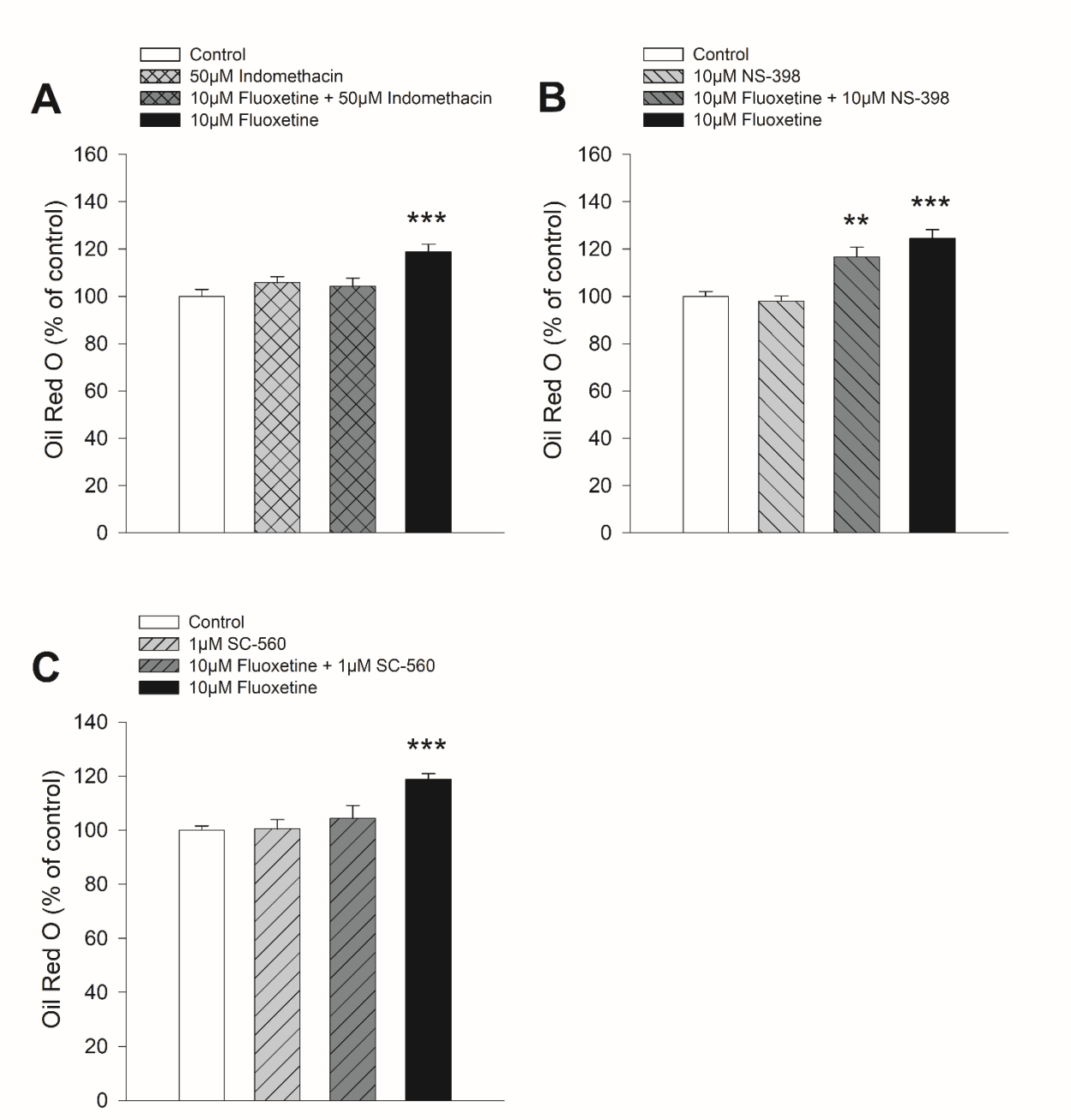

Figure 4. Lipid accumulation was determined in H4-II-E-C3 cells by Oil Red O staining following 24 h treatment with fluoxetine plus (**A**) the non-selective PTGS1 and PTGS2 inhibitor indomethacin (50 μM) (**B**) the selective PTGS2 inhibitor NS-398 (10 μM) and (**C**) the selective PTGS1 inhibitor SC-560 (1 μM). Outcome measures between control and treatments were analyzed using a one-way ANOVA ($\alpha = 0.05$) **, $P \leq 0.01$ vs. control group and ***, $P \leq 0.001$ vs. control group Data represent mean ± SEM. (n = 9-10 independent experiments).

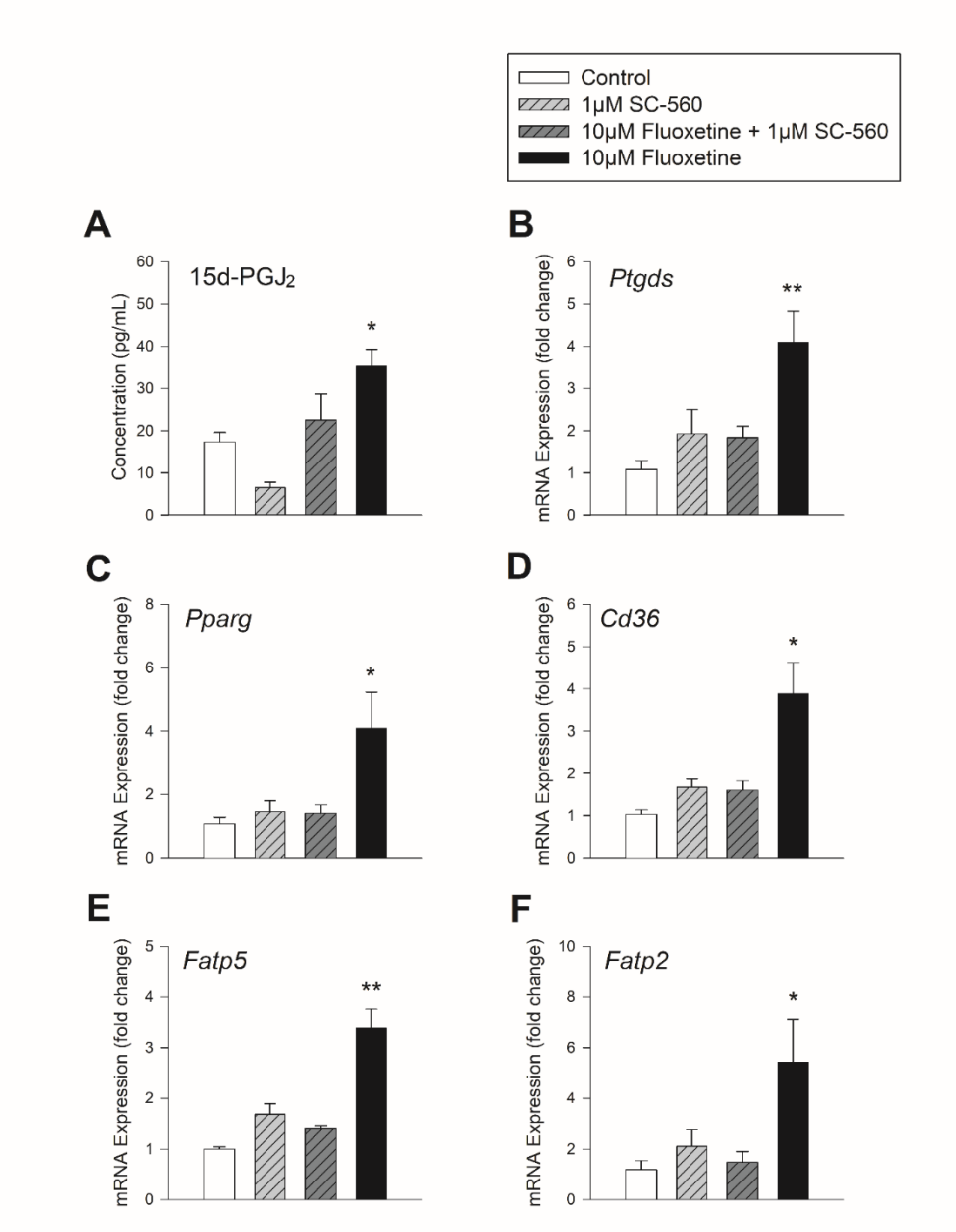

Figure 5. (A) 15d-PGJ_2 output in media following 24 h treatment with fluoxetine with and without the selective PTGS1 inhibitor SC-560 (1 μM). Relative mRNA expression of (**B**) *Ptgds*, (**C**) *Pparg*, and PPARG targets involved in fatty acid uptake including (**D**) *Cd36*, (**E**) *Fatp5* and (**F**) *Fatp2* in H4-II-E-C3 cells following 24 h treatment with fluoxetine with and without the selective PTGS1 inhibitor SC-560 (1 μM). Outcome measures between control and treatments were analyzed using a one-way ANOVA (α = 0.05). *, $P \leq 0.05$ vs. control group, **, $P \leq 0.01$ vs. control group, and ***, $P \leq 0.001$ vs. control group followed by the Bonferroni multiple comparisons test. Data represent mean ± SEM. (n = 4-5 independent experiments).

4 Chapter 4:

Full title: Elevated miR-122 is Mediated by Peroxisome Proliferator-activated Receptor γ and is Among microRNAs Altered as a Consequence of Fluoxetine-induced Hepatic Lipid Accumulation.

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4.1 Introduction

Major Depressive Disorder (MDD) is a complex, costly, and common psychiatric disorder, affecting nearly 322 million people or 4.4% of the population worldwide (Kessler et al., 2005; World Health Organization, 2017). With a lifetime prevalence of approximately 13%, MDD is the leading cause of disability worldwide, is among the leading causes of disease burden globally, and is associated with significant morbidity and mortality (J. Alonso et al., 2004; Bromet et al., 2011; Emslie et al., 2005; Greenberg et al., 2015; Pundiak et al., 2008). Several lines of evidence from clinical studies in humans suggest that altered serotonergic neurotransmission might contribute to the pathophysiology of MDD (Cowen, 2008; Sharp & Cowen, 2011; K. A. Smith et al., 1997). Thus, selective serotonin reuptake inhibitors (SSRIs), antidepressant drugs which act to enhance serotonergic neurotransmission, have been the first-line pharmacotherapy for the treatment of MDD (Clevenger et al., 2018; T. Kato et al., 2018; Thaler et al., 2012). Notably, there is increasing evidence that SSRIs can cause perturbations in lipid metabolism which may lead to metabolic disease with long-term use (Feng et al., 2012; Fjukstad et al., 2016; S. Pan et al., 2018a; Xiong et al., 2014). In particular, SSRIs may be contributing to aberrant hepatic lipid accumulation, an association that requires further exploration (A. Ayyash & Holloway, 2021a, 2021b; Deuschle, 2013; Labenz et al., 2020; A. Pan, Sun, et al., 2012; Rubin et al., 2008; Shaheen et al., 2021).

Cell culture, animal, and clinical studies have demonstrated that SSRIs, including fluoxetine, have been linked to altered epigenome and microRNA (miRNA) expression (Baudry et al., 2010a; Hansen & Obrietan, 2013a; Launay et al., 2011a; Miao et al., 2018b). Other studies have shown that epigenetic changes, in particular, altered miRNA expression may underlie altered hepatic lipid homeostasis (Y. Y. Feng et al., 2014; Hanin et al., 2018a; W. Liu et al., 2015; F. Xiao et al., 2014; Zarfeshani et al., 2015). Epigenetic alterations refer to changes in gene expression that are not caused by DNA sequence variation and include processes such as histone modification, DNA methylation, non-coding RNAs (ncRNAs), and chromatin remodeling (Saavedra et al., 2016; Torres-Berrío et al., 2019). These regulatory mechanisms are fundamental for diverse cellular functions, including transcription of messenger RNA (mRNA), miRNA expression, and regulation of physiological and pathological processes (Saavedra et al., 2016). miRNA are small non-coding RNAs, ~22 nucleotides in length, that can modulate gene expression at the post-transcriptional level by targeting mRNAs and inhibiting their translation or promoting their degradation (Ambros, 2004; Hammond, 2015). Importantly, miRNAs have recently been implicated for their role in the progression of liver steatosis, inflammation, fibrosis, and cirrhosis (Bala et al., 2009; Szabo & Bala, 2013; Szabo & Csak, 2016).

Micro-RNA-122 (miR-122) is the most abundant miRNA in the liver, comprising 70% of the total liver miRNAs pool, and plays a fundamental role in liver lipid metabolism (Deng et al., 2014; Esau et al., 2006; Laudadio et al., 2012; H. Xu et al., 2010). A study by Esau et al. demonstrated that inhibition of miR-122 in both normal and high-fat-fed mice with an antisense oligonucleotide was associated with a significant reduction in hepatic steatosis

and plasma cholesterol levels, uncovering an unexpected role for miR-122 in the regulation of hepatic lipid metabolism (Esau et al., 2006). Furthermore, a cross-sectional study reported that levels of miR-122 were higher in the serum of participants with NAFLD, with serum levels of miR-122 correlating with the severity of liver steatosis in both sexes (men: healthy vs mild NAFLD $P < 0.001$, mild NAFLD vs severe NAFLD $P = 0.047$, women: healthy vs mild NAFLD $P = 0.002$, mild NAFLD vs severe NAFLD $P = 0.035$) (Yamada et al., 2013).

Inhibition of miR-122 was accompanied by a reduction in hepatic sterol and fatty-acid synthesis rates, stimulation of hepatic fatty-acid oxidation, and a significant decrease in the mRNA levels of many key genes that regulate lipid metabolism, including fatty acid synthase (*Fasn)*, acetyl-CoA carboxylase 2 (*Acc2*), Stearoyl-CoA desaturase-1 (*Scd1*), and ATP citrate synthase (*Acly*) (Esau et al., 2006). This finding was further confirmed by other studies which found that miR-122 targets genes that regulate cholesterol and lipid metabolism, and inhibition of miR-122 led to indirect downregulation of FASN and decreased accumulation of intracellular triglycerides (Baffy, 2015; Cermelli et al., 2011; H.-Y. Lin et al., 2020; Long et al., 2019; J.-L. Torres et al., 2018). For example, the flavonoid silibinin was shown to reduce hepatic miR-122 expression both *in vivo* and *in vitro*, and co-treatment with palmitic acid, miR-122 mimic and silibinin resulted in a reduction of triglyceride content, reduced mRNA and protein expression of FASN and ACC and an increased mRNA and protein expression of carnitine palmitoyl transferase 1A (CPT1A)(L. Yang et al., 2021). Similarly, pharmacological inhibition of miR-122 in mice, resulted in reduced levels of plasma cholesterol, increased hepatic fatty acid oxidation, and decreased synthesis of hepatic fatty acid and cholesterol (Li, 2012). Further, silencing of miR-122 in HFD-fed mice reduced hepatic steatosis and miR-122 deficient mice had lower serum cholesterol, low-density lipoprotein (LDL), triglyceride, and high-density lipoprotein (HDL) levels (Szabo & Csak, 2016). Additionally, treatment of hepatocytes with free fatty acids elevated the expression levels of major lipogenic genes, such as sterol regulatory element-binding protein 1 (*Srebp1*), *Fasn*, *Scd1*, *Acc1* and Apolipoprotein AV (*ApoA5*); however, their expression was suppressed by a miR-122 inhibitor (Long et al., 2019).

