ABSTRACT

Recent epidemiological studies have suggested that mothers with onset allergies or asthma during pregnancy are at an increased risk for having a child with autism spectrum disorders (ASD). A novel mouse model of maternal allergic asthma (MAA) has shown ASD-like behavioral deficits in offspring of MAA dams in two genetically distinct mouse strains. However, the mechanisms underlying MAA remain unknown. One hypothesis is that induced allergic asthma may negatively impact the developing fetal brain by disrupting gene expression in the placenta. This study investigated alterations in gene expression in two distinct pathways critical for neurodevelopment in the placenta of MAA dams: the tryptophan (TRP) pathway and the long-chain polyunsaturated fatty acid (LC-PUFA) pathway. Specifically, C57Bl/6J (C57) and FVB.129P2-Pde6b(+) Tyr(c-ch)/Ant (FVB) female mice, strains with distinct behavioral responses to MAA, were sensitized and exposed to ovalbumin during pregnancy on gestational day (G)9.5,12.5, and 17.5 and placentae were extracted on G17.5 four hours after the final OVA exposure. Placental RNA samples were analyzed for differences in expression of *solute carrier family 7 member 5 (LAT1)* and *indoleamine 2,3-dioxygenase (Ido1)* in the TRP pathway and *fatty acid transport protein 4 (FATP4)* and *Acyl-CoA synthetase long-chain family member 3* (*Acsl3)* in the LC-PUFA pathway using quantitative reverse transcription PCR. Results reveal increased expression of *Ido1* in C57 but not FVB MAA placentae, implicating strain-specific differences in TRP metabolism and its associated metabolic constituents, kynurenine and serotonin, as important mechanisms that may underlie the ASD-like behavioral deficits associated with MAA.

Placental gene expression and maternal allergic asthma:

Investigating neurodevelopmental pathways in a mouse model of autism spectrum disorders

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# INTRODUCTION

## Autism Spectrum Disorders

**Overview.** Autism spectrum disorders (ASD) are a class of neurodevelopmental disorders that currently affect around 1 in 68 children in the United States, with males being 4.5 times more likely to develop ASD than females (Center for Disease Control, 2014). Individuals with ASD exhibit social-communication deficits and restricted, repetitive patterns of behavior that begin during early childhood and result in significant impairments in personal, social, academic, or occupational functioning (American Psychiatric Association, 2013). Care for an autistic child is estimated to cost families an additional $17,000 per year (Center for Disease Control, 2014), underscoring the immense public health cost of the disorder. While ASD etiology remains largely unknown, it is now understood that ASD development is likely due to a complex combination of genetic and environmental factors and that in order to fully understand ASD development, research must examine the interaction between these factors.

**Genetics and autism spectrum disorder predisposition.** While most cases of ASD arise idiopathically, about 20% of cases have direct genetic causes (Lukose, Beebe, & Kulesza, 2015). Of these, Fragile-X syndrome is the most common genetic cause of ASD and results from a mutation that causes more than 200 copies of a cytosine-guanine-guanine (CGG) trinucleotide repeat in the promoter of the *fragile X mental retardation 1* (*FMR1*) gene (van Karnebeek, Bowden, & Berry-Kravis, 2016). This mutation causes decreased expression of fragile X mental retardation protein (FMRP), which is necessary for synaptic development. Rett

syndrome, a rare disorder that resembles ASD, is caused by genetic mutations that result in a loss of function of the X-linked *Methyl-CpG-binding protein 2 (MeCP2*) (van Karnebeek et al., 2016). In both cases, *FMRP* and *MeCP2* are involved in the dendritic translation pathway, and when they function normally they allow for the translation of synaptic proteins necessary for successful synaptic transmission (van Karnebeek et al., 2016) (See Figure 1A). Similarly, either *de novo* or inherited mutations of the *neurexin-1 (NRXN1*) gene, and specifically of neurexin-1α, the longer *NRXN1* transcript, have been shown to be responsible for approximately one half percent of autism cases (Etherton, Blaiss, Powell, & Südhof, 2009). Neurexins are cellular adhesion molecules on pre-synaptic membranes that are postulated to connect pre- and post-synpatic membranes by interacting with neuroligins on the post-synaptic side (See Figure 1B). They allow synapses to become fully functional and have roles in the operation of voltage-gated calcium channels and neurotransmitter release (Reichelt, Rodgers, & Clapcote, 2012). Overall, it is clear that causal genetic mutations linked to ASD often lead to synaptic defects, suggesting that idiopathic ASD cases may be caused by alterations in similar pathways during brain development.

While these examples show a clear genetic basis for some ASD cases, most ASD diagnoses cannot be explained by a single genetic mutation. Even keeping genetics constant, ASD is concurrent in only about 50-70% of monozygotic (identical) twins (Hallmayer et al., 2011). Many current studies suggest that a combination of certain common alleles may predispose an individual to developing ASD (Devlin, Melhem, & Roeder, 2011), part of the common disease-common variant hypothesis (Rybakowski et al., 2016). However, considering that not all individuals with these common variants will develop ASD and that the concordance rate among twins for ASD development is not 100%, it is clear that something other than