There is also evidence that miR-122 expression may be altered following exposure to SSRI antidepressants. For example, Fang et al., (2020) compared the serum exosome miRNA profile in rats subjected to stress + vehicle and rats subjected to stress + fluoxetine and found that miR-122 was expressed at significantly higher levels in the fluoxetine-treated animals relative to controls and was the miRNA with the most significantly altered p-value (P = 5.44 E-08) following fluoxetine treatment (Fang et al., 2020). It has also been suggested that miR-122 may be involved in the toxicological response to environmental contaminants, including fluoxetine (Disner et al., 2021). Interestingly, previous research from our group found that fluoxetine treatment led to a significant increase in steady-state mRNA expression of PPAR gamma (*Pparg)* in rat hepatoma cells, along with a concurrent increase in PPARG target genes involved in hepatic lipid accumulation (Ayyash & Holloway, 2021b). In hepatocytes, PPARG is a central regulator of lipid metabolism, targeting genes involved in *de novo* lipogenesis and free fatty acid uptake (Skat-Rørdam et

al., 2019); PPARG expression was found to be significantly elevated in the liver of obese rats and involved in the development of NAFLD (Edvardsson et al., 2006; Gavrilova et al., 2003; Matsusue et al., 2008; Pettinelli & Videla, 2011; Schadinger et al., 2005). Interestingly, PPARG was found to be associated with sites in the *miR-122* gene promoter, and the binding of PPARG agonists enhanced *miR-122* gene transcription in human hepatocellular carcinoma cells (Song et al., 2013). Knockdown of PPARG decreased miR-122 levels, conversely, overexpression of PPARG increased miR-122 expression thus supporting the hypothesis that in hepatocytes miR-122 expression is positively regulated by PPARG (Song et al., 2013). Given the link between PPARG and miR-122 and the fact that fluoxetine increased the expression of PPARG target genes, I hypothesized that *fluoxetine exposure would alter miRNA signatures in hepatic cells which would contribute, in part, to increased lipid accumulation.* To address this question, I first assessed the effects of fluoxetine treatment on the expression of the PPARG-regulated miRNA, miR-122, then employed a literature search strategy to identify miRNAs that have been shown to upregulated in NAFLD as well as having been differentially expressed following fluoxetine exposure.

4.2 Objective A: Investigating the effects of fluoxetine on miR-122 expression

4.2.1 Objective A: Rationale

In order to investigate the link between PPARG and miR-122 following fluoxetine treatment, the miRNA expression of miR-122 and mRNA expression of *Pparg* and the miR-122 target gene, *Prkra* were examined. To further determine whether fluoxetine could be altering miR-122 expression via a PPARG mediated pathway, hepatocytes were treated with PPARG antagonist GW9662 ± 10 μM fluoxetine, and relative miRNA expression of miR-122 was examined.

4.2.2 Objective A: Experimental Methods

4.2.2.1 Cell culture maintenance and treatment

H4-II-E-C3 hepatoma cells were grown in Corning™ Dulbecco's modified Eagle's medium (DMEM) (Corning, NY) supplemented with 10 % heat-inactivated fetal bovine serum (Hyclone, South Logan, UT), 2% L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin (Gibco, Long Island, NY) at 37 °C in a humidified atmosphere of 95 % O2 and 5 % CO2. All cell treatments were made in supplemented DMEM as described above. When H4-II-E-C3 cells reached 80% confluence, they were treated for 24 h with vehicle or 10 μM fluoxetine hydrochloride (Toronto Research Chemicals, North York, ON), a dose previously shown to increase lipid accumulation in this cell line (A. Ayyash & Holloway, 2021a; X.-M. Feng et al., 2011; Xiong et al., 2014). Cells were then collected for either miRNA or mRNA isolation.

To determine whether fluoxetine could be altering miR-122 expression via a PPARG mediated pathway, cells were treated with fluoxetine in the presence of the selective PPARG antagonist GW9662, Briefly, cells were grown as above and treated with 1) vehicle, 2) 10 µM GW9662 ± 10 µM fluoxetine, 3) 10 µM fluoxetine. This concentration of GW9662 was selected based on other studies which had reported that it could block PPARG-mediated signaling in hepatocytes (H.-J. Lee et al., 2009; Ou et al., 2017; Tay et al., 2010). Cells were also treated with rosiglitazone (10 µM), a known PPARG agonist as a positive control (Jarrar & Lee, 2021; K. Kim et al., 2009; L. Ma et al., 2012).

4.2.2.2 miRNA extraction and cDNA synthesis

Following 24 h treatment, the cell-culture medium was aspirated, and cells were washed with phosphate-buffered saline (PBS) (N = 5 independent experiments). Cells were then directly lysed with the addition of 450 µl of Buffer RLT, before being collected into a microcentrifuge tube. miRNA was extracted from the lysate using the miRNeasy Tissue/Cells Advanced Mini Kit (Cat# 217604, Qiagen, Toronto, ON) according to the manufacturer's instructions. The miRCURY LNA RT Kit (Cat# 339340, Qiagen) was then used to synthesize cDNA from the miRNA. Reactions were performed using the iCycler Thermocycler (Bio-Rad, Mississauga ON) for 60 min at 37°C followed by 5 min at 95°C to allow cDNA synthesis. These cDNA samples made from extracted miRNA were diluted with RNAse-free water at a 1:60 dilution before quantification.

4.2.2.3 Quantification of miRNA-122 expression

To determine the effect of fluoxetine treatment on the expression of miR-122 we used real-time quantitative PCR (RT_qPCR) and miRCURY LNA SYBR Green PCR Kit (Cat# 339346, Qiagen) and rat-validated commercial primers (Qiagen; Table 1). RT-qPCR was completed using CFX384 Touch™ Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA). The PCR cycling settings included polymerase activation (95 °C for 2 m), followed by 40 cycles of denaturing (95 °C for 10 s), and annealing/elongation (56 °C for 1 m). Levels of miRNA expression were calculated using the ΔΔCt method and normalized using the geometric means of two reference miRNAs (Livak & Schmittgen, 2001). Reference miRNAs were selected based on those reported in the literature and the analysis tool 'RefFinder'; this tool combines the results from three commonly used programs for selecting ideal housekeepers (GeForce, BestKeeper, NormFinder). We selected miR-191 and miR-16 as the ideal housekeepers for this experiment. Use of these housekeepers has been reported in other studies of miRNA expression in liver tissues and cells (Albracht-Schulte et al., 2019; Auguet et al., 2016b; Hoekstra et al., 2012; Karimi-Sales et al., 2021; Lardizábal et al., 2012; Millán et al., 2019; Soubeyrand et al., 2021).

4.2.2.4 microRNA-122 target prediction and target mRNA expression

To reveal miRNA target genes that may be dysregulated by the fluoxetine-induced alteration in miRNA expression we used target prediction software followed by experimental confirmation of altered mRNA expression of these targets. The online miRNA database miRTarBase 2020 (http://miRTarBase.cuhk.edu.cn/) was selected as the most suitable for this study as it provides up-to-date, comprehensive information on experimentally validated miRNA–target interactions (H.-Y. Huang et al., 2020). Using this information, we assessed changes in the relative mRNA expression of *Prkra*, a validated target of miR-122. Primer sequences for mRNA targets and housekeeping genes are provided in Table 2.

4.2.2.5 mRNA extraction and cDNA synthesis

Cells were treated for 24h with fluoxetine (10µM) or vehicle as described above (N=5 independent experiments). After 24h, media was removed, cells were washed with with phosphate-buffered saline (PBS), and total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA). RNA concentrations were determined using the NanoDrop One TM Microvolume UV-Vis Spectrophotometer (Thermo Scientific, Waltham, MA). Complementary DNA (cDNA) was made from 2 µg of mRNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) as per the manufacturer's instructions. The resulting cDNA made from the extracted mRNA was then diluted at a 1:10 dilution with RNAse-free water before quantification.

4.2.2.6 Quantification of validated miR-122 *target gene*

The resulting cDNA was a template for RT-qPCR, which was carried out using PerfeCTa® SYBR® Green FastMix® (Quanta Biosciences, Gaithersburg, MD) on the CFX384 Touch™ Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA). The PCR cycling settings included polymerase activation (95 °C for 10 m), followed by 40 cycles of denaturing (95 °C for 15 s), and annealing/elongation (60 °C for 1 m). Levels of gene expression were calculated using the ΔΔCt method and normalized using the geometric means of two reference genes glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*) and beta-actin (*Actb*) (Livak & Schmittgen, 2001).

4.2.2.7 Statistical Analysis

All statistical analyses were conducted using SigmaPlot (v.11.2, Systat Software, San Jose, CA). Data were tested for outliers (Grubbs' test), normality, and equal variance. Comparisons among the two groups were analyzed using Student's t-tests. Comparisons among multiple groups were analyzed using One-Way Analysis of Variance (ANOVA) followed by the Bonferroni multiple comparisons test. When normality or equal variance failed, the Mann-Whitney Rank Sum Test or Student-Newman-Keuls One-Way ANOVA on Ranks were used to determine significance. All data are presented as mean ± SEM and were considered significant when $P \leq 0.05$.

4.2.3 Objective A: Results

4.2.3.1 Role of PPARG in Fluoxetine induced upregulation of miR-122

The expression of *miRNA-122* in the H4-II-E-C3 hepatocytes was significantly elevated following 24 h treatment with fluoxetine (Figure 1A). Moreover, the expression of the validated mRNA target of miR-122, *Prkra* was also significantly decreased following the same treatment (Figure 1B), as had previously been demonstrated (H.-Y. Huang et al., 2020). Given that miR-122 has been reported to be positively regulated via PPARG activation (Yarushkin et al., 2017), we tested the ability of the known PPARG agonist, rosiglitazone, to increase miR-122 expression. Indeed, as predicted, rosiglitazone treatment caused a significant increase in miR-122 expression (Figure 2A). To examine whether the increase in miR-122 expression following fluoxetine treatment was linked to activated PPARG, cells were treated with fluoxetine ± a selective PPARG antagonist, GW9662. While fluoxetine treatment caused a significant upregulation of miR-122, this effect was attenuated with the addition of GW9662 (Figure 2B) such that the expression of miR-122 in the fluoxetine + GW9662 treatment group was not significantly different from controls.

4.3 Objective B: Discovery of miRNA involved in the pathogenesis of NAFLD and altered as a result of fluoxetine treatment

4.3.1 Objective B: Rationale

Results demonstrating fluoxetine's increase in miR-122 prompted us to explore other candidate miRNA that may have also been altered following treatment with the SSRI and play a role in the progression of NAFLD. To further test our hypothesis, we began by employing a literature review to identify other potential candidate miRNAs to investigate those that have been reported to be dysregulated with both SSRI treatment and NAFLD.

4.3.2 Objective B Experimental Methods: Identifying common miRNA targets between SSRI exposure and NAFLD

4.3.2.1 SSRIs exposure and altered miRNA

A literature search was conducted in NCBI using MeSH to search for articles with the following terminology as a major topic in the headings: "Serotonin Uptake Inhibitors [Pharmacological Action]" OR "Serotonin Uptake Inhibitors" AND "MicroRNAs". We searched between the years 2010 and 2022 as the first relevant article on the topic was published in 2010. Our search strategy is outlined in Appendix 1. We identified a total of 38 publications using this search strategy. Records were screened to remove reviews (n=2), systematic reviews, meetings, letters, books, documents, and non-english texts (n=2), resulting in a remaining total of 34 full-text articles to be assessed for eligibility by AA. Studies that did not investigate altered miRNA expression as a direct result of SSRI

treatment were excluded. After excluding studies that were deemed irrelevant or not directly related, a total of 25 studies met the eligibility criteria. All of the studies selected show statistically significant differences in the expression level of specific miRNAs between cases and controls. 202 miRNAs were reported to be differentially expressed in the 25 studies that compared SSRI treatment with either healthy or depressed controls (Appendix 2).

4.3.2.2 NAFLD and miRNA

A literature search was conducted in NCBI using MeSH to search for articles with the following terminology as a major topic in the headings: "Non-alcoholic Fatty Liver Disease" AND "MicroRNAs". We searched between the years 2013 and 2022 as the first relevant article on the topic was published in 2013. We identified a total of 280 publications using this search strategy. Our search strategy is outlined in Appendix 3. Records were screened to remove reviews (n=31), systematic reviews (n=3), meetings, letters, books, documents, and non-English texts (n=9), resulting in a remaining total of 237 full-text articles to be assessed for eligibility by AA, CR and SJ. Studies that did not investigate altered miRNA expression as a direct result of NAFLD were excluded. Studies designed to alter the expression of miRNA and examine changes in pathogenesis of NAFLD rather than to investigate miRNAs which were altered in animals/humans with NAFLD were also excluded. After applying our exclusion criteria, a total of 168 studies met the eligibility criteria. All of the studies selected show statistically significant differences in the expression level of specific miRNAs between cases and controls. 160 miRNAs were reported to be differentially expressed in the 168 studies that compared NAFLD to controls (Appendix 4).

When comparing the miRNAs which were altered with SSRI exposure or NAFLD, we identified a total of 54 miRNAs which were differentially expressed in both conditions (Figure 3). A comprehensive list of these shared miRNA targets is provided in Table 3.

4.3.2.3 Final miRNA target selection and quantification

Two investigators reviewed the results separately and each independently identified 2 miRNAs as potential targets based on the following criteria, in no particular order of importance: (i) the number of studies that reported a miRNA as differentially expressed, (ii) the number of studies that reported a miRNA as differentially expressed in the same direction, (iii) the degree to which the miRNA has been reported to be expressed in the liver, (iv) the reported implication of the miRNA in NAFLD progression and/or lipid accumulation in the liver, (v) the function of the miRNA and/or the function of the miRNA's target messenger RNA(s) (mRNA). A third independent investigator examined the presented rationale for each potential miRNA target, and it was determined that all presented miRNA targets would be experimentally investigated. One of the miRNA targets were duplicated by the independent investigators, therefore a total of 3 miRNAs were chosen: miR-21, miR-29a*, and miR-34a (Appendix 5).

miR-34a has been suggested to modulate lipid metabolism and is highly expressed in patients with steatosis, NASH, and in experimental animal models of NAFLD (Baffy, 2015; Ding et al., 2015a). miR-29a* may be involved in mitigating hepatic lipid accumulation associated with NAFLD progression, as miR-29a* transgenic mice fed a high-fat diet revealed that an overexpression of miR-29a* reduced fat accumulation in the liver (H.-Y. Lin et al., 2020; Mattis et al., 2015; Su et al., 2018). miR-21 has previously been implicated for its role in hepatic lipid accumulation and activation of the NLRP3 Inflammasome (Kong et al., 2019; Ning et al., 2017; Sun et al., 2017; Xue et al., 2019). A cross-sectional investigation revealed miR-21 was elevated in the liver and plasma of patients with NAFLD, while another study found a significant increase in miR-21 in mice treated with fluoxetine (Yamada et al., 2013; T. Zhang et al., 2020). Importantly, activation of the NLRP3 inflammasome has been shown to contribute to the progression of NAFLD to its more severe stages, including NASH (Mridha et al., 2017; Wan et al., 2016). As such, changes to key components of the NLRP3 inflammasome were also investigated in the hepatocytes following treatment with fluoxetine.

Following selection of these 3 target miRNAs, the expression of the selected candidate miRNAs (miR-21, miR-29a*, miR-34a) was determined via RT-qPCR following 24 h fluoxetine treatment as described above. Assessment of changes in the relative mRNA expression of the miR-21 validated target, *Tiam1*, along with *Casp1*, *Asc*, *Nlrp3* and *Il-1b* (key components of the NLRP3 inflammasome) were also determined via RT-qPCR as outlined above.

4.3.3 Objective B: Results

4.3.3.1 Exploration of Fluoxetine induced alterations in miRNA's related to pathogenesis of NAFLD

We measured the relative expression of miR-21, miR-29a* and miR-34a in H4-II-E-C3 following 24 h treatment with 10μM fluoxetine. While there were no significant changes in the expression of miR-29a* and *miR-34* (Figure 4 A&B), miR-21 was significantly upregulated and the steady-state mRNA expression of miR-21's validated target *Tiam1* was significantly decreased (Figure 4 C&D).

4.3.3.2 Fluoxetine induced hepatic inflammasome response may be mediated by increased miR-21

Previously, our lab demonstrated that fetal and neonatal exposure to fluoxetine led to the development of mild to severe NASH, increased inflammation and significantly elevated mRNA expression of core components of the NLRP3 inflammasome in the livers of adult offspring (De Long, Barry, et al., 2015; De Long et al., 2017). Given miR-21's importance in activation of the NLRP3 inflammasome (Sun et al., 2017; Xue et al., 2019), mRNA targets relevant to NLRP3 activation, *Casp1*, *Asc*, *Nlrp3* and *IL-1B* were further explored (Figure 5). H4-II-E-C3 cells had a significant increase in the expression of *Casp1*, *Nlrp3* and *Il-1b* following 24 h treatment with 10μM fluoxetine.

4.4 Discussion

Although miRNAs have been implicated as having a role in a myriad of physiological processes and pathologies, including cancer, cardiovascular and metabolic diseases, the specific roles of these miRNAs in disease pathophysiology are still largely unknown. miRNA arise from long hairpin pri-miRNA structures that are initially cleaved into pre-miRNA and subsequently cleaved into mature miRNA (Hansen & Obrietan, 2013b; Torres-Berrío et al., 2019). Mature miRNA binds to the incomplete complementary regions of the 3' untranslated region of mRNA within the RNA-inducing silencing complex, thus repressing mRNA translation or inducing deadenylation and degradation of mRNA (Hansen & Obrietan, 2013b; Torres-Berrío et al., 2019). miRNA expression can be regulated by some mRNAs, and each miRNA can regulate hundreds of mRNAs; this signifies the highly coordinated system by which protein translation can be regulated (Hansen & Obrietan, 2013b; Torres-Berrío et al., 2019).

In recent years the role of miRNAs in MDD pathophysiology, synaptic plasticity, and gene regulation critical to signaling pathways involved in MDD has gained increasing attention (Issler et al., 2014; Saavedra et al., 2016; Torres-Berrío et al., 2019). In addition, there is now increasing evidence that demonstrates that antidepressants used for the treatment of MDD can also affect miRNA expression in multiple tissues (Craig et al., 2014b; Oved et al., 2012a). Indeed, in our literature review, we identified 202 miRNAs that had been altered following exposure to SSRI antidepressants (Appendix 2). Given the link between exposure to SSRI antidepressants and increased hepatic lipid accumulation found in our studies (A. Ayyash & Holloway, 2021a, 2021b), the aim of this objective was to investigate miRNA changes in a model of fluoxetine-induced hepatic lipid accumulation especially since there are numerous studies which have reported changes in the miRNA profile associated with NAFLD (Appendix 1).

Results from this study have shown that treatment with the SSRI fluoxetine led to elevated miR-122 expression in H4-II-E-C3 cells. The effects of fluoxetine to induce lipid abnormalities appear to be mediated via PPARG (A. Ayyash & Holloway, 2021b), which directly regulates the expression of miR-122 (K. Song et al., 2013). Indeed, treatment with the PPARG agonist rosiglitazone has been reported to directly increase miR-122 expression in mouse and human hepatocytes (K. Song et al., 2013; Yarushkin et al., 2017); a result confirmed in this study. Previously, we demonstrated that the SSRI fluoxetine increased mRNA expression of *Pparg*, the key transcriptional regulator that drives lipogenesis, and its downstream targets in the same cell line (A. Ayyash & Holloway, 2021b) suggesting that fluoxetine can act as a direct PPARG agonist. Further support to suggest that fluoxetine can act as a direct PPARG agonist comes from *in silico* binding experiments which demonstrate that fluoxetine (-8.1 kcal/mol) and the known PPARG agonist rosiglitazone (-7.9 kcal/mol) and antagonist GW9662 (-7.8 kcal/mol) have similar binding energies to PPARG (Jamshed and Holloway, unpublished data). A threshold of −7.0 kcal/mol works well to discriminate between putative specific and nonspecific binding with PPARG, with the greatest binding energies having the most negative value (Chang et al., 2007).

To examine whether upregulated miR-122 expression following fluoxetine treatment was linked to PPARG activation, we investigated if treatment with a known selective PPARG antagonist (GW9662) could block the effect of fluoxetine to increase miR-122 expression. Indeed, treatment with GW9662 significantly attenuated *miR-122* expression (Figure 2). The exact mechanism by which the inhibition of miR-122 leads to a downregulation of genes involved in hepatic steatosis is not clear, with one possibility suggesting that miR-122 negatively regulates SIRT1 (Esau et al., 2006, Long et al., 2019). As SIRT1 is a transcriptional inhibitor of lipogenic genes, inhibition of SIRT1 by overexpression of miR-122 could result in increased transcription of lipogenic genes. Interestingly SIRT1 has been reported to be altered in MDD (Kishi et al., 2010) and by fluoxetine exposure (Sharma et al., 2021), however, whether or not SIRT1 is involved in a miR-122-fluoxetine-lipid accumulation pathway remains to be determined.

Clinical studies have also demonstrated that miR-122 has been correlated with the severity of NAFLD; targets of miR-122 also include genes involved in fibrosis and inflammation which may contribute to non-alcoholic steatohepatitis (NASH) and hepatic fibrosis (Farrell et al., 2012; Y. Liu, Song, et al., 2020; Yamada et al., 2013). Interestingly, based on our literature review we identified miR-21 as a miRNA that has been shown to be upregulated in NAFLD and is altered with SSRI exposure, and is associated with activation of the NLRP3 inflammasome (Table 3) (Ning et al., 2017; Yamada et al., 2013; Y. Zhang et al., 2020a). Importantly, activation of the NLRP3 inflammasome has been shown to contribute to the progression of NAFLD to its more severe stages, including NASH (Mridha et al., 2017; Wan et al., 2016) and we have previously reported that fetal and neonatal exposure to fluoxetine in a rat model resulted in increased hepatic expression of NLRP3 inflammasome components and lipid accumulation in the adult offspring (De Long et al., 2017). In the current study, the expression of miR-21 was significantly upregulated by fluoxetine exposure. Consistent with an increased miR-21 expression we also saw a significant decrease in its validated target gene, *Tiam1* (Figure 4 C&D), and an upregulation of the mRNA expression of the inflammasome components *Casp1*, *Nlrp3,* and *Il-1b* (Figure 5 A, 5 C, and 5 D). While further studies are required to examine this association, our results do suggest the presence of a fluoxetine-miR-21-NLRP3 inflammasome axis which may underlie the increased risk of lipid accumulation and inflammation associated with SSRI antidepressant use (De Long et al., 2017; Miao et al., 2018a; Xue et al., 2019; Zhao et al., 2021). Taken together the results of this study have demonstrated that fluoxetine exposure can significantly alter the expression of miRNAs in hepatic cells. Importantly, the 2 miRNAs which were increased by this exposure (i.e. miR-122 and miR-21) have been shown to play key roles in lipid accumulation and inflammation; key aspects of NAFLD. While the exact mechanisms by which fluoxetine can affect the expression of these miRNAs remains unknown, this study provides proof of concept that the effects of fluoxetine to affect hepatic lipid accumulation may be mediated, in part, via changes in the epigenome.

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4.6 Tables and Figures

4.6.1 Tables:

Table 1. Real-time PCR miRNA primers used in the study, all validated in *Rattus norvegicus.*

Mature miR	Primer Sequence (5'-3')	GeneGlobe ID	Accession #
hsa-miR-16	UAGCAGCACGUAAAUAUUGGCG	YP00205702	MIMAT0000069
hsa-miR-21	UAGCUUAUCAGACUGAUGUUGA	YP00204230	MIMAT0000076
*hsa-miR-29a**	UAGCACCAUCUGAAAUCGGUUA	YP00204698	MIMAT0000086
hsa-miR-34a	UGGCAGUGUCUUAGCUGGUUGU	YP00204486	MIMAT0000255
hsa-miR-122	UGGAGUGUGACAAUGGUGUUUG	YP00205664	MIMAT0000421
hsa-miR-191	CAACGGAAUCCCAAAAGCAGCUG	YP00204306	MIMAT0000440

Table 2. Real-time PCR *Rattus norvegicus* mRNA primers used in the study.

Gene	Forward primer Sequence (5'-3')	Reverse Primer Sequence (5'-3')	Genebank accession #
Actb	CACAGCTGAGAGGGAAAT	TCAGCAATGCCTGGGTAC	NM_031144.3
Asc	TGGTTTGCTGGATGCTCTGT	CACGAACTGCCTGGTACTGT	NM_172322.1
Casp1	AACACCCACTCGTACACGTC	TGAGGTCAACATCAGCTCCG	NM_012762.3
Gapdh	TGGAGTCTACTGGCGTCTTCAC	GGCATGGACTGTGGTCATGA	NM_017008.4
Il-1b	GCAGTGTCACTCATTGTGGC	AAGAAGGTGCTTGGGTCCTC	NM_031512.2
Nlrp3	CACAACTCACCCAAGGAGGA	ACAGGCAACATGAGGGTCTG	NM_001191642.1
Prkra	GTGCCCACTTTCACCTTCAG	TGCTTCGCCAGCTTCTTACT	XM_032903531.1
Tiam1	AGGTGAGACCCCGATGGA	ATACTGAGGCTGGAGATGGTG	NM_001100558.2

Table 3. Comprehensive list of miRNA altered in the literature as a result of both NALFD and following SSRI exposure.

miRNA Altered as a Result of NAFLD and SSRI Treatment	
miR-1	miR-140
miR-9	miR-144
miR-15b	miR-145
miR-16	miR-146b
miR-17	miR-150
miR-20b	miR-155
miR-21	miR-181a
miR-22	miR-182
miR-22*	miR-183
miR-221	miR-185
miR-23b*	miR-192
miR-26a	miR-194
miR-27a*	miR-200a*
miR-27b*	miR-200b*
miR-29a*	miR-205
miR-29b	miR-206
miR-29b*	miR-212
miR-29c*	miR-214*
miR-30b	miR-218
miR-30d	miR-328
miR-34a	miR-335
miR-92a	miR-361
miR-103	miR-378*
miR-128	miR-451a
miR-130b	miR-486
miR-132	miR-505
miR-139	miR-1290

4.6.2 Figures:

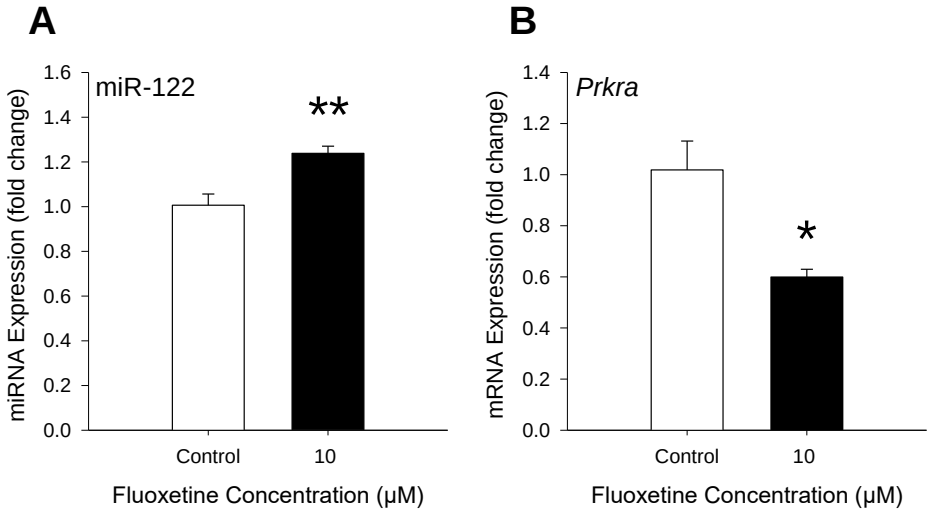


Figure 1: (**A**) Measurement of miR-122 relative miRNA expression in H4-II-E-C3 treated with 10 μM fluoxetine (n = 4-5), for 24 h. (**B**) H4-II-E-C3 were treated with 10μM fluoxetine for 24h and expression of the validated mRNA target of miR-122, *Prkra* was examined (n = 4-5). Outcome measures between control and treatment were analyzed using Student's t-test ($\alpha = 0.05$) * $P \leq 0.05$, ** $P \leq 0.01$. Data represents mean ± SEM.