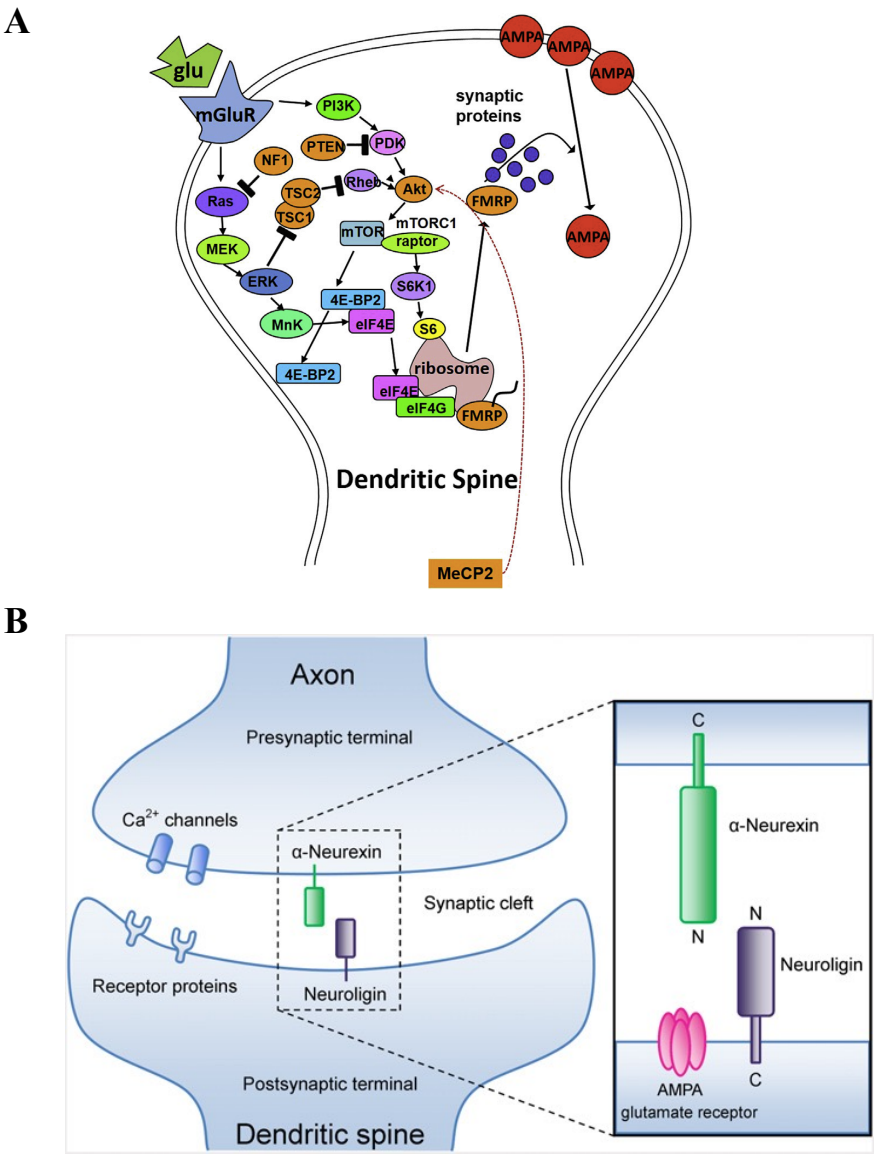


*Figure 1*. Mutations linked to autism spectrum disorders (ASD) commonly disrupt synaptic transmission by affecting genes that code for proteins involved in the (A) dendritic translation pathway, such as fragile X mental retardation protein (FMRP) or Methyl-CpG-binding protein 2 (MeCP2), and (B) synaptic adhesion molecules such as neurexin-1 (NRXN-1). Figures A and B adapted from van Karnebeek et al., 2016 and Reichelt et al., 2012, respectively.

genetics plays a role in ASD development in many cases. Specifically, environmental factors during pregnancy and early life are thought to contribute to risk of ASD diagnosis along with genetics (Rybakowski et al., 2016), potentially by changing gene expression rather than the genetic code itself. Accordingly, many ongoing studies investigate gene expression changes,

rather than genetic mutations, and have elucidated thousands of genes as differentially expressed in proband ASD patients compared to non-ASD siblings (Ansel,Rosenzweig, Zisman, Melamed, & Gesundheit, 2017). Although complex, these studies may have the power to explain idiopathic ASD development that does not arise from distinct genetic mutations, but rather is likely due to a combination of genetic predisposition and environmental factors resulting in changes in gene expression across development. However, while the idea that ASD development often arises due to a combination of genetic and environmental factors is commonly accepted with the ASD research field, these gene by environment interactions are rarely directly studied either in human epidemiology or in mouse models (Kim & Leventhal, 2015). Considering this, the current study examined how genetic susceptibility as modeled by mouse strain may interact with environmental factors to produce distinct placental gene expression profiles during gestation.

**Environment.** While genetics and gene expression analyses are key in describing ASD, it is becoming increasingly clear that environmental insults, and particularly disturbances *in utero*, play a substantial role in the development of ASD. Specifically, the notion that maternal immune activation (MIA) influences brain development has emerged as one of the most promising hypotheses for risk of ASD. Mounting evidence suggests that when a mother's immune system is activated during pregnancy due to a bacterial, viral, or fungal infection, her child has an increased risk of developing ASD (for review see Estes & McAllister, 2016) (See Figure 2). These environmental insults are postulated to increase the risk of ASD, especially in individuals who already possess increased susceptibility due to genetics (Parker-Athill & Tan, 2010). For example, mothers who contract the influenza virus or other viral infections during the first trimester of pregnancy have up to double the risk of having a child with ASD (Atladóttir et

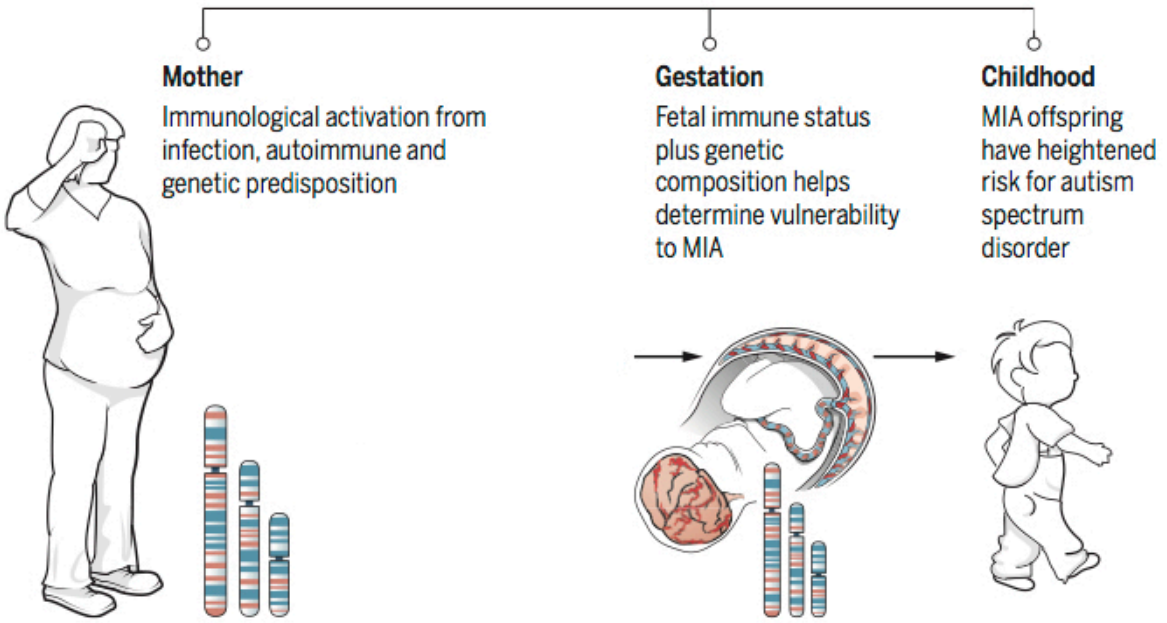


*Figure 2.* Chart illustrating how maternal immune activation (MIA) can lead to disturbances *in utero* that, combined with genetic predisposition, can lead to increased risk for autism spectrum disorder (ASD) development in offspring. Adapted from Estes & McAllister, 2016.

al., 2010; Atladóttir, Henriksen, Schendel, & Parner, 2012). Similarly, mothers who are hospitalized with a bacterial infection, especially during the second trimester of pregnancy, have almost a three-fold increased risk of having a child with ASD (Atladóttir et al., 2010; Zerbo et al., 2015). These infections stimulate the mother's immune system by first activating the innate immune system, the body's initial response to pathogens and eventually the adaptive immune system, which poses a response specific to the individual invader by forming memories and eliminating particular pathogens (Mogensen, 2009). This results in an elevation of classical pro-inflammatory cytokines, including interleukin (IL)-1β (Meyer et al., 2006), IL-6, and IL-17 (Estes & McAllister, 2016), which are postulated to negatively affect fetal neurodevelopment.

Importantly, the severity of ASD symptomology as a result of maternal infection has been shown to be dependent on genetic susceptibility. Specifically, autistic individuals with

ASD-associated copy number variants (CNVs) born to a mother who experienced an infection during pregnancy were found to have more severe social deficits and increased restricted repetitive behaviors compared to individuals with ASD who were either born to infected mothers and lacked CNVs, or who had CNVs and were not born to an infected mother (Mazina et al., 2015). While this study lacked non-ASD controls and was therefore limited in showing a gene by environment interaction in receiving an ASD diagnosis, it provides strong evidence for the interaction of genetics with maternal infection, suggesting that a similar interaction may increase likelihood for initial ASD development.