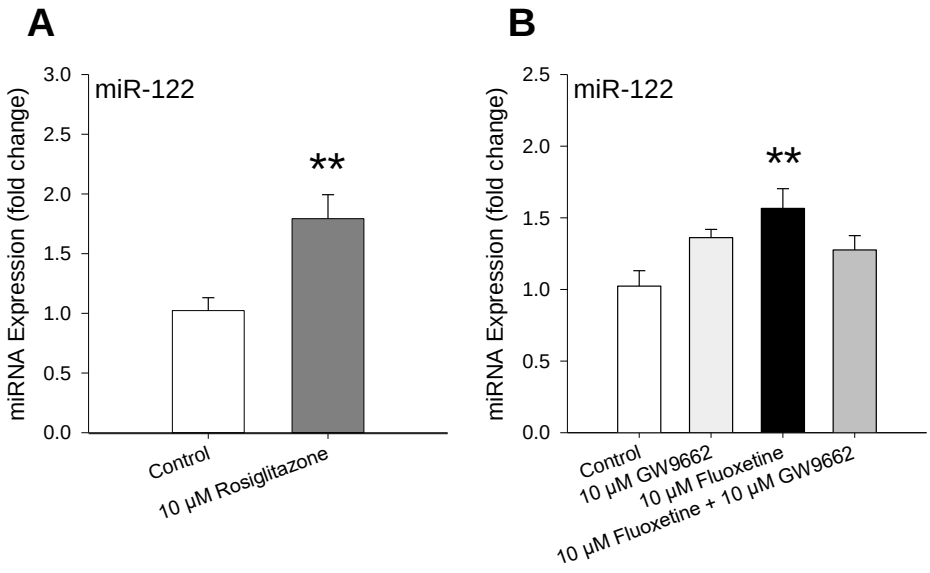


Figure 2: (**A**) Relative miRNA expression of miR-122 in H4-II-E-C3 treated with 10 μM Rosiglitazone for 24 h was quantified (n = 4-5). Statistical significance between control and 10μM Rosiglitazone was assessed via Student's T-test *, $P \leq 0.05$. (**B**) Relative *miRNA-122* expression was determined in H4-II-E-C3 cells treated for 24h with 10 μM GW9662, co-treated with 10 μM fluoxetine + 10 μM GW9662, and 10μM fluoxetine alone (n = 4-5). Outcome measures between control and treatments were analyzed using a one-way ANOVA ($\alpha = 0.05$). **, $P \leq 0.01$ vs. control group, followed by the Holm-Sidak multiple comparisons test. Data represent mean ± SEM.

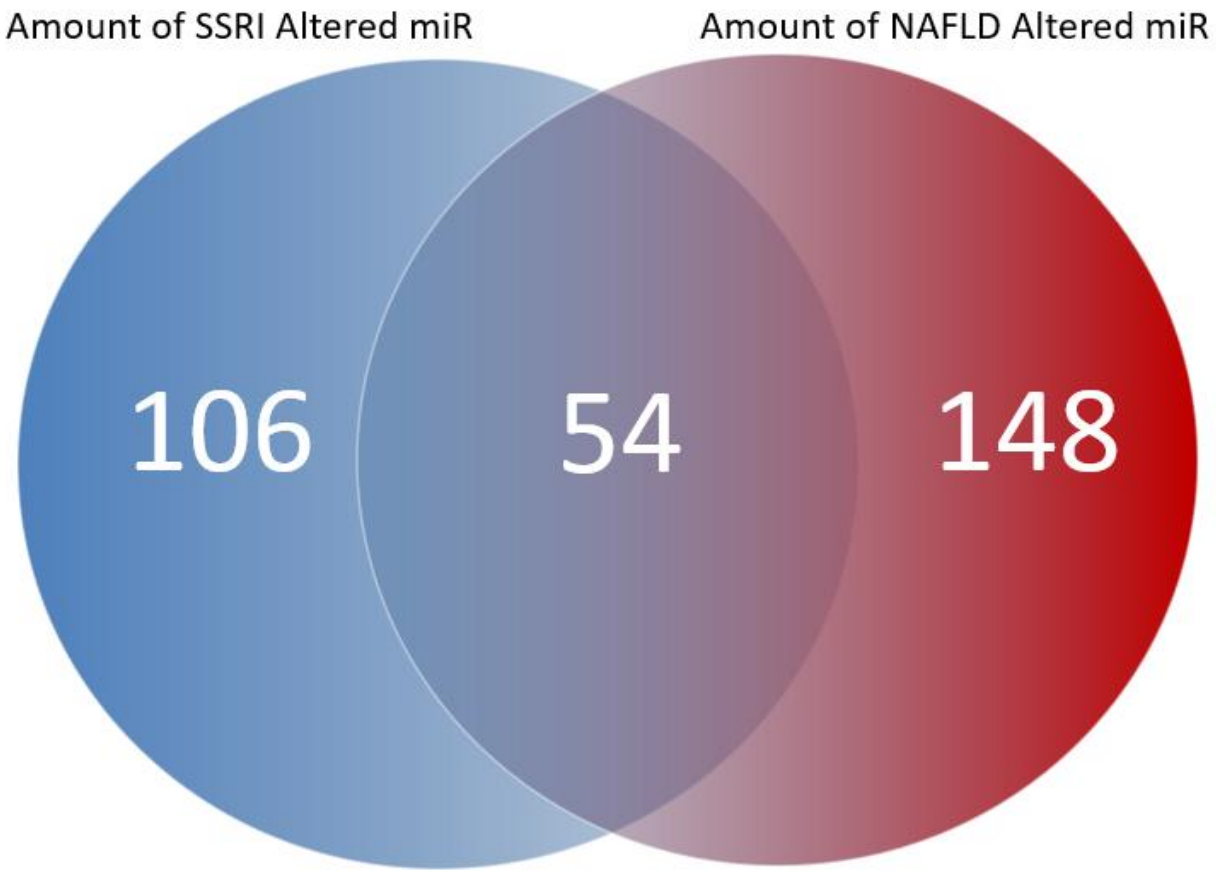


Figure 3. Venn Diagram representing the amount of overlapping expressed microRNAs in association with SSRI treatment and NAFLD.

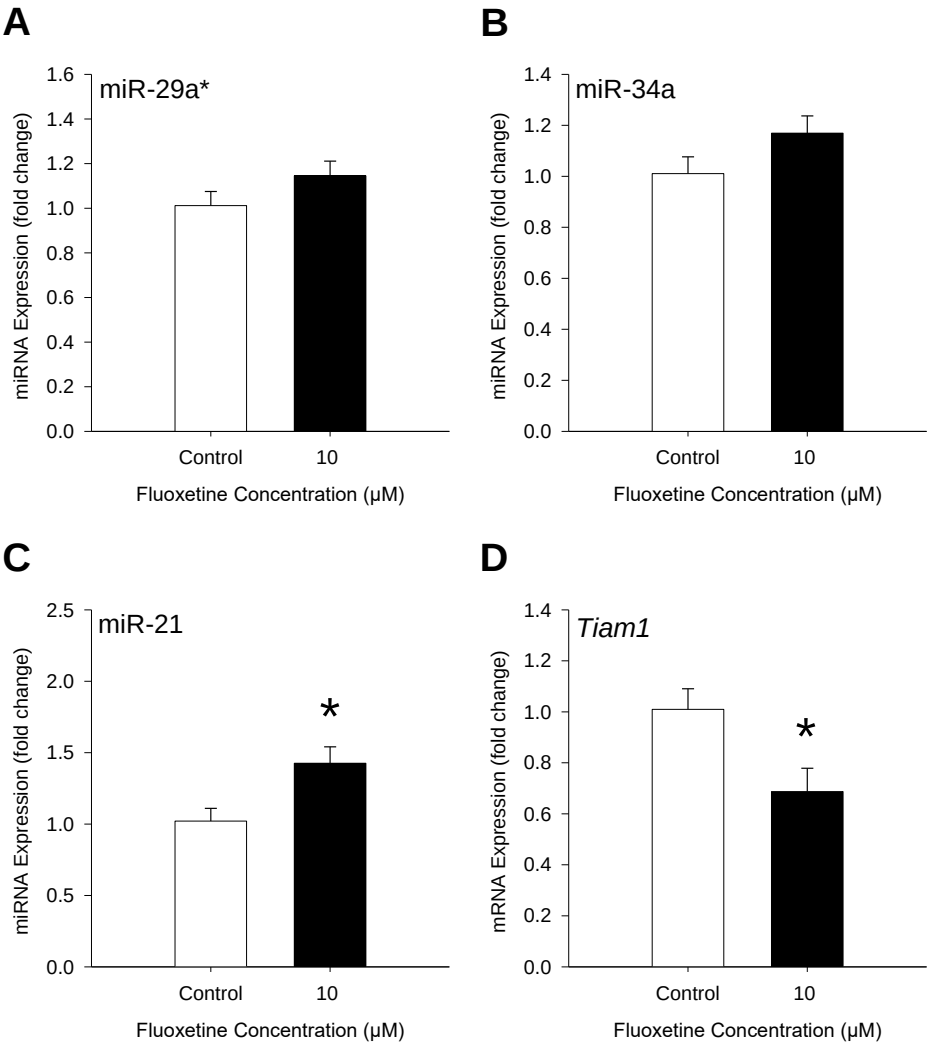


Figure 4: H4-II-E-C3 were treated with 10 μM fluoxetine for 24 h and relative expression of the candidate miRNAs (**A**) miR-29a*, (**B**) miR-34a and (**C**) miR-21 were determined (n = 4-5). (**D**) Quantification of miR-21's validated mRNA target, *Tiam1* expression in H4-II-E-C3 treated with 10 μM fluoxetine for 24 h (n = 4-5)**.** Outcome measures between control and treatment were analyzed using Student's t-test ($\alpha = 0.05$) * $P \leq 0.05$, Data represents mean ± SEM.

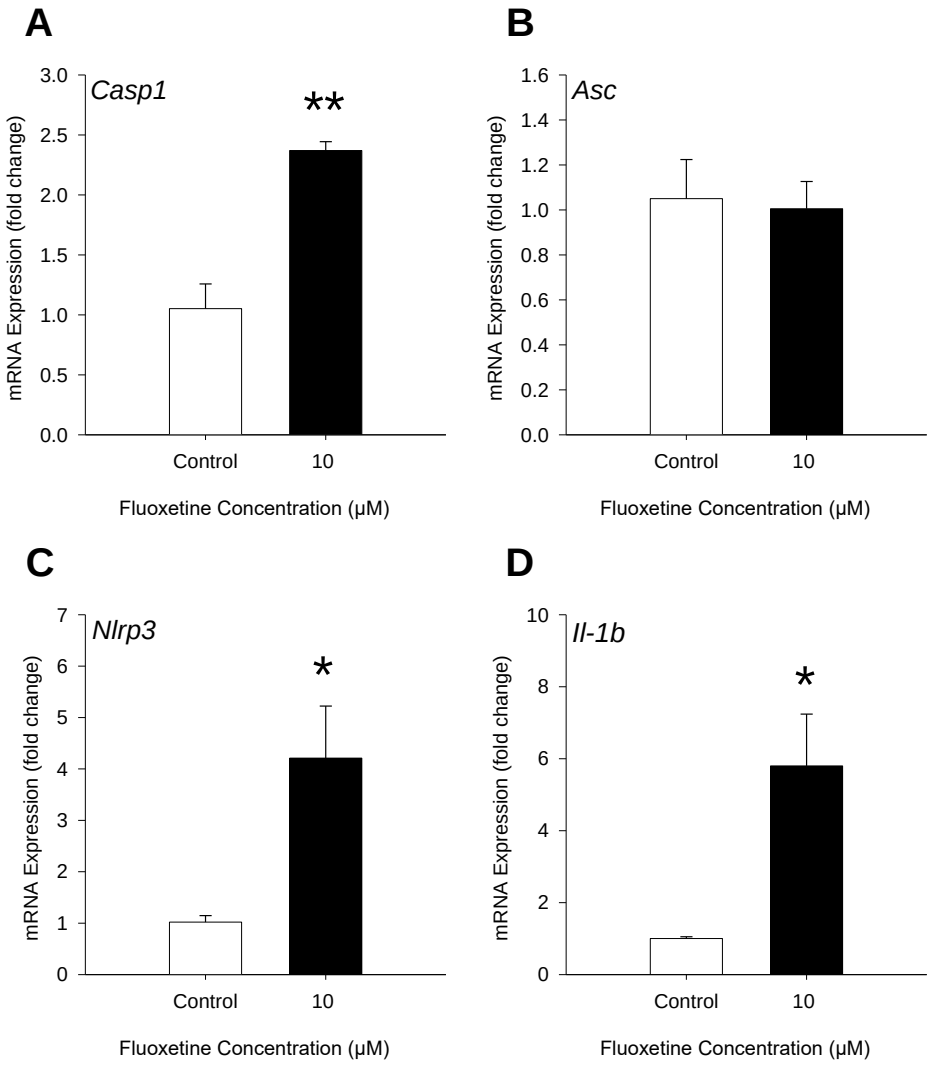
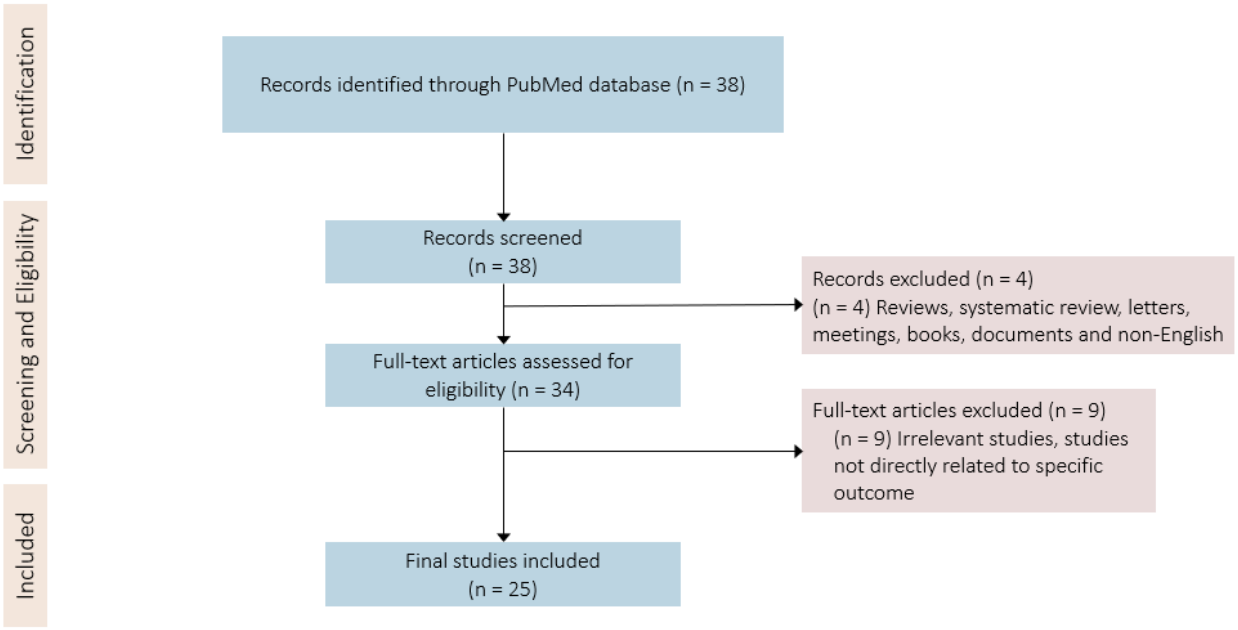


Figure 5: H4-II-E-C3 were treated with 10 μM fluoxetine for 24 h and relative mRNA expression of (**A**) *Casp1*, (**B**) *Asc*, (**C**) *Nlrp3* and (**D**) *Il-1b* was determined (n = 4-5). Outcome measures between control and treatment were analyzed using Student's t-test (α = 0.05) * $P \leq 0.05$, ** $P \leq 0.01$.

4.7 Appendix



Appendix 1. Flow chart to identify studies of miRNA changes with SSRI exposure.

Appendix 2. Comprehensive list of miRNA altered following exposure to SSRIs

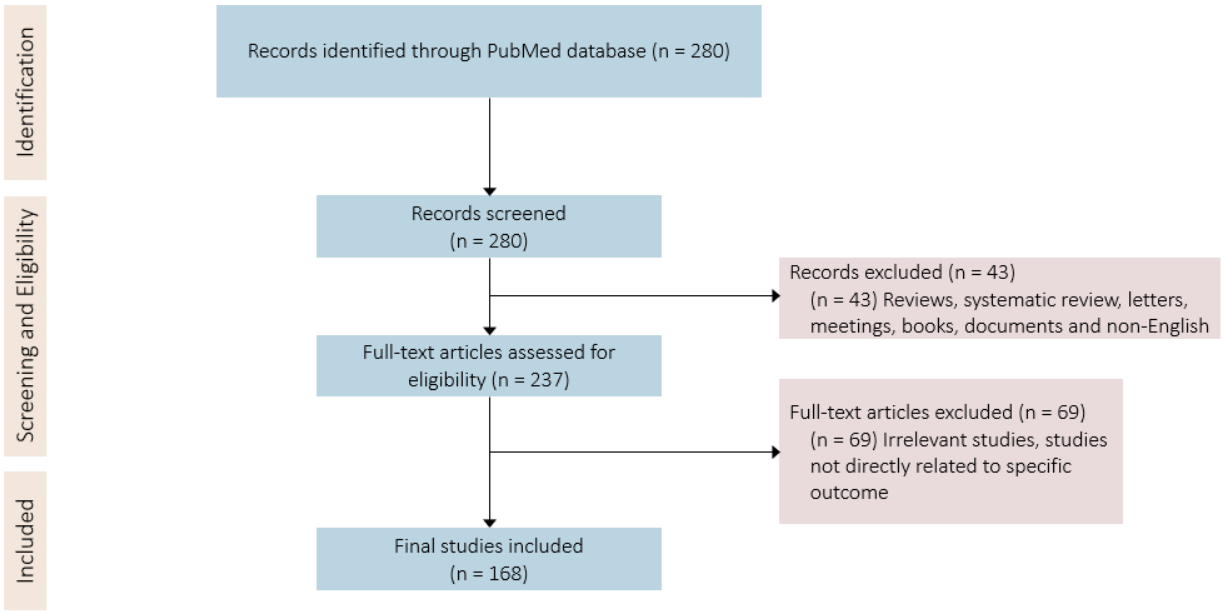
miRNA Altered	Reference
miR-1	(Min et al., 2019)
miR-7a	(Miao et al., 2018a; W. Song et al., 2019)
miR-9	(Miao et al., 2018a)
miR-10b	(W. Song et al., 2019)
miR-15a	(Yrondi et al., 2020)
miR-15b	(Yrondi et al., 2020)
miR-16	(Baudry et al., 2010; Fiori et al., 2017, p. 1202; Launay et al., 2011; Lin et al., 2018; Miao et al., 2018)
miR-17	(Yrondi et al., 2020)
miR-19a*	(Craig et al., 2014a)
miR-20a	(Yrondi et al., 2020)
miR-20b	(Yrondi et al., 2020)
miR-20b*	(Oved et al., 2012b)
miR-21	(Miao et al., 2018a)
miR-21*	(Oved et al., 2012b)
miR-22	(Bocchio-Chiavetto et al., 2013)
miR-22*	(Miao et al., 2018a)
miR-22b	(Craig et al., 2014a)
miR-23a*	(Miao et al., 2018a)
miR-23b*	(Miao et al., 2018a)
miR-25	(Yrondi et al., 2020)
miR-25*	(Min et al., 2019; Yrondi et al., 2020)
miR-26a	(Bocchio-Chiavetto et al., 2013; Maffioletti et al., 2017)
miR-26b	(Bocchio-Chiavetto et al., 2013; Miao et al., 2018)
miR-27a*	(Yrondi et al., 2020)
miR-27b*	(Miao et al., 2018a)
miR-28c	(Miao et al., 2018a)
miR-29a*	(Miao et al., 2018a)
miR-29b	(Bocchio-Chiavetto et al., 2013)
miR-29b*	(Miao et al., 2018a)
miR-29c*	(Miao et al., 2018a; Oved et al., 2013)
miR-30a	(Miao et al., 2018a; Yrondi et al., 2020)
miR-30b	(Miao et al., 2018; Yrondi et al., 2020; Oved et al., 2012)
miR-30b*	(Oved et al., 2012b)
miR-30c-1*	(M. Kato et al., 2022; Yrondi et al., 2020)
miR-30d	(Bocchio-Chiavetto et al., 2013; Miao et al., 2018)

miR-32*	(W. Song et al., 2019)
miR-34a	(Kuang et al., 2018; Lo Iacono et al., 2021)
miR-34c	(Bocchio-Chiavetto et al., 2013)
miR-92a	(Yrondi et al., 2020)
miR-92b*	(Miao et al., 2018a)
miR-93	(Yrondi et al., 2020)
miR-103	(Bocchio-Chiavetto et al., 2013)
miR-103*	(Miao et al., 2018a)
miR-103a*	(Yrondi et al., 2020)
miR-103b	(Yrondi et al., 2020)
miR-106a	(Yrondi et al., 2020)
miR-106b	(Bocchio-Chiavetto et al., 2013)
miR-106b*	(Yrondi et al., 2020)
miR-107*	(Miao et al., 2018a)
miR-124	(Y. Fang et al., 2018)
miR-126a*	(Miao et al., 2018a)
miR-128	(Bocchio-Chiavetto et al., 2013)
miR-129*	(Miao et al., 2018a)
miR-130b	(Bocchio-Chiavetto et al., 2013)
miR-132	(Bocchio-Chiavetto et al., 2013; Fang et al., 2018; Miao et al., 2018)
miR-132*	(Miao et al., 2018a)
miR-133b	(Miao et al., 2018a)
miR-135	(Fiori et al., 2017)
miR-135a	(Y. Liu et al., 2017)
miR-139	(Miao et al., 2018a)
miR-140	(Craig et al., 2014a)
miR-140*	(Bocchio-Chiavetto et al., 2013; Miao et al., 2018a; Yrondi et al., 2020)
miR-144	(Min et al., 2019)
miR-145	(Yrondi et al., 2020)
miR-146b	(Oved et al., 2012b)
miR-148	(Min et al., 2019)
miR-148a*	(Oved et al., 2012b)
miR-148b*	(Yrondi et al., 2020)
miR-149*	(Miao et al., 2018a)
miR-150	(Miao et al., 2018a)
miR-151	(Miao et al., 2018a)
miR-151*	(Oved et al., 2012b)
miR-151a	(Yrondi et al., 2020)
miR-154	(Miao et al., 2018a)
miR-155	(J. Dai et al., 2020; X. Wang et al., 2018)

miR-181a	(Miao et al., 2018a)
miR-181a*	(W. Song et al., 2019)
miR-181c	(Craig et al., 2014a)
miR-181c*	(Oved et al., 2012b)
miR-182	(Yrondi et al., 2020)
miR-183	(Bocchio-Chiavetto et al., 2013; C.-C. Lin et al., 2018)
miR-185	(Yrondi et al., 2020)
miR-185*	(Yrondi et al., 2020)
miR-186	(Miao et al., 2018a)
miR-191	(Bocchio-Chiavetto et al., 2013)
miR-191*	(Yrondi et al., 2020)
miR-192	(Oved et al., 2012b)
miR-193a	(Craig et al., 2014; Oved et al., 2012)
miR-194	(Oved et al., 2012b)
miR-195	(X. Huang et al., 2021)
miR-195a	(Miao et al., 2018a)
miR-200a*	(W. Song et al., 2019)
miR-200b*	(Yrondi et al., 2020)
miR-203a*	(W. Song et al., 2019; Tsoporis et al., 2022)
miR-205	(Craig et al., 2014a)
miR-206	(Oved et al., 2012b)
miR-210	(Craig et al., 2014a)
miR-210*	(Yrondi et al., 2020)
miR-212	(C.-C. Lin et al., 2018; Oved et al., 2012b)
miR-212*	(Miao et al., 2018a)
miR-214*	(W. Song et al., 2019)
miR-218	(Miao et al., 2018a)
miR-221	(Oved et al., 2013)
miR-221*	(Kuang et al., 2018)
miR-222*	(Yrondi et al., 2020)
miR-300*	(Miao et al., 2018a)
miR-301a	(Craig et al., 2014a)
miR-324	(Yrondi et al., 2020)
miR-326	(Y. Zhang et al., 2015, p. 2)
miR-328	(Oved et al., 2012b)
miR-328*	(Miao et al., 2018a; Yrondi et al., 2020)
miR-329*	(Miao et al., 2018a)
miR-331	(Yrondi et al., 2020)
miR-335	(Bocchio-Chiavetto et al., 2013; J. Li et al., 2015)
miR-341	(Miao et al., 2018a)

miR-342*	(Miao et al., 2018a)
miR-346	(Miao et al., 2018a)
miR-361	(Bocchio-Chiavetto et al., 2013)
miR-363	(Oved et al., 2012b)
miR-363*	(Oved et al., 2012b)
miR-374b	(Bocchio-Chiavetto et al., 2013)
miR-378*	(Oved et al., 2012b)
miR-411*	(Miao et al., 2018a)
miR-425*	(Oved et al., 2012b)
miR-433*	(Miao et al., 2018a)
miR-451a	(Kuang et al., 2018; Min et al., 2019)
miR-457b	(Craig et al., 2014a)
miR-466	(Oved et al., 2012b)
miR-466m	(Miao et al., 2018a)
miR-466m*	(Miao et al., 2018a)
miR-483	(M. Kato et al., 2022)
miR-485*	(Miao et al., 2018a; Oved et al., 2012b)
miR-486	(Bocchio-Chiavetto et al., 2013; Oved et al., 2012b)
miR-489	(Mundalil Vasu et al., 2016)
miR-494	(Bocchio-Chiavetto et al., 2013)
miR-500	(Oved et al., 2012b)
miR-500a*	(Yrondi et al., 2020)
miR-501*	(Oved et al., 2012b)
miR-502*	(Bocchio-Chiavetto et al., 2013; Oved et al., 2012b; Yrondi et al., 2020)
miR-505	(Bocchio-Chiavetto et al., 2013; Yrondi et al., 2020)
miR-532	(Yrondi et al., 2020)
miR-550*	(Oved et al., 2013)
miR-551b*	(Miao et al., 2018a)
miR-572	(Mundalil Vasu et al., 2016)
miR-574*	(Bocchio-Chiavetto et al., 2013)
miR-584	(Yrondi et al., 2020)
miR-589	(Bocchio-Chiavetto et al., 2013; Yrondi et al., 2020)
miR-625	(Oved et al., 2012b)
miR-629	(Bocchio-Chiavetto et al., 2013; Oved et al., 2012b)
miR-629*	(Oved et al., 2012b)
miR-653	(W. Song et al., 2019)
miR-660	(Min et al., 2019; Yrondi et al., 2020)
miR-663a	(Mundalil Vasu et al., 2016)

miR-664	(Bocchio-Chiavetto et al., 2013; Oved et al., 2013)
miR-669f	(Miao et al., 2018a)
miR-671*	(Oved et al., 2012b)
miR-739	(Craig et al., 2014a)
miR-744	(Yrondi et al., 2020)
miR-758	(Miao et al., 2018a)
miR-766	(Oved et al., 2012b)
miR-770	(Bocchio-Chiavetto et al., 2013)
miR-1301*	(Yrondi et al., 2020)
miR-1202	(Fiori et al., 2017)
miR-1246	(Oved et al., 2013)
miR-1249	(M. Kato et al., 2022)
miR-1263	(Oved et al., 2013)
miR-1290	(Oved et al., 2013)
miR-1903	(Miao et al., 2018a)
miR-3095*	(Miao et al., 2018a)
miR-3151	(M. Kato et al., 2022)
miR-3158	(Yrondi et al., 2020)
miR-3158*	(Yrondi et al., 2020)
miR-3168	(Yrondi et al., 2020)
miR-3178	(Oved et al., 2013)
miR-3195	(Oved et al., 2013)
miR-3962	(Miao et al., 2018a)
miR-3963	(Miao et al., 2018a)
miR-4315	(Oved et al., 2012b)
miR-4534	(M. Kato et al., 2022)
miR-5099	(Miao et al., 2018a)
miR-5121	(Miao et al., 2018a)
miR-6215	(W. Song et al., 2019)
miR-6360	(Miao et al., 2018a)
miR-6769*	(M. Kato et al., 2022)
miR-6769a*	(M. Kato et al., 2022)
miR-6807	(M. Kato et al., 2022)
miR-6896*	(Miao et al., 2018a)
miR-7081	(Miao et al., 2018a)
miR-7109	(M. Kato et al., 2022)
miR-7111*	(M. Kato et al., 2022)
miR-7235*	(Miao et al., 2018a)
miR-7661*	(Miao et al., 2018a)



Appendix 3. Flow chart to identify studies of miRNA changes with NAFLD.