While most attention to MIA is given to infections during pregnancy, recent epidemiological studies suggest that the unique immune response to allergies and asthma may be especially relevant to ASD development. Specifically, mothers who had onset allergies or asthma during pregnancy were significantly more likely to have a child with ASD (Croen, Grether, Yoshida, Odouli, & Van de Water, 2005). The immune response to allergies/asthma is distinct from infection, because allergies/asthma primarily activate the adaptive immune system, where antibodies have already been formed against specific allergens (Zakeri & Yazdi, 2017). This allergy/asthma response is associated with elevated levels of IL-4, IL-5, and interferon gamma (IFN-γ) (KleinJan, 2016), molecules that have also been shown to be elevated in serum samples taken from mothers during mid-gestation who would later have a child with ASD (Goines et al., 2011). Importantly, these cytokines were shown to be associated with ASD risk specifically, while IL-6, a cytokine commonly elevated in MIA infection models (Smith, Li, Garbett, Mirnics, & Patterson, 2007) was found to be associated generally with developmental disabilities other than autism (Goines et al., 2011). These distinct developmental outcomes in response to maternal allergy/asthma cytokines compared to pathogen-associated immune

responses suggest that the unique immune response to allergies/asthma during pregnancy may be a risk factor for ASD specifically. These findings are especially convincing considering the parallel increases in both allergies and asthma (American Academy of Allergy Asthma & Immunology) and ASD (Center for Disease Control) over the past few decades in the developed world. While these clinical findings provide compelling evidence linking maternal allergic asthma (MAA) with an increased risk of ASD, they are limited in identifying a direct causal link between MAA and ASD.

## Mouse Models of ASD

**Overview.** Mouse models are powerful research tools that allow for controlled genetic and environmental manipulations and clear evaluation of behavioral and biological data. Genetic mouse models of ASD alter genes including *Mecp2* and *Fmr1* (Hulbert and Jian, 2016), the genes implicated in Rett Syndrome and Fragile X respectively, and provide a way for researchers to study the physiology underlying these mutations that are known to be impacted in human ASD. However, classical genetic models of ASD fail to elucidate the causative mechanisms underlying the majority of ASD cases – those that arise idiopathically. By translating the human epidemiological data correlating MIA and ASD into mouse models, researchers create a resource for demonstrating a causative linkage, taking a first step at ultimately developing prophylactic measures to prevent idiopathic ASD development.

MIA mouse models stimulate the immune system of a pregnant mouse (dam) and elicit social behavioral deficits in offspring reminiscent of ASD. Offspring are tested for these deficits using a variety of behavior tasks aimed at assessing social behavior and restricted, repetitive behaviors, two characteristic symptoms of ASD in humans (American Psychiatric Association, 2013). Specifically, the social approach task tests for preference for a social stimulus over a non-

social one, the reciprocal social interaction task tests for frequency and duration of specific types of social interactions, and grooming, marble burying, and ultrasonic vocalizations are markers of repetitive behaviors (Crawley, 2007). These tasks have reliably demonstrated the link between maternal inflammation and offspring ASD-like behavior deficits across various animal models.

**Pathogen-driven maternal immune activation models.** Pathogen-driven MIA mouse models elicit maternal inflammation with polyinosinic:polycytidylic (PolyI:C), a viral-mimic, or lipopolysaccharide (LPS), a component of the bacterial cell wall which mimics a bacterial infection (for review see Meyer, 2014). The immune response of dams following LPS and PolyI:C injection produces a robust increase in levels of the canonical pro-inflammatory cytokines, IL-1β, IL-6, and tumor necrosis factor alpha (TNF-α) in peripheral serum samples (Meyer et al., 2006). Pups born to MIA dams exhibit decreased sociability and increased restricted, repetitive behaviors (Malkova, Yu, Hsiao, Moore, & Patterson, 2012). Along with these key behavioral deficits, synaptic transmission is altered, specifically in serotonergic and dopaminergic pathways (Estes & McAllister, 2016), both of which are associated with ASD in humans (Pagan et al., 2014; Anderson et al., 2008). Importantly, similar behavioral deficits have been seen in non-human primate models of MIA, emphasizing the translational power of these models. Specifically, offspring of rhesus monkeys injected with PolyI:C during pregnancy showed species atypical socialization and increased repetitive behaviors compared to controls (Bauman et al., 2014).

Interestingly, the effects of MIA are influenced by genetics, supporting the idea that a gene by environment interaction contributes to ASD development. Specifically, male offspring of contactin- associated protein-like 2 mice, a model ASD, showed increased social behavioral deficits and decreased ultrasonic vocalizations when born to MIA dams compared to wild-type

mice born to MIA dams (Schaafsma et al., 2017). Similarly, BTBR $T^{+}$ tf/J mice, a strain with low sociability, showed more extreme social deficits and increased repetitive behaviors when born to MIA dams compared to the behavioral deficits seen in MIA offspring of C57Bl/6J (C57) mice, a strain that typically exhibits high sociability (Schwartzer et al., 2013).

**Limitations of pathogen-driven maternal immune activation models.** While PolyI:C and LPS models reliably elicit ASD-like behavioral deficits in offspring, their translational power is limited. Viruses and bacteria activate both the innate and adaptive immune system, however PolyI:C and LPS are not true pathogens and activate the innate immune system alone, failing to successfully model the entire immune response seen in infected pregnant mothers. In contrast, the novel MAA model primarily activates the adaptive immune system in the same mechanism as in humans with allergies/asthma, providing a more accurate model of the immune signaling that would occur naturally. Additionally, considering that ASD rates are rising in the developed world, it seems unlikely that idiopathic development of ASD could be explained by pathogen-driven MIA alone. Rather, it seems more likely that a variety of environmental insults, including the traditional viral/bacterial infections and allergic asthma during pregnancy, may all contribute to risk for ASD development. In fact, one hypothesis is that the type of immune insult may not matter as much as the immune response it elicits. Accordingly, it is believed that the neurodevelopmental effects on offspring are likely due to effects of specific cytokines and maternal inflammation (Spencer & Meyer, 2017), which may be elicited by several types of immune insults rather than one specific insult. While it remains contentious whether the type of immune insult is significant, the mechanism of action of pathogen-driven MIA and its commonalities or divergences from MAA pathogenesis has not yet been uncovered. Given the rising rates of ASD and asthma in developed countries, the strong human correlation between

onset of allergies/asthma and risk of ASD development, and the power of the MAA model in directly activating the adaptive immune system, further investigation must be done to uncover the causative link between ASD and allergies/asthma during pregnancy and the mechanism of action of MAA specifically.

**Maternal allergic asthma mouse model.** To examine the link between allergies/asthma and ASD experimentally, a mouse model of MAA was created in which female C57 mice are sensitized with ovalbumin (OVA) and challenged with aerosolized OVA at three time points during pregnancy (Schwartzer, Careaga, Chang, Onore, & Ashwood, 2015). MAA pups showed characteristic ASD-like behavioral deficits including species atypical sociability, increased marble burying, and altered grooming, behavioral characteristics with validity for ASD. Importantly, MAA-exposed C57 dams showed increased levels of IL-4, IL-5, and IFN-γ in their serum (Schwartzer et al., 2016), consistent with the allergy/asthma associated cytokines reported in human mothers with increased risk for having a child with ASD (Goines et al., 2011), and further validating the translational value of the MAA mouse model.