Appendix 4. Comprehensive list of miRNA altered as a result of NALFD

miRNA Altered	Reference
let-7d	(López-Pastor et al., 2021)
let-7e	(Y.-J. Zhang et al., 2017)
miR-1	(F. Jiang et al., 2020)
miR-9	(Ao et al., 2016; H. Liu et al., 2021)
miR-15b	(Du et al., 2015)
miR-16	(X.-L. Liu et al., 2016; Pillai et al., 2020; D. Zhang et al., 2016)
miR-17	(Leti et al., 2015; Ye, Lou, et al., 2018; Ye, Zhang, et al., 2018, p. 17)
miR-19a	(Pirola et al., 2015)
miR-19b	(Pirola et al., 2015)
miR-20a	(Ando et al., 2019; Ye, Lou, et al., 2018; Ye, Zhang, et al., 2018, p. 17)
miR-20b	(Y. H. Lee et al., 2021; Ye, Zhang, et al., 2018, p. 17)
miR-21	(Alhasson et al., 2018; Becker et al., 2015; Dattaroy et al., 2015; Lendvai et al., 2014; H. Lin et al., 2022; J. Liu et al., 2018; X.-L. Liu et al., 2016; Pillai et al., 2020; Pourhoseini et al., 2015; Rodrigues et al., 2017; Salman et al., 2022; C. Sun et al., 2015; Takeuchi-Yorimoto et al., 2016; X.-M. Wang et al., 2019; H. Wu et al., 2016; X. Zhang et al., 2021)
miR-21a	(Escutia-Gutiérrez et al., 2021)
miR-22	(Z. Yang et al., 2021, p. 22)
miR-22*	(H. Lin et al., 2022; López-Pastor et al., 2021)
miR-23b	(H. Li et al., 2021)
miR-23b*	(Y.-J. Zhang et al., 2017)
miR-26a	(Ali et al., 2018; Q. He et al., 2017; H. Xu et al., 2021)
miR-26b	(López-Pastor et al., 2021)
miR-27a	(Ando et al., 2019; Teimouri et al., 2020)
miR-27a*	(H. Lin et al., 2022; D. Zhang et al., 2016)
miR-27b	(J. Zhang et al., 2021)
miR-27b*	(López-Pastor et al., 2021; Tan et al., 2014)
miR-29a	(Jampoka et al., 2018; H.-Y. Lin et al., 2019; J. Liu et al., 2018; M.-X. Liu et al., 2017)
miR-29a*	(Cui et al., 2022)

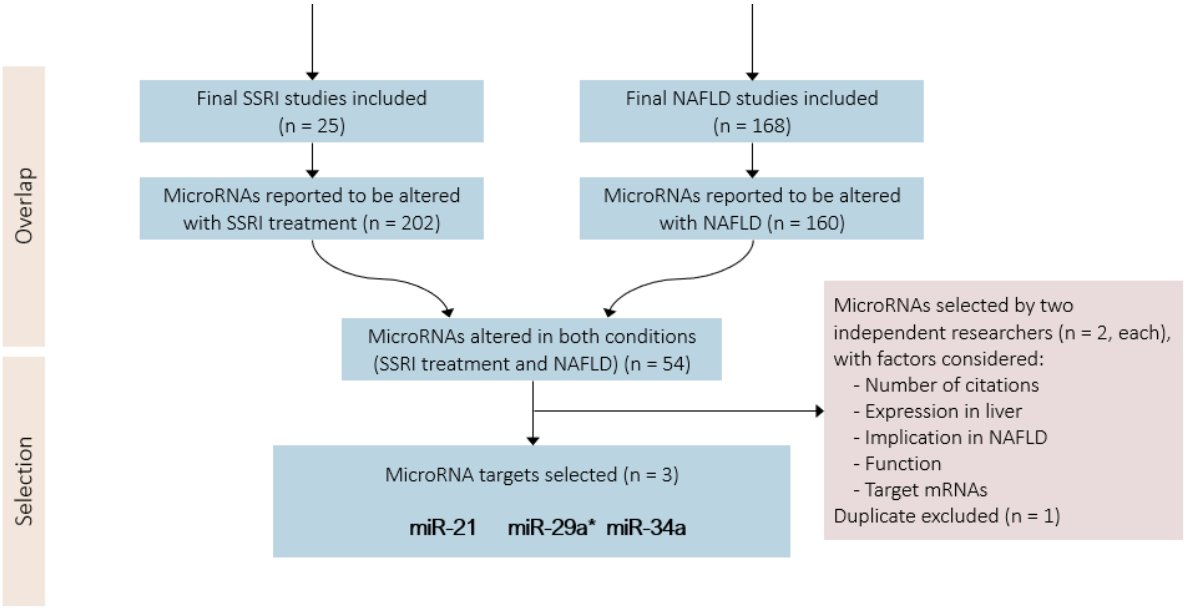
miR-29b	(Z. He et al., 2019; X. Jin et al., 2017)
miR-29b*	(Bissoondial et al., 2021; Nie et al., 2018)
miR-29c	(H. Wang et al., 2017)
miR-29c*	(Braza-Boïls et al., 2016; D. Zhang et al., 2016)
miR-30a*	(D.-R. Wang, Wang, et al., 2020)
miR-30b	(L.-L. Dai et al., 2019; Latorre et al., 2017)
miR-30c	(J. Fan et al., 2017; Zarrinpar et al., 2016)
miR-30d	(H. Wang et al., 2017)
miR-31	(Leti et al., 2015)
miR-31a	(D. Zhang et al., 2016; Y. Zhang et al., 2020b)
miR-33	(Ghareghani et al., 2018; J. H. Pan et al., 2021; Pang et al., 2017; D. Zhang et al., 2016)
miR-33a	(Erhartova et al., 2019; Karimi-Sales et al., 2018; Lendvai et al., 2014; Vega-Badillo et al., 2016)
miR-33b*	(Auguet et al., 2016a)
miR-34a	(Braza-Boïls et al., 2016; Ding et al., 2015b; X.-Y. Guo et al., 2018; Y. Guo et al., 2016; Harrison et al., 2020; Karimi-Sales et al., 2018; J. Liu et al., 2018; X.-L. Liu et al., 2016; López-Pastor et al., 2021; Pang et al., 2017; Pillai et al., 2020; Salman et al., 2022; Salvoza et al., 2016; Simão et al., 2019; L. F. Torres et al., 2019; L. Wang, Sun, et al., 2020; Y. Xu et al., 2015, 2021)
miR-34a*	(Zarrinpar et al., 2016)
miR-92a	(Di Mauro et al., 2016)
miR-96	(El-Derany & AbdelHamid, 2021; H. Zhang et al., 2020; Y. Zhang et al., 2020b)
miR-99a	(Estep et al., 2015)
miR-99a*	(Zhu et al., 2018)
miR-99b	(Estep et al., 2015)
miR-100	(Estep et al., 2015; Smolka et al., 2021)
miR-101*	(Meroni et al., 2019)
miR-103	(X. Wang & Wang, 2018)
miR-103a*	(Soronen et al., 2016)
miR-122	(Akuta et al., 2016; Auguet et al., 2016a; Becker et al., 2015; Brandt et al., 2018; Braza-Boïls et al., 2016; Chai et al., 2020; Clarke et al., 2014; Csak et al., 2015; Escutia-Gutiérrez et al., 2021; Jampoka et al., 2018; H. Jiang et

	al., 2021, p.; S.-S. Jin et al., 2022a; Kalaki-Jouybari et al., 2020; Latorre et al., 2017; Lendvai et al., 2014; J. Liu et al., 2018; X.-L. Liu et al., 2016; Long et al., 2019, p. 1; López-Pastor et al., 2021; Miyaaki et al., 2014; Naderi et al., 2017; J. H. Pan et al., 2021; Pang et al., 2017; Panzarin et al., 2022; Pillai et al., 2020; Pirola et al., 2015; Povero et al., 2014; Salman et al., 2022, p. 1; Salvoza et al., 2016; Tan et al., 2014; Yamada et al., 2015; L. Yang et al., 2021; Ye, Zhang, et al., 2018, p. 17; Zarrinpar et al., 2016; B. Zhang et al., 2014; D. Zhang et al., 2016)
miR-122*	(Bissoondial et al., 2021)
miR-124*	(G. Wang, Zou, et al., 2020)
miR-125b	(Cai et al., 2020; Pirola et al., 2015; Q. Zhang et al., 2021)
miR-126	(Di Mauro et al., 2016)
miR-127	(Okamoto et al., 2016)
miR-128	(Teimouri et al., 2020)
miR-128*	(Y.-J. Zhang et al., 2017)
miR-129	(Y. Wang et al., 2021)
miR-129b	(H. Jiang et al., 2021)
miR-130a	(J. Liu et al., 2019)
miR-130b	(X. Liu et al., 2020)
miR-130b*	(B. Guo et al., 2022; Y.-J. Zhang et al., 2017)
miR-132	(Hanin et al., 2018b; Zong et al., 2020)
miR-134	(Tryndyak et al., 2016)
miR-135a	(H. Jiang et al., 2021)
miR-136	(Okamoto et al., 2016; X. Wang & Wang, 2018)
miR-138	(L. Wang et al., 2022)
miR-139	(S.-S. Jin et al., 2022b; Latorre et al., 2017)
miR-140	(Y. Sun et al., 2019)
miR-141	(Yousefi et al., 2020)
miR-142	(Teimouri et al., 2020; Zhou et al., 2020)
miR-144	(Vega-Badillo et al., 2016)
miR-144*	(Zhu et al., 2018)
miR-145	(D. Zhang et al., 2016)
miR-146	(X. Jin et al., 2017)
miR-146a	(X. Chen et al., 2019; Du et al., 2015; Y. Y. Feng et al., 2014; K. Li et al., 2020)

miR-146b	(Y. Y. Feng et al., 2014; S. He et al., 2018; Latorre et al., 2017; Leti et al., 2015; X.-L. Liu et al., 2016; López-Pastor et al., 2021)
miR-148a	(X. Wang & Wang, 2018)
miR-149	(An et al., 2017; S. Chen et al., 2020; Z. Chen et al., 2020; J. Xiao, Lv, et al., 2016)
miR-150	(Di Mauro et al., 2016; Leti et al., 2015; H. Lin et al., 2022; Zhuge & Li, 2017)
miR-152	(Y. Y. Feng et al., 2014)
miR-153	(Teimouri et al., 2020)
miR-155	(H. Lin et al., 2022; L. Wang et al., 2016; Y. Zhang et al., 2020b)
miR-181a	(R. Huang et al., 2019)
miR-181b	(López-Pastor et al., 2021; Y. Wang et al., 2017)
miR-182	(Leti et al., 2015; Nie et al., 2018; H. Zhang et al., 2020; Y. Zhang et al., 2020b)
miR-183	(Leti et al., 2015; H. Zhang et al., 2020)
miR-185	(X.-C. Wang et al., 2014)
miR-188	(Y. Liu, Zhou, et al., 2020; Riaz et al., 2021)
miR-190b	(M. Xu et al., 2018)
miR-192	(Becker et al., 2015; Y. Lin et al., 2017; J. Liu et al., 2018; X.-L. Liu et al., 2016, 2017, p. 1; X.-L. Liu, Pan, et al., 2020; Pirola et al., 2015; Povero et al., 2014; Tan et al., 2014)
miR-193*	(Y. Zhang et al., 2020b)
miR-194	(López-Pastor et al., 2021; L. F. Torres et al., 2019; D. Zhang et al., 2016)
miR-199a	(Y. Li et al., 2020; B. Zhang et al., 2014)
miR-200	(X. Chen et al., 2018; Y. Wang, Zeng, et al., 2020)
miR-200a	(Y. Y. Feng et al., 2014)
miR-200a*	(D. Zhang et al., 2016)
miR-200b	(Y. Y. Feng et al., 2014)
miR-200b*	(Zhu et al., 2018)
miR-200c	(Y. Y. Feng et al., 2014)
miR-200c*	(T.-T. Zhang et al., 2022; Zhu et al., 2018)
miR-203*	(Du et al., 2015)
miR-205	(Y. Hu et al., 2019)
miR-206	(X. Chen, Tan, et al., 2021; H. Wu et al., 2017)
miR-211	(Y.-J. Zhang et al., 2017)
miR-212	(J. Xiao, Bei, et al., 2016)

miR-214*	(D.-H. Lee et al., 2021, p. 214)
miR-218	(J. He et al., 2019)
miR-219a	(Leti et al., 2015)
miR-221	(Lendvai et al., 2014)
miR-222	(J.-J. Wang et al., 2019; Z.-X. Xu et al., 2022, p. 608)
miR-223	(Di Mauro et al., 2016; Y. He et al., 2019, 2021; Hou et al., 2021)
miR-223*	(Y. Zhang et al., 2020b)
miR-224	(Lendvai et al., 2014; Leti et al., 2015)
miR-291b*	(Pang et al., 2017)
miR-328	(H. Wang et al., 2017)
miR-331*	(Zarrinpar et al., 2016)
miR-335	(G.-H. Fan et al., 2021; Y. Zhang et al., 2020b)
miR-361	(Z. Zhang et al., 2018)
miR-367	(D.-D. Li et al., 2017)
miR-370	(Panzarin et al., 2022)
miR-375	(Y. Guo et al., 2016; Lei et al., 2018; Pillai et al., 2020; Pirola et al., 2015)
miR-375*	(López-Pastor et al., 2021)
miR-376c	(López-Pastor et al., 2021)
miR-378	(Okamoto et al., 2016)
miR-378*	(Leti et al., 2015)
miR-379	(Okamoto et al., 2016, 2020)
miR-380	(X. Chen, Ma, et al., 2021)
miR-409*	(Okamoto et al., 2016; Tryndyak et al., 2016)
miR-410	(Tryndyak et al., 2016)
miR-411	(Okamoto et al., 2016)
miR-421	(Cheng et al., 2016)
miR-422a	(Latorre et al., 2017)
miR-423	(W. Yang et al., 2017, p. 2)
miR-451	(Gan et al., 2019; Hur et al., 2015; Karimi-Sales et al., 2018)
miR-451a	(Y.-J. Zhang et al., 2017)
miR-486	(Al Azzouny et al., 2021)
miR-488	(X. Wang & Wang, 2018, p. 3)
miR-495	(Okamoto et al., 2016; Tryndyak et al., 2016)
miR-504-3p	(H. Jiang et al., 2021)
miR-504*	(Y.-J. Zhang et al., 2017)
miR-505	(J. Liu et al., 2018; X. Wang & Wang, 2018)
miR-506	(X. Chen et al., 2019, p. 1; Q. Xu et al., 2015)

miR-552*	(L. Fan et al., 2021)
miR-576	(Soronen et al., 2016)
miR-590	(Hanson et al., 2019; Leti et al., 2015)
miR-599	(L. Kong et al., 2022)
miR-650	(Matboli et al., 2021)
miR-669b*	(Y.-J. Zhang et al., 2017)
miR-741*	(Nie et al., 2018)
miR-871*	(Y. Zhang et al., 2020b)
miR-873	(Fernández-Tussy et al., 2019)
miR-881*	(Y. Zhang et al., 2020b)
miR-892a	(Soronen et al., 2016)
miR-1205	(Matboli et al., 2021)
miR-1290	(Tan et al., 2014)
miR-1296	(Yu et al., 2019)
miR-3666	(Mittal et al., 2020)



Appendix 5. Flow chart of the literature review to identify overlapping differentially expressed microRNAs in association with SSRI treatment and NAFLD.

5 Chapter 5: Discussion

5.1 Summary of the work

The goal of this dissertation was to contribute to the literature regarding adverse metabolic side effects of SSRI antidepressant use with a particular focus on NAFLD. I aimed to delineate the mechanism by which SSRI exposure could lead to hepatic metabolic disturbance, with an emphasis on its contribution to steatosis and dysregulated lipid homeostasis. Overall, I have demonstrated using *in vitro* cell culture models that the SSRI fluoxetine can cause excess hepatocyte lipid accumulation an effect which was mediated by elevated serotonin (chapter 2) and prostaglandin production (chapter 3). I also found that fluoxetine's ability to cause hepatic lipid accumulation appears to be mediated, in part, via PPARG (chapters 3 & 4) and changes in miRNA expression.

5.2 The Two-Hit Hypothesis and NAFLD

An estimated 25% of Canadians currently suffer from NAFLD, making it the most common liver disease in Canada (Morris, 2014). With the growing prevalence of obesity, the incidence of NAFLD is also on the rise (Glasgow et al., 1997). This poses a significant clinical and economic burden as the rising rate of NAFLD is compounded with the lack of treatment (Younossi et al., 2016). NAFLD is classified as a range of diseases varying from simple hepatic steatosis to inflammatory non-alcoholic steatohepatitis (NASH) with different levels of fibrosis. These illnesses are observed in the absence of other known etiologies of hepatic injury such as significant alcohol consumption and viral hepatitis (Chalasani et al., 2012). The initial stage of NAFLD, known as nonalcoholic fatty liver (NAFL) is defined by steatosis, which is the presence of lipid droplets (LDs) in ≥5% of hepatocytes (Reeder & Sirlin, 2010). This can be identified by excess accumulation of triglycerides within hepatocytes. Hepatic steatosis can be a result of one or more mechanisms including elevated *de novo* fatty acid synthesis; diminished fatty acid oxidation; elevated transport of fatty acids from the peripheral organs to the liver; and reduced transport of fatty acids from the liver to the general circulation and peripheral organs (Cimini et al., 2017; Mallat et al., 2011). In accordance with the 'two-hit' hypothesis of NAFLD, the progression from a healthy liver to NASH occurs in a stepwise fashion beginning with the development of steatosis which leads to hepatic inflammation (Paschos & Paletas, 2009). As steatosis persists, the increased inflammation may develop into inflammation, fibrosis, and even cirrhosis of the liver (Purohit et al., 2010). As such, examining exogenous compounds' contributions to hepatic steatosis ('first-hit' of NAFLD) is important as it may prove useful in preventing the subsequent inflammation, fibrosis, and even cirrhosis of the liver associated with severe NAFLD (Cholankeril et al., 2017; Mallat & Lotersztajn, 2008). While many research studies focus on environmental pollutants or ubiquitous contaminants and their role in the development of NAFLD, medication use has also been associated with liver damage and the development of NAFLD (S. David &

Hamilton, 2010). Importantly, psychotropic medication use has commonly been associated with hepatotoxicity (Telles-Correia et al., 2017). There is evidence from clinical studies that depressed individuals are more likely to have NAFLD than non-depressed individuals (D. Kim et al., 2019). Although this association may be due to the underlying pathology of MDD, it is also possible that this relationship is influenced by the drugs used to treat MDD. SSRIs are the first-line medication for MDD treatment (Israel-Elgali et al., 2021; Yuan et al., 2020; Zai, 2021) and there is now a growing body of evidence from clinical studies, animal experiments, and cell culture studies that SSRIs can cause perturbations in lipid metabolism which may lead to metabolic disease with long term use (Beigi et al., 2022; X.-M. Feng et al., 2012; Fjukstad et al., 2016; S. Pan et al., 2018a; Xiong et al., 2014)

It is well documented in the literature that steatosis precedes NASH under most circumstances (Mashek, 2021). This thesis aimed to examine how the SSRI fluoxetine contributes to the 'first-hit' of NAFLD. In doing so, this thesis uncovered novel mechanisms by which the widely prescribed SSRI antidepressant fluoxetine contributed to drug-induced steatosis.

5.3 NAFL & Hepatic Steatosis

5.3.1 Fluoxetine-induced steatosis is mediated by serotonin

In Chapter 2 I found that fluoxetine treatment caused excess hepatic lipid accumulation via altered peripheral serotonin production (A. Ayyash & Holloway, 2021a). To date, this is the only study addressing the effects of fluoxetine on hepatic serotonin production. Treatment of H4-II-E-C3 hepatoma cells with serotonin in this study resulted in a significant increase in lipid accumulation and expression of fatty acid synthase, which was consistent with what has been reported in Hep-G2 and SK-Hep-1 hepatic cell lines (Niture et al., 2018). Additionally, PCPA treatment abolished the fluoxetine-induced increase in serotonin synthesis and lipid accumulation suggesting that hepatic serotonin production was mechanistically linked to the lipid perturbations reported following fluoxetine exposure (A. Ayyash & Holloway, 2021a). Interestingly, inhibiting peripheral serotonin synthesis or signaling has been suggested to be a potential avenue for treating obesity, type 2 diabetes, and NAFLD (Yabut et al., 2019). Although our results from this chapter suggested a pivotal role for increased serotonin production underlying fluoxetine-induced hepatic lipid accumulation, it may also involve changes in the expression of serotonin receptors or signaling by other monoamines, as has previously been shown (Owens et al., 2001; Zwartsen et al., 2017).

5.3.2 Fluoxetine elevates prostaglandin production, PPARG, and Steatosis

In Chapter 3, I demonstrated that fluoxetine treatment led to a significant increase in hepatic production of the PGD_2 prostaglandin metabolite 15d-PGJ2. 15d-PGJ2 acts as a potent

endogenous ligand for PPARG and was crucial to SSRI-induced hepatic lipid accumulation (A. Ayyash & Holloway, 2021b). This study also found that fluoxetine induced hepatic lipid accumulation in association with an elevated mRNA expression of the PPARG downstream targets involved in fatty acid uptake (*Cd36, Fatp2*, and *Fatp5*) (A. Ayyash & Holloway, 2021b). Importantly, the effects of fluoxetine to induce lipid accumulation was attenuated with a PTGS1 specific inhibitor (SC-560), which also attenuated 15-deoxy-Δ12,14PGJ2 production and expression of PPARG downstream target genes (A. Ayyash & Holloway, 2021b). My findings that both serotonin and prostaglandins are involved in fluoxetine-induced steatosis are consistent with studies demonstrating that serotonin signaling and prostaglandin production are interrelated. For example, activation of the 5-HT_{2A} receptor by serotonin can stimulate the release of AA from membrane phospholipids through the activity of cPLA2 (Choi et al., 2018; Felder et al., 1990; Niture et al., 2018; Qu et al., 2003). AA serves as the precursor to several biologically active acid lipids, including prostaglandins, leukotrienes, and thromboxanes. In my thesis, I only explored the effects of fluoxetine treatment on prostaglandin synthesis as there is considerable literature that implicates prostaglandins in the progression of NAFLD (Calder, 2020; Chung et al., 2014; Kumar et al., 2020; Maciejewska et al., 2020; Qin et al., 2015). However, AA is also the substrate for the production of other eicosanoids including thromboxane A2 which has also been reported to be involved in the development of hepatic steatosis (Q. Li et al., 2020; Maciejewska et al., 2015; Marchix et al., 2020; Nanji et al., 1997; W. Wang et al., 2021).

5.3.3 Fluoxetine altered miRNA and Steatosis

In Chapter 3 I found that gene targets of PPARG activation were upregulated following fluoxetine exposure. To follow up on this finding Chapter 4 explored the link between PPARG activation to changes in miRNA signatures. Of particular interest was miR-122 which is regulated by PPARG and is central to the progression of NAFLD (Akuta et al., 2016; Long et al., 2019; K. Song et al., 2013). I found that fluoxetine treatment increased miR-122 expression an effect which was attenuated by treatment with the selective PPARG antagonist (GW9662). These results as well as in silico binding data strongly suggest that fluoxetine can act as a PPARG agonist. This hypothesis is also supported by the observation that the increase in miR-122 following fluoxetine treatment is similar to that seen with the known PPARG agonist rosiglitazone. However, there remain questions about the exact mechanism by which fluoxetine and miR-122 interact to result in increased hepatic lipid accumulation. Interestingly, several other miRNAs have been shown to be altered by fluoxetine treatment and play a role in the pathogenesis of NAFLD (Baudry et al., 2010a; Craig et al., 2014; Launay et al., 2011; Miao et al., 2018). In chapter 4, I attempted to identify miRNAs that could be involved in the observed increase in lipid accumulation following fluoxetine treatment. I found that miR-21 and its validated mRNA target *Tiam1* were significantly altered following fluoxetine treatment. Future studies should extend this observation to investigate the mechanism by which fluoxetine-induced increases in miR-21 expression can contribute to hepatic steatosis. Previous studies found that miR-21

promotes hepatic lipid accumulation by interacting with several lipogenic factors, such as sterol regulatory element-binding protein, 3-hydroxy-3-methylglutaryl-co-enzyme A reductase and fatty acid-binding protein 7 (T. Zhang et al., 2020). Additionally, hepatocyte-specific knockout of miR-21 in mice has been shown to improve steatosis through upregulation of multiple miR-21 target pathways relating to lipid metabolism (Baffy, 2015; Wu et al., 2016a).

5.3.4 Is fluoxetine a PPARG agonist?

Results from chapters 3 and 4 strongly suggested a pivotal role of PPARG in fluoxetine-induced hepatic steatosis (A. Ayyash & Holloway, 2021b). Future studies should aim to better understand whether fluoxetine binds to PPARG directly and activates downstream signaling pathways or if fluoxetine exposure is acting indirectly to activate PPARG signaling via the production of 15d-PGJ2, a known PPARG ligand, or a combination of these two mechanisms. I have shown that fluoxetine exposure resulted in the upregulation of the PPARG ligand 15d-PGJ2 suggesting an indirect effect of fluoxetine with respect to PPARG activation (Álvarez-Almazán et al., 2017; A. Ayyash & Holloway, 2021b; Fujitani et al., 2010; J. Li et al., 2019). However, *in silico* docking simulations suggest a direct effect of fluoxetine. These docking experiments found that fluoxetine binds to PPARG with greater binding energy than a known PPARG agonist (i.e. rosiglitazone) and antagonist (i.e. GW9662) (Jamshed and Holloway, unpublished data). To confirm whether or not fluoxetine is a direct agonist of PPARG, the *in silico* docking experiments would need to be confirmed via ligand binding activity assays.

5.3.5 Does fluoxetine exposure alter hepatic lipid composition?

In hepatocytes, triacylglycerols, cholesteryl esters, and other neutral lipids are stored in liposomes around the central nucleus or within organelles called LDs, which are subject to expansion and an increase in abundance as a result of excess lipid accumulation(Scorletti & Carr, 2022). In this thesis, hepatic lipid accumulation was examined using a commonly used technique known as 'Oil Red O' (ORO) staining (A. Ayyash & Holloway, 2021a, 2021b). ORO remains a robust, commonly used semiquantitative method for examining lipid staining *in vitro,* as it stains neutral lipids but not biological membranes (Ramírez-Zacarías et al., 1992b). Although ORO staining is frequently used as an accurate method for measuring hepatic steatosis both in mouse and human liver biopsies, this technique is not without its shortcomings (Catta-Preta et al., 2011; Levene et al., 2012). Specifically, ORO can be used to semi-quantitatively compare the abundance, localization, and size of the LDs, but the different lipid species within LDs cannot be characterized using this technique (Mehlem et al., 2013). The fat-soluble ORO dye is only capable of staining the most hydrophobic and neutral lipids, such as cholesterol esters, diacylglycerols, and

triglycerides, whereas polar lipids including phospholipids, sphingolipids, and ceramides are left unstained (Fowler & Greenspan, 1985; Mehlem et al., 2013, 2013). Lipotoxicity is characterized by the accumulation of harmful lipid species, such as triglycerides, cholesterol, ceramides, diacylglycerols, and lysophosphatidyl choline species leading to chronic inflammation and progression from hepatic steatosis to NASH (M. S. Han et al., 2008; Ibrahim et al., 2011; Luukkonen et al., 2016; Mauer et al., 2017; Perry et al., 2014). Due to ORO's selective staining of neutral lipids, lipotoxic lipid species such as ceramides that may be contributing to the development of NASH are left unmeasured (Andrés-Manzano et al., 2015; Neuschwander-Tetri, 2010). To address this limitation, more recent studies have been integrating the use of high-performance liquid chromatography-mass spectrometry (HPLC-MS), gas chromatography-mass spectrometry (GC-MS), fluorescence microscopy, or commercially available enzymatic kits to quantify the total amount of triglycerides and other lipid species (Fuchs et al., 2010; Yen et al., 2010; Y. Zhang et al., 2013). Importantly, other studies investigating the metabolic consequences of exposure to antidepressants including fluoxetine, have made use of more advanced lipidomic analysis techniques such as LC-MS/MS. These studies have reported that concentrations of many different lipid molecules were markedly changed following fluoxetine treatment (L. H.-W. Lee et al., 2009; Pinto et al., 2022; S.-S. Xue et al., 2020). These more advanced lipidomic analysis techniques may provide unique insight into fluoxetine-induced steatosis, particularly since the results in Chapter 3 suggested that fluoxetine treatment resulted in altered prostaglandin production, yet the production of other eicosanoids derived from AA such as lipoxins, leukotrienes, and thromboxanes was not investigated.