**Genetic variability in the maternal allergic asthma model.** Using this new model, one study examined the role of genetic variation in predisposition to ASD-like behaviors induced by MAA by utilizing two genetically distinct mouse strains (Schwartzer et al., 2016). Given the significant role that genetics play in human predisposition to ASD, Schwartzer et al. explored whether the genetics of mouse strains could model ASD predisposition similarly to the common genetic variants known to play a role in human ASD (2016). The study compared the traditional C57 mice to FVB.129P2-Pde6b(+) Tyr(c-ch)/Ant (FVBs), a sighted mutant of the FVBn/J strain. The FVBn/J strain has previously been shown to exhibit a more exaggerated airway hyperreactivity in response to OVA sensitization and challenge compared to C57s, which show a

muted response (Whitehead, Walker, Berman, Foster, & Schwartz, 2003). Interestingly, while MAA reduced sociability in C57 offspring, it induced inappropriately increased sociability in FVB offspring (Schwartzer et al., 2016). Specifically, in the reciprocal social interaction task, C57 juveniles born to MAA dams exhibited significantly decreased total time spent socializing and time spent sniffing the body of a novel mouse compared to C57s born to PBS-exposed dams, however FVBs showed the opposite response. FVB juveniles born to MAA dams spent significantly more total time socializing and sniffing the body of a novel mouse compared to FVBs born to PBS-exposed dams (Schwartzer et al., 2016). These findings support the hypothesis that genetics and the maternal immune challenge of allergic asthma interact to produce distinct behavioral phenotypes in offspring.

While these phenotypes are seemingly different, MAA elicited species atypical socialization in both strains. Considering that ASD is a phenotypically diverse disorder in humans and that genetics play a key role in predisposition to ASD, these data indicate that MAA can induce a variety of ASD-like behavioral phenotypes dependent on genetics. Both strains showed characteristic alterations in repetitive behaviors, with significantly increased marble burying and decreased grooming. Biologically, C57 and FVB dams exposed to OVA both showed significantly increased levels of the cytokines IL-4, IL-5, and IFN-γ in serum samples, validating that they exhibited a classic allergic asthma immune response, however, only C57 MAA dams showed elevated levels of IL1β, IL-2, IL-6, IL-17, and TNF-α (Schwartzer et al., 2016). These intriguing strain-specific behavioral and biological effects suggest that mouse genetics play a substantial role in predisposition to specific phenotypic development of ASD-like behavioral deficits and give rise to the question of what similar or distinct biological mechanisms may be acting during pregnancy to lead to this dichotomy of behavioral responses.

## Placental Mediated Maternal-Fetal Interactions

**Mechanisms of maternal immune activation pathogenesis.** The current study aimed to identify potential mechanisms underlying MAA and further analyze whether those mechanisms remain consistent across mouse strains of C57 and FVB. While relatively little is known about the mechanisms of MIA that produce deficits in social behavior, it is well established that in response to an immune challenge, classic inflammatory cytokines are elevated in the mother (Estes & McAllister, 2016). In fact, injecting pregnant rodents with IL-2, -6, or -17 alone can produce ASD-like behavioral deficits in offspring (Meyer, 2014; Choi et al., 2016; Ponzio, Servatius, Beck, Marzouk, & Kreider, 2007), and the cytokine profile of fetal brains is altered shortly after MIA (Estes & McAllister, 2015; Meyer, 2014). While these reports demonstrate a link between maternal and fetal cytokine levels, it remains unknown whether maternal cytokines can cross the placental barrier to directly alter fetal immune signaling (Estes & McAllister, 2016). Rather, there is a novel hypothesis that maternal cytokine signaling may instead alter biological properties of the placenta such as gene expression or protein levels in important neurodevelopmental pathways, which may ultimately impact the developing fetal brain (Burton, Fowden, & Thornburg, 2016). Given the current gap in knowledge, this study aimed to investigate whether MAA alters placental gene expression in key pathways relevant to neurodevelopment.

**Role of the placenta.** The placenta is the key mediating organ between the maternal immune response and neurobehavioral development in offspring (For review of anatomy, See Figure 3). It is the master regulator of fetal development, providing nutrients that the fetus needs to survive, secreting fetal hormones (Burton et al., 2016), and acting on the mother's immune system to prevent the rejection of the pregnancy (Parker-Athill & Tan, 2010). The placenta also

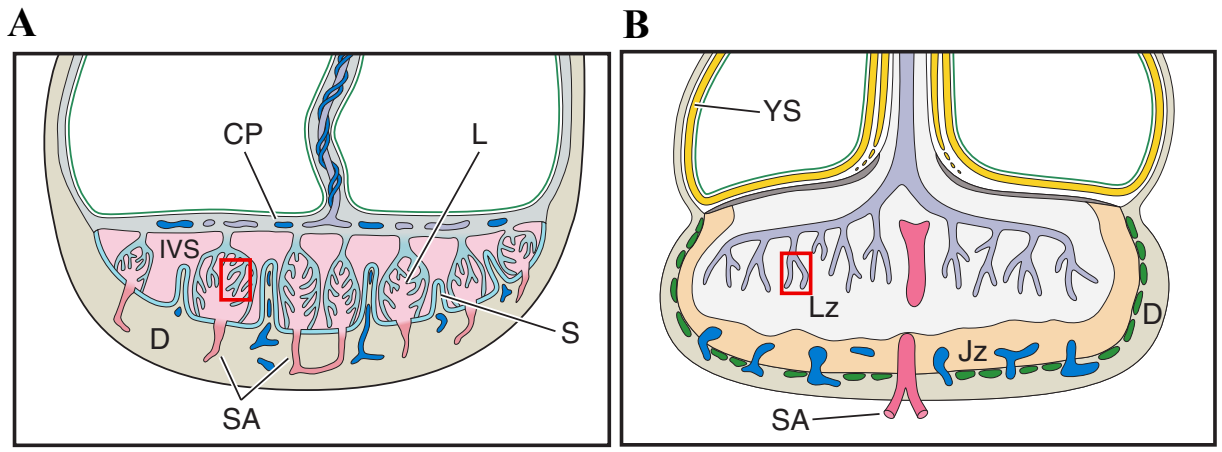


*Figure 3*. Comparative anatomy between the (A) human and (B) mouse placenta. In the human placenta, maternal blood enters through spiral arteries (SA), fills the intervillous space (IVS), and exits through sepatae (S), which are folds from the basal maternal decidua (D). The chorionic plate (CP) folds into lobules (L), each of which have villi containing the fetal-maternal placental interface, most importantly the synctiotrophoblast. While maternal blood still enters through SA in the mouse, the placenta is divided into the endocrine junctional zone (Jz) and exchange labyrinth zone (LZ), with the LZ being the murine equivalent to the human L, and importantly still containing the synctiotrophoblast membrane. The yolk sac (YS) is adjacent to the fetal-facing side of the placenta in mice. Adapted from Burton et al., 2016.

plays a protective role by preventing toxins and maternal hormones from reaching the fetus (Burton et al., 2016). This is accomplished by creating a physical barrier between maternal and fetal circulation, using efflux transporters to flush out unwanted chemicals, and using enzymes to break down potentially harmful materials (Burton et al., 2016).

**The placenta and the environment.** Importantly, the activity of the placenta can be altered by a host of genetic or environmental insults, often compromising the nutrient supply or protective effects of the placenta (Burton et al., 2016). For example, gross abnormalities in human placental size and shape are associated with increased likelihood of asthma, hypertension, coronary heart disease, heart failure, diabetes, lung cancer, Hodgkin's lymphoma, and reduced life span in offspring (Burton et al., 2016).

Environmental alterations may act through several potential mechanisms to alter fetal development and prime a developing fetus for a disorder later in life (See Figure 4), including

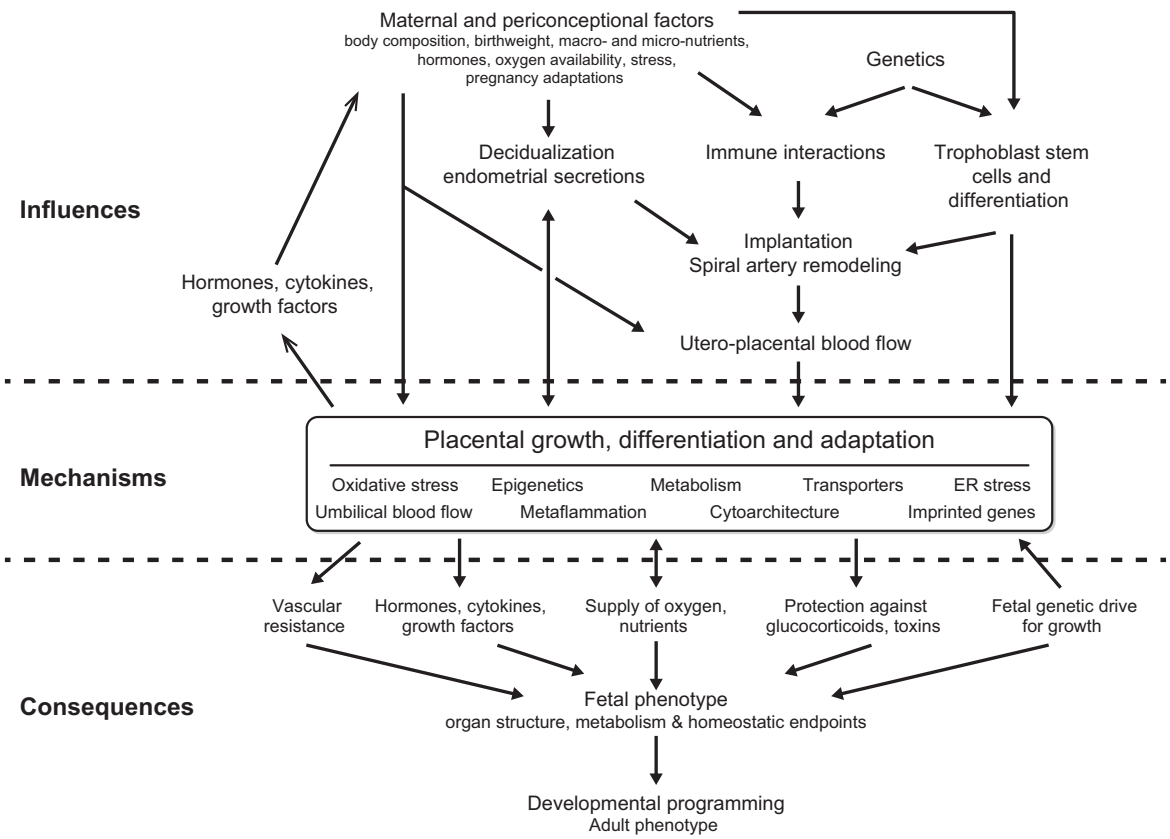


*Figure 4.* Potential pathways through which factors during pregnancy may act on the placenta to impact fetal development. Adapted from Burton et al., 2016.

oxidative stress, endoplasmic reticulum stress, and inflammation of the placenta itself (Burton et al., 2016). Of particular interest is the notion that alterations of gene transcription in the placenta, likely caused by elevated cytokine signaling in the mother or placenta itself, can lead to downstream effects that are detrimental to fetal neurodevelopment (Burton et al., 2016). Placental gene expression can be altered by numerous factors, including maternal diet, hormones, oxygen availability, stress (Burton et al., 2016), and exposure to air pollution (Perera et al., 2009). For example, expression of *SLC6A4,* the gene that codes for the serotonin transporter (SERT) protein, has been shown to be increased in the placenta of mothers who experienced anxiety and depression during pregnancy (Ponder et al., 2011). Taken together, it is clear that gene expression can be altered in the placenta due to environmental factors during

pregnancy, raising the question of what genes may be differentially expressed due to MAA.

**Transporter and metabolism genes in the placenta.** Among the myriad of genes that may be differentially expressed due to environmental factors, two types are of particular interest: transporter and metabolism genes. The placenta possesses numerous transporter proteins that allow nutrients such as sugars, proteins, lipids, and amino acids to enter the placenta from maternal circulation (Burton et al., 2016) through the synctiotrophoblast, the major cellular separation between maternal and fetal tissue (See Figure 5). Genes that code for these proteins can be differentially expressed according to epigenetic or regulatory processes (Burton et al., 2016). Transporters like amino acid transporters and fatty acid transport proteins (FATPs) (See Figure 5) are some of the most highly regulated proteins in the placenta (Jansson, Myatt, & Powell, 2009), making them prime candidates of genes to be altered in response to environmental insults.

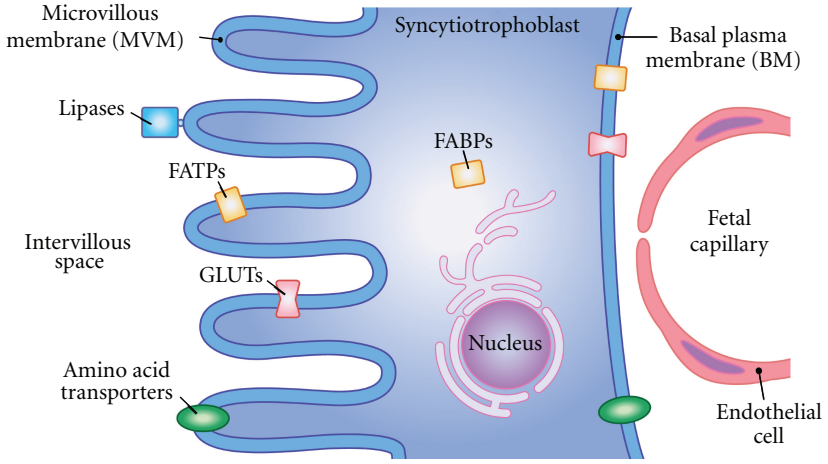


*Figure 5*. The human placental synctiotrophoblast and its two membranes, the maternal-facing microvillous membrane and fetal-facing basal plasma membrane, both of which contain transporters to pump nutrients from the mother to fetus. Importantly, while human and mouse placentae share many structural similarities, the synctiotrophoblast in the human placenta is composed of a single layer, while the mouse placenta has two layers of synctiotrophoblast (Burton et al., 2016). FATPs, fatty acid transport proteins; GLUTs, glucose transporters; FABPs, fatty acid binding proteins. Adapted from Lager & Powell, 2012.

Another subset of genes prone to regulatory mechanisms from environmental insult includes genes involved in metabolism (Burton et al., 2016), and some genes involved in metabolism have previously been shown to be altered by MIA (Goeden et al., 2016). Given the importance of both transporter and metabolism genes in placenta function and their susceptibility to environmental insults, the current study investigated whether transporter and metabolism genes are differentially expressed in the MAA mouse model. In order to accomplish this, the current study explored one transporter and one metabolism gene in two distinct pathways both relevant to neurodevelopment and linked to ASD: the tryptophan (TRP) pathway and the long-chain polyunsaturated fatty acid (LC-PUFA) pathway.

## Tryptophan Pathway

**Serotonin and neurodevelopment.** Serotonin, also known as 5-hydroxytryptamine (5-HT), is a neurotransmitter derived from TRP which is critical for both neurodevelopment and neurotransmission (Brummelte, Glanaghy, Bonnin, & Oberlander, 2017). Early in fetal development, serotonin serves as a trophic factor, promoting growth of the brain by regulating synapse formation, myelination, dendritic pruning, and cell division and differentiation (Brummelte et al., 2017). 5-HT also promotes the development of the hypothalamic-pituitary-adrenal (HPA) axis and the locus coeruleus-norepinephrine (LC-NE) stress response systems, which are known to be involved in psychiatric disorders (Brummelte et al., 2017). Later in fetal development and in adulthood, 5-HT acts as a neurotransmitter, with roles in emotion, attention, cognition, pain, sleep, and arousal (Brummelte et al., 2017).