5.4 A Further Delve into Fluoxetine & NAFLD – Examining NASH

5.4.1 Second hit & NASH

NAFLD severity is often measured in histological sections using the NAFLD activity score, which evaluates progression based on hepatic scores for ballooning, steatosis, and lobular inflammation (Kleiner & Brunt, 2012; Satapathy et al., 2015). The aforementioned two-hit hypothesis posits that the 'first hit' of hepatic steatosis increases the vulnerability of the liver to injury mediated by 'second hits', such as inflammatory cytokines/adipokines, oxidative stress, and mitochondrial dysfunction which in turn lead to NASH and/or fibrosis (Day, 2006; Dowman et al., 2010). If left untreated, inflammation and fibrosis in the liver are further exacerbated via activation of Kupffer cells, recruitment of circulating immune cells, and subsequent activation of hepatic stellate cells ultimately leading to fibrogenesis (Bence & Birnbaum, 2020; Hart et al., 2017; Heymann & Tacke, 2016; Kazankov et al., 2019). Throughout this thesis, I focused primarily on the contribution of fluoxetine to excess hepatocyte lipid accumulation, however, a more comprehensive examination of SSRI's contribution to NAFLD should examine the role and mechanism by which fluoxetine contributes to hepatic inflammation.

5.4.2 Fluoxetine, lipotoxicity, and NASH

The specific lipid species responsible for promoting hepatocyte inflammation and NASH pathogenesis is of considerable debate (Farrell et al., 2018). According to lipidomic analysis, the liver contains the following lipid categories: fatty acyls, glycerolipids, glycerophospholipids, sphingolipids, sterol lipids, and prenol lipids, some of which are more lipotoxic in the hepatocyte than others (Fahy et al., 2005, 2009). Previous research from our lab demonstrated that fetal and neonatal exposure to fluoxetine resulted in NAFLD, with significantly elevated levels of hepatic triglycerides and hepatic cholesterol in exposed offspring relative to controls (De Long, Barry, et al., 2015). Triglycerides are the most prevalent lipid that accumulates in the liver leading to steatosis and is significantly elevated in the liver of NAFLD patients when compared to control subjects (Magkos et al., 2012; Thomas et al., 2005). Studies have also suggested that the delivery of excess FFA to the liver or the generation of other lipotoxic species within the liver can result in hepatocyte injury and inflammation (Marra & Svegliati-Baroni, 2018; Sharma et al., 2015).

5.4.2.1 Fluoxetine-induced serotonin and NASH

In chapter 2, I demonstrated that fluoxetine treatment led to a significant increase in hepatic serotonin production (A. Ayyash & Holloway, 2021a). In addition to serotonin's role as a regulator of metabolism and energy storage by promoting insulin secretion, hepatic *de novo* lipogenesis, and decreasing lipolysis in adipose tissue, serotonin acts as a proinflammatory agent (H. Wu et al., 2019; Yabut et al., 2019). It has been suggested that peripheral 5-HT plays a pivotal role in inflammatory conditions of the gut, allergic airway inflammation, and rheumatoid arthritis (Shajib & Khan, 2015). Interestingly, serotonin has recently been reported to result in the progression of NASH via the liver HTR2A/PPARγ2 Pathway (Choi et al., 2018; Niture et al., 2018; L. Wang, Fan, et al., 2020). Additionally, serotonin mediates oxidative stress and mitochondrial toxicity in a murine model of NASH, an effect that was related to the reactive oxygen species generated from serotonin degradation by monoamine oxidase A (Nocito et al., 2007). Moving forward, future research should aim to examine hepatic oxidative stress and related inflammatory markers that may be linked to fluoxetine-induced elevated serotonin production.

5.4.2.2 Fluoxetine elevated inflammatory prostaglandins

In Chapter 3, I examined the importance of the rate-limiting enzymes in the prostaglandin synthesis pathway, *Ptgs1*, and *Ptgs2*, for their role in hepatic steatosis and identified the importance of *Ptgs1* in fluoxetine-induced lipid accumulation. Importantly, prostaglandins are key components of the inflammatory response, and the literature suggests that increased levels of certain prostaglandins are associated with NAFLD (Chung et al., 2014; Kumar et al., 2020; Maciejewska et al., 2020; Qin et al., 2015). AA serves as a precursor to several biologically active eicosanoids including prostaglandins, thromboxanes, lipoxins, prostacyclin, leukotrienes, hydroxyeicosatetraenoic acids (HETEs), and

epoxyeicosatrienoic acids (EETs) (Bieren, 2017). Many of these eicosanoids are pro-inflammatory and leukotrienes, EETs, HETEs, prostacyclin, and other prostaglandins have been implicated in the progression of NASH and more severe NAFLD (Banaszczak et al., 2020; J. Fan et al., 2004, 2004; Gai et al., 2018; Hardwick et al., 2013; Kumei et al., 2018; S. Li et al., 2021; K. Ma et al., 2017; Maciejewska et al., 2020; Raffaele et al., 2019; Schuck et al., 2014; X. Wang et al., 2019; Wells et al., 2016). Notably, it has been reported that chronic treatment with the SSRI fluoxetine resulted in enhanced PLA2 expression, as well as altered expression of some eicosanoids (B. Li et al., 2009; Ramadan et al., 2014; Yaron et al., 1999). Therefore, future studies should evaluate the effects of fluoxetine exposure on the production of a wider range of eicosanoids to determine if other classes of these bioactive molecules are also involved in the hepatic steatosis and inflammation observed following fluoxetine exposure (Higgins & Lees, 1984; Maciejewska et al., 2020).

5.4.2.3 Fluoxetine induced miR-21 and NASH

A growing body of literature implicates epigenetic modifications including but not limited to DNA methylation, histone modifications, and activity of miRNAs to the progression of NASH (Buzzetti et al., 2016). Results from Chapter 4 suggest a significant increase in the expression of miR-21 following fluoxetine treatment, a result that was supported by other research (Baudry et al., 2010c; Craig et al., 2014b; Launay et al., 2011a; Miao et al., 2018b). Importantly, it has been suggested that miR-21 may play an important role in the inflammatory response (Madhyastha et al., 2021; Nakamura et al., 2015; Sheedy, 2015; Shi et al., 2019). Interestingly, miR-21 is well documented to be involved in hepatic fibrosis as well, a component of more severe NAFLD, via the TGF-B1/SMAD and SPRY2/ HNF4A/ERK1 signaling pathways (K. Wu et al., 2016; F. Yang et al., 2016; J. Zhao et al., 2014). In the liver, it is well documented that fluoxetine causes hepatic inflammation via several mechanisms including but not limited to elevated oxidative stress and increased pro-inflammatory cytokines such as IL-1β, a component of the NLRP3 inflammasome (Elgebaly et al., 2018; Karimi-Sales et al., 2021; Mohamed Kamel, 2021). The inflammasome is activated by danger-associated molecular patterns (DAMPs), which include products of *de novo* lipogenesis, such as saturated fatty acids (Csak et al., 2011). Further, activation of the NLRP3 inflammasome has been shown to contribute to the progression of NAFLD to its more severe stages, including NASH (Mridha et al., 2017; Wan et al., 2016). In Chapter 4, the expression of miR-21 was significantly upregulated by fluoxetine exposure; upregulation of this miRNA has been associated with activation of the NLRP3 inflammasome (Ning et al., 2017; Yamada et al., 2013; Y. Zhang et al., 2020a). I also found increased expression of related inflammasome components *Casp1*, *Nlrp3*, and *Il-1b*. Moreover, work from our group has previously reported that fetal and neonatal exposure to fluoxetine in a rat model resulted in increased hepatic expression of NLRP3 inflammasome components and lipid accumulation in the adult offspring (De Long et al., 2017). Taken together these results suggest that fluoxetine activates a miR-21-NLRP3 inflammasome pathway, but this remains to be confirmed using miRNA silencing experiments or inhibition of the inflammasome.

Interestingly, there is no consensus in the literature with regards to fluoxetine being an inflammatory or an anti-inflammatory agent (Abdel-Salam et al., 2004; Alboni et al., 2016; Coccaro et al., 2015; Creeden et al., 2021; Duda et al., 2017; D. Liu et al., 2011). In the brain, fluoxetine appears to have anti-inflammatory effects, being touted as a neuroprotective agent (Caiaffo et al., 2016; H.-M. Hu et al., 2018). Conversely, fluoxetine causes hepatic inflammation, as previous research has demonstrated that fetal and neonatal exposure to fluoxetine resulted in elevated expression of pro-inflammatory cytokines *Tnfα* and *Il-6*, both of which have been elevated in serum and hepatic samples from patients and animal models of NASH (De Long, Barry, et al., 2015; Haukeland et al., 2006; Klover et al., 2005; Tarantino et al., 2009). Future investigations into the development of NASH cannot comprehensively be completed without a more appropriate model that incorporates the inflammatory response of activated Kupffer cells and macrophages, which play a central role in exacerbating liver inflammation (Mridha et al., 2017).

5.5 Limitations of the model

In this thesis, I used a cell line to investigate the molecular mechanisms by which fluoxetine could increase hepatic lipid accumulation. Although these cell models are a convenient robust tool for pharmacological research due to their ease of use, availability, and ability to scale up for high throughput experiments, however, they come with significant limitations including the fact that NAFLD involves multiple cell types in the liver which are not modeled using a single cell line (Allen et al., 2005; Müller & Sturla, 2019). Moreover, adipose tissue function, the gut microbiome, and other risk factors such as metabolic disorders, genetics, and drug and environmental exposures are also important contributors to the pathogenesis of the NAFLD: effects that cannot be modeled using a single cell line (Safari & Gérard, 2019; Tamura & Shimomura, 2005; Vanni et al., 2010). Recently hepatic 3D organoid models, including 3D co-cultures with hepatocytes and Kupffer cells or stellate cells, have been developed to examine the development of NAFLD. These 3D models provide cell-cell relationships that more closely replicate the *in vivo* tissue structure (Gamboa et al., 2021; Jensen & Teng, 2020; Leite et al., 2016; Panwar et al., 2021). Alternatively, animal models address some of the major drawbacks of *in vitro* cell line research but these models also cannot fully account for the continuous interplay of several risk factors including overnutrition and/or an inappropriate dietary pattern, inadequate energy expenditure due to a sedentary lifestyle and genetic susceptibility observed in a clinical population of NAFLD (Kanuri & Bergheim, 2013). Animal models of NAFLD can be broadly categorized as diet-induced, genetic or a combination both (Santhekadur et al., 2018). Commonly used genetic animal models of NASH are the *ob/ob* (leptin deficient) and *db/db* (leptin receptor deficient) mice, the Zucker *fa/fa* rat, and several transgenic or conditional knockout mice (Santhekadur et al., 2018). Future research of fluoxetine's effect on NAFLD, should aim to make use of these models and examine its role on hepatic inflammation and NASH (Friedman et al., 2018). This will also allow for a more comprehensive understanding of fluoxetine's effect on altered lipids as levels of triglycerides, total cholesterol, HDL and LDL/VLDL cholesterol and free fatty acids can

be determined in serum and hepatic samples using commercially available kits, as well as full lipid profiling (i.e., lipidomics) of serum samples using reversed-phase LC-MS (Fuchs et al., 2010).

5.6 An innovative way to rethink NAFLD

The "two-hit" hypothesis of NAFLD was formulated by Day and James in 1998, yet more recently the multiple-hit hypothesis has been proposed (Buzzetti et al., 2016; Day & James, 1998; Yilmaz, 2012). The multiple-hit hypothesis posits that the underlying mechanism for the development and progression of NAFLD is complex and multifactorial and does not necessarily begin at steatosis and then develop into NASH in a step-wise fashion (Buzzetti et al., 2016; Peng et al., 2020). There are multiple parallel factors, including the gut microbiome, gut-liver axis, insulin resistance, hormones secreted from the adipose tissue, and hyperlipidemia all of which can act synergistically in genetically predisposed individuals resulting in changes to energy homeostasis and systemic inflammation both of which can extend to NAFLD progression (C. Alonso et al., 2017; Buzzetti et al., 2016). In the liver, NASH is characterized by dysfunctional unfolded protein response, endoplasmic reticulum (ER) stress, activation, enhanced wound response, and activation of the inflammasome and apoptotic pathways (Guy et al., 2012; J. Han & Kaufman, 2016; Puri et al., 2008; Szabo & Petrasek, 2015). Although these manifestations of NASH are most commonly due to hepatic steatosis and lipotoxic lipids, several other factors can contribute to NASH and lead to these adverse outcomes, including epigenetic changes such as DNA methylation and histone methylation/acetylation (Juanola et al., 2021). Importantly, the comorbid state of MDD has been associated with more severe histological liver steatosis, and its widely prescribed pharmacological treatment option, fluoxetine has also been implicated in contributing to NAFLD (A. Ayyash & Holloway, 2021a, 2021b; De Long et al., 2017; Elgebaly et al., 2018; X.-M. Feng et al., 2011; Mohamed Kamel, 2021; S. Pan et al., 2018a; Tomeno et al., 2015).

Recent advances in our understanding of NAFLD have also led to an attempt to redefine this disease to a name that reflects the knowledge that currently exists about the metabolic dysfunction associated with NAFLD (Byrne & Targher, 2015). Furthermore, there has been a recent push to redefine NAFLD from being a disease defined by exclusion to one of inclusion (Eslam, Sanyal, et al., 2020). As such Eslam et al. recently proposed renaming the disease metabolic-associated fatty liver disease (MAFLD) as it is a more encompassing, overarching term that better defines this multisystem disorder (Byrne & Targher, 2015; Eslam, Sanyal, et al., 2020). This new and improved definition puts dysmetabolism and fat accumulation in the liver, the primary driver of disease progression, at the epicenter of the disease, and does not require the exclusion of alcoholic liver disease or viral hepatitis (Dongiovanni et al., 2018; Eslam, Newsome, et al., 2020; Nasr et al., 2020). MAFLD can be diagnosed in patients based on observed hepatic steatosis and the presence of any one of the following three conditions, including diabetes mellitus, obesity,

or evidence of metabolic dysregulation (S. Lin et al., 2020). Importantly, the contribution of pharmacotherapies, including SSRI antidepressants, to liver disease can be better explored using the construct of MAFLD where the effects of SSRI exposure on multiple metabolic pathways should be explored.

5.7 Conclusion

Given the high prevalence of MDD and the increasing use of SSRIs for both on-label and off-label use, the results of this thesis may have significant implications to improve our understanding of metabolic adversities associated with the use of these SSRIs (Skånland & Cieślar-Pobuda, 2019; Wong et al., 2017). While the data in this dissertation suggests that SSRI use can lead to metabolic perturbations associated with NAFLD, I am not advising individuals with MDD to discontinue medication as the benefits of therapy may outweigh any metabolic risk. Additionally, while this study consistently demonstrated hepatic lipid accumulation following fluoxetine treatment the same cannot be said for all SSRIs as this association may be unique to this medication and may not extend to other drugs in the class. That being said, the results of this thesis do identify significant metabolic liability associated with SSRI use and do identify potential molecular pathways which might be potential targets for therapeutic interventions to prevent fluoxetine-induced lipid accumulation.

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