**Serotonin and autism spectrum disorders.** Misregulation of serotonin is known to occur in individuals with ASD and in ASD animal models. In fact, the first biomarker identified in humans with ASD was elevated 5-HT levels in whole blood samples (Schain and Freedman,

1961), a marker now thought to be shared among 25% of individuals with ASD (Gabriele, Sacco, & Persico, 2014). Strong correlations have been found between alterations in the SERT gene, *SLC6A4*, and ASD (Muller, Anacker, & Veenstra-Vanderweele, 2016). Specifically, the short allele of the serotonin-transporter-linked polymorphic region, a region in the promoter of *SLC6A4*, has been associated with ASD (Muller et al., 2016), as well as certain rare genetic mutations that alter an amino acid in the transmembrane domain of the SERT protein (Sutcliffe et al., 2005). Additionally, serotonin misregulation during development is thought to be a potential risk factor for ASD. For example, boys born to mothers who took selective serotonin reuptake inhibitors (SSRIs), a common treatment for depression and anxiety, during pregnancy were found to be at an increased risk for developing ASD (Harrington et al., 2014). Similar serotonergic misregulation can be seen in mouse models of ASD. MAA offspring show increased levels of serotonin transporter (SERT) protein levels in the cortex (Schwartzer et al., 2015), suggesting that misregulation of the serotonin pathway may be a promising hypothesis for investigation in MAA placentae.

**Tryptophan in the placenta.** The little work that has been done on the serotonin pathway in the placenta of MIA mice has focused on TRP, an amino acid that is transported into the placenta from maternal tissue (Kudo & Boyd, 2001) and is critical for fetal neurodevelopment. Once in the placenta, TRP can either be converted to 5-HT by the enzyme tryptophan hydroxylase (TPH1), or to kynurenine (KYN) by the enzyme indoleamine 2,3-dioxygenase (IDO1) (Goeden et al., 2016). The production of 5-HT provides an exogenous source of serotonin to the developing fetal brain, which is essential for neurodevelopment as exogenous concentrations remain high until the fetal brain is able to synthesize high enough endogenous sources (Bonnin et al., 2011) (See Figure 6). KYN is also essential during

pregnancy and required to prevent the mother's immune system from rejecting the fetus (Badawy, 2015), however its metabolites are known to be neurotoxic in microglia, the resident immune cell of the brain (Dantzer, O'Conner, Lawson, & Kelley, 2011).

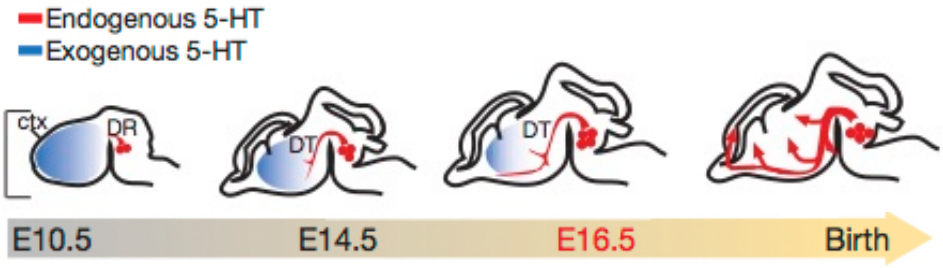


*Figure 6.* Use of endogenous (produced by the fetal brain) and exogenous (produced by the placenta) 5-HT by the developing mouse brain throughout gestation. E, embryonic day; ctx, cortex; DR, dorsal raphe; DT, dorsal thalamus. Adapted from Bonnin et al., 2011.

**Misregulation of the tryptophan pathway in maternal immune activation models.**

While these pathways are critical for fetal development, too much TRP can inappropriately alter 5-HT and KYN output to the fetal brain, potentially affecting neurodevelopment (Goeden et al., 2016). Specifically, the alteration of genes involved in TRP metabolism in the placenta have been shown to alter neurogenesis and are associated with autism-like behaviors in mice and humans (Kanai et al., 2009; Miller et al., 2009; Nabi, Serajee, Chugani, Zhong, & Huq, 2004). Importantly, pregnant mice injected with PolyI:C have higher TRP concentrations and levels of *Ido1* expression in the placenta 24 hours after injection, higher levels of KYN in the fetal hindbrain 24 hours after injection, and higher levels of both KYN and 5-HT in the fetal forebrain 48 hours after injection (Goeden et al., 2016). These results indicate that MIA alters the TRP pathway in the placenta, calling into question whether the same pathway is affected by MAA treatment.

The current study examined differences in the expression of *Ido1* and *solute carrier*

*family 7 member 5 (LAT1)*, a gene that codes for an integral membrane protein amino acid transporter on the microvillous membrane of the synctiotrophoblast that transports TRP and other large neutral amino acids into the placenta from maternal circulation (Kudo & Boyd, 2002) (See amino acid transporters in Figure 5). Investigating *LAT1* and *Ido1* expression together is key because *LAT1* is believed to regulate *Ido1* based on extracellular TRP concentrations (Kudo & Boyd, 2002), suggesting their expression may be altered in similar ways. Given the importance of the TRP/5-HT pathway in brain development as well as its demonstrated alterations in various models of MIA, it was hypothesized that MAA would disrupt gene expression in these two key regulators of the TRP pathway in the placenta.

**Long-chain Polyunsaturated Fatty Acid Pathway**

**Fatty acids and neurodevelopment.** Another pathway hypothesized to be altered in the MAA mouse model is the LC-PUFA pathway, which has been implicated in both ASD and asthma. LC-PUFAs and in particular, omega-3 fatty acids, are essential for both neurodevelopment and neural maintenance throughout life, with roles in gene expression, neurotransmitter production, and immune molecule production (Schuchardt, Huss, Stauss-Grabo, & Hahn, 2010). Docosahexaenoic acid (DHA), an omega-3 LC-PUFA, and arachidonic acid (AA), an omega-6 LC-PUFA, are structural and functional components of cellular membranes, which means that in neurons, their abundance directly influences neuronal membrane conductance and signal transduction (Schuchardt et al., 2010). In fact, omega-3 deficiency in rats has been shown to lead to deficits in the serotonergic pathway (Schuchardt et al., 2010), which is commonly affected in mouse models of ASD. Omega-3 and omega-6 are also precursors to immune molecules including eicosanoids and interleukins, with anti-inflammatory or pro-inflammatory roles, respectively. Given the importance of these molecules to

neurodevelopment and the immune system, they arise as prime candidates to study in neurodevelopmental disorders.

**Fatty acids and autism spectrum disorders.** Importantly, malfunctions in LC-PUFAs have already been linked to neurodevelopmental disorders, including ASD (Schuchardt et al., 2010). Many studies have found that a lack of omega-3, and more specifically, a low omega-3 to omega-6 ratio, is associated with ASD, bipolar disorders, and Attention-deficit/hyperactivity disorder (ADHD) (Schuchardt et al., 2010; Jory, 2016). Additionally, decreases in the omega-3 to omega-6 ratio seen in modern diets are postulated to be contributing to the recent increase in ASD rates (van Elst et al., 2014). In fact, omega-3 supplementation is currently being considered as a potential treatment for ASD patients, as studies have found that autistic children given omega-3 demonstrated decreased hyperactivity and stereotypies compared to the placebo group (Amminger et al., 2007).

It has been suggested that individuals with ASD may have single nucleotide polymorphisms (SNPs) or other factors such as mineral imbalances or increased oxidative stress, which alter their ability to metabolize LC-PUFAs (Schuchardt et al., 2010). Interestingly, sex hormones play a role in the metabolism of LC-PUFAs; specifically, young women are able to metabolize alpha lipoic acid (ALA), the precursor to omega-3s that is obtained from food, much more efficiently than young men, likely because testosterone has been shown to inhibit LC-PUFA synthesis. These discrepancies in LC-PUFA metabolism have been suggested to partially explain the sex differences in prevalence of ASD, wherein males are much more likely to develop the disorders than females (Schuchardt et al., 2010).

Deficient LC-PUFA intake during pregnancy has also been linked to increased likelihood of developing a neurodevelopmental disorder. Both human and non-human primate models have

demonstrated that DHA deficiency during pregnancy can negatively impact offspring neurodevelopment and increase ASD susceptibility (Neuringer, Connor, Lin, Barstad, & Luck, 1986; Schultz et al., 2006; Schuchardt et al., 2010). Importantly, offspring of MIA mice that received DHA supplementation during pregnancy did not develop the ASD-like behavioral deficits characteristic of MIA (Weiser et al., 2016), further implicating DHA, and more generally omega-3, in protection of offspring from MIA pathogenesis.

**Fatty acids in the placenta.** The developing fetus is dependent upon maternal transfer of both essential fatty acids and LC-PUFAs through the placenta (Larqué et al., 2011). Normally, humans take up essential fatty acids through their food and convert them to LC-PUFAs. However, the placenta lacks the essential enzymes Δ5 and Δ6 desaturases for this conversion, and the desaturase activity of the fetus is not high enough to account for the LC-PUFA supply needed, making the direct import of LC-PUFAs through the placenta critical for fetal development (Larqué et al., 2011). Significantly, LC-PUFAs, especially the omega-3 DHA, are transferred preferentially across the placenta with higher concentrations in placental tissue than maternal circulation, stressing their importance to fetal development (Larqué et al., 2011). As the fetus develops it is thought to be more dependent on LC-PUFAs in order to develop neurotypically, suggesting the end of gestation is the best time to look at differences in LC-PUFA pathways that may impart neurodevelopmental phenotypes (Schuchardt et al., 2010).

Taking together the role of LC-PUFAs in brain development, links between ASD and LC-PUFAs, and the critical role of the placenta in LC-PUFAs supply to the fetus, the current study investigated the expression of two genes involved in the transport and metabolism of LC-PUFAs in the placenta of MAA dams: *fatty acid transport protein 4 (FATP4)* and *Acyl-CoA synthetase long-chain family member 3* (*Acsl3)*. *FATP4* codes for an integral membrane

transporter in the microvillous membrane of the synctiotrophoblast (See FATPs in Figure 5), allowing for the selective import of fatty acids into the placenta with a preference for LC-PUFAs, especially DHA (Larqué et al., 2011). *Acsl3* codes for an enzyme that catalyzes the conversion of long-chain fatty acids to acyl-CoA (Coleman & Lee, 2004), and the expression of which is altered in mice exposed to dust mite-induced asthma (Shang et al., 2013) and in human placenta samples from mothers exposed to polycyclic aromatic hydrocarbons (Perera et al., 2009), a component of air pollution and a risk factor for ASD (Raz et al., 2015; Volk, Hertz-Picciotto, Delwiche, Lurmann, & McConnell, 2011). Given the importance of omega-3 LC-PUFAs in neurodevelopment, the increased risk for ASD development among children born to DHA-deficient mothers, and the critical link between MIA mice and the omega-3 pathway, it was hypothesized that MAA would disrupt LC-PUFA transfer to the fetus by downregulating these essential fatty acid genes in the placenta.

**Summary**

Recent epidemiological studies have suggested that mothers who experience allergies or asthma during pregnancy are more likely to have a child with ASD (Croen et al., 2005), however the causal mechanisms leading to ASD development remain unknown. While investigating these mechanisms in human studies would be ethically and logistically challenging, mouse models allow for the rapid generation of data to answer crucial questions. The current study used the novel MAA mouse model which has provided causal evidence supporting the link between allergic asthma during pregnancy and the development of ASD-like behavioral deficits (Schwartzer et al., 2015).

Given the lack of mechanistic understanding of MAA-induced ASD-like behavioral deficits in mice, the strain-specific effects of MAA, the crucial role of the placenta in fetal

development, and the importance of the TRP and LC-PUFA pathways in ASD and MIA mouse models, the current study investigated differences in the expression of genes in the TRP and LC-PUFA pathways in the placenta of C57 and FVB MAA dams. It was hypothesized that mRNA levels of *LAT1* and *Ido1* would show increased expression in MAA placentae compared to controls, indicating that TRP transport and metabolism may be increased by MAA. Similarly, it was hypothesized that if the LC-PUFA pathway is altered in MAA, then mRNA levels of *FATP4* and *Acsl3* would be decreased in MAA placentae, suggesting that fatty acid transport and metabolism may be decreased by MAA.

To answer these questions, placenta samples were harvested from pregnant mice following sensitization and exposure to OVA or phosphate-buffered saline (PBS) control. RNA was extracted from placenta samples and used to create complementary (c)DNA. Gene expression was then quantified using quantitative reverse transcription (qRT)-PCR. Because MAA has been shown to induce distinct biological and behavioral markers in different mouse strains (Schwartzer et al., 2016), the current study used two commonly studied and genetically distinct strains of mice: C57 and FVB. It was hypothesized that there may be strain-specific biological effects of MAA such that genetic susceptibility may exacerbate or protect against placental changes following MAA exposure. Moreover, these findings could explain the phenotypic differences in behavioral profiles previously shown to result from MAA exposure across mouse strains. The results have important implications for the understanding of the mechanistic steps leading to ASD-like behaviors in MAA offspring, and ultimately could elucidate novel mechanisms that could be relevant to the development of prophylactic measures to prevent ASD development.

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## METHOD

In order to assess alterations in gene transcription in MAA placenta samples compared to controls, the current study utilized the established MAA protocol (Schwartzer et al., 2015), harvested placentae, extracted RNA, synthesized cDNA, and performed qRT-PCR (for overview of experimental procedures, see Figure 7).

### Animals

Male and female C57 and FVB mice (Jackson Laboratory) were bred and cared for at Mount Holyoke College. Mice were maintained at room temperature on a 12 hour light/dark cycle, with lights on at 0800. All animals were housed in static plastic cages with same-sex littermates and were given food and water *ad libitum*. Mice were housed two to four females per cage, with male breeders and pregnant females single housed. All mice were given nestlets for enrichment, and all pregnant females were provided with plastic tubes and/or domes for additional enrichment. All procedures were approved by Mount Holyoke College's Institutional Animal Care and Use Committee in accordance with the guidelines provided by the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

### Maternal Allergic Asthma Induction

Eight C57 and eight FVB sexually naïve female mice were randomly assigned to either the allergic asthma (OVA) or the control group (PBS) with 4 mice per group and sensitized with either 10 μg OVA (Sigma) in 1 mg $Al(OH)_3$ (InvivoGen) dissolved in 200 μl PBS or PBS alone on postnatal day (P)42 and again one week later at P49. One week after the final injection,

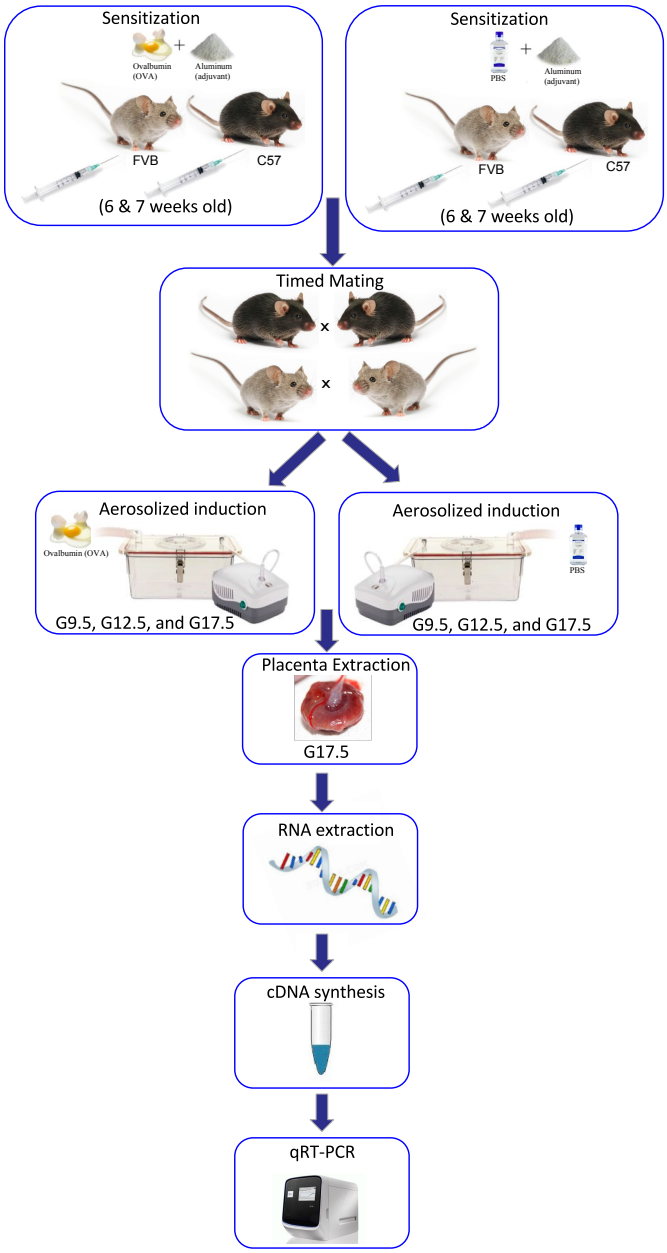


*Figure 7.* Overview of experimental procedures. Female C57 and FVB mice were sensitized to ovalbumin (OVA) or phosphate-buffered saline (PBS), mated, and exposed to aerosolized OVA or PBS on gestational day (G)9.5, G12.5, and G17.5. On G17.5, placentae were extracted and prepared for gene expression analysis using quantitative reverse transcription (qRT)-PCR.

sensitized females were mated overnight with male littermates, and the following morning females were checked for the presence of a seminal plug. The presence of a plug was marked as

gestational day (G)0.5, and pregnant females were housed separately from G0.5 for the entirety of their pregnancies. As previously described (Schwartzer et al., 2016), pregnant females from the OVA and PBS groups were exposed to either aerosolized 1% (wt/vl) OVA in PBS or PBS alone, respectively, for 45-minute inductions on G9.5, G12.5, and G17.5 of their pregnancies. All inductions were performed within the first four hours of the light cycle.

**Placenta Extraction**

Pregnant females were euthanized with $CO_2$ and dissected for placenta extraction four hours after the final induction (G17.5), at a time period when heightened immune activation is known to be present (Schwartzer et al., 2015). Specifically, a vertical incision was made at the xiphoid process and the diaphragm was cut as a secondary method of euthanasia. The incision was extended caudally and laterally to reveal both uterine horns. Fetuses were removed from their yolk sacs, umbilical cords cut, and the placentae isolated. The spiral artery was severed and excess decidua basalis was removed, and on the fetal side, the placenta was separated from the yolk sac and umbilical cord. Importantly, the labyrinth zone, containing the synctiotrophoblast, always remained intact. Dissected placenta samples were placed in PBS, then weighed and added to TRIzol (ThermoFisher) at a concentration of 1 mL TRIzol per 100 mg tissue, with all placentae from one dam pooled together. The samples were then homogenized and frozen at -80°C until RNA extraction. A total of four dams per group (C57 OVA/PBS and FVB OVA/PBS) were used for this study, totaling 16 placenta homogenates.

**RNA Extraction**

RNA was extracted from a single 1 mL aliquot (100 mg tissue) of each placenta homogenate using the TRIzol Plus RNA Purification Kit per the manufacturer's specifications (ThermoFisher). Briefly, samples were added to chloroform and centrifuged to isolate the

aqueous layer containing RNA. RNA was bound to a spin column, washed, and eluted for purification. Quality and quantity of RNA was tested using a NandoDrop Spectrophotometer (ThermoFisher), and the 260/280 ratio and RNA quantity in ng/µl were recorded. Samples were only used if the 260/280 ratio was in the acceptable range, 1.8-2.2 (Goeden et al., 2016). RNA was stored at -80°C until DNase treatment.

**DNase Treatment**

To remove genomic (g)DNA contamination from RNA samples, each RNA sample was DNase-treated before cDNA synthesis using the DNA-*free*™ Kit per manufacturer's specifications (Ambion). Briefly, RNA samples were diluted to contain 10 µg nucleic acid in 50 µl total volume before the reaction to reduce the amount of DNA contamination per sample. Then 5 µl 10X DNase I Buffer and 1 µl rDNase I were added to each RNA sample, and the reagents were mixed gently and incubated at 37°C for 30 minutes. After incubation, 5 µl DNase Inactivating Reagent was added to each sample and mixed. The samples were then incubated at room temperature for 2 minutes while mixed and centrifuged at 10,000 x g for 1.5 minutes. The RNA was transferred to a new tube and the pellet containing DNase Inactivating Reagent was discarded.

**cDNA Synthesis**

1 µg of RNA from each sample was reverse transcribed using the SuperScript First-Strand Synthesis Kit per the manufacturer's specifications (Invitrogen). Briefly, 1 µg of RNA was added to dNTP mix, DEPC-treated water, and 2 µl of random hexamers at a concentration of 50 ng/µl. The RNA/primer mixture was incubated at 65°C for 5 minutes, then put on ice and a mixture of buffer, $MgCl_2$, DTT, and RNaseOUT was added to each RNA/primer mix. The final mixture was incubated at room temperature for 2 minutes, and then the SuperScript II Reverse

Transcriptase enzyme was added to each mix before incubating at room temperature for 10 minutes, 42°C for 50 minutes, and 70°C for 15 minutes. To test for gDNA contamination, one sample was prepared using the same protocol of cDNA synthesis described above, but with water substituted for the reverse transcriptase enzyme as a no reverse transcriptase (No-RT) control. Reactions were chilled on ice and RNase H was added to each tube. cDNA was stored at -20°C until used for qRT-PCR.

**Primer Testing**

Primers were selected for the four genes of interest and *Polr2a*, a housekeeping gene shown to be the most stable in the mouse placenta after MIA (Solano, Thiele, Kowal, & Arck, 2016). Primers were designed using the NCBI database primer design tool for *LAT1, FATP4,* and *Polr2a*, and sequences were used from previous literature for *Ido1* (Metz et al., 2014) and *Acsl3* (Paczkowski, Schoolcraft, & Krisher, 2014) (See Table 1). All primers were designed as cDNA specific to minimize gDNA amplification by either possessing one primer that spanned exon-exon boundaries or by possessing primers in separate exons separated by a large intron. Once received as lyophilized powder, primers were resuspended and diluted to 5 μM working concentrations and stored at -20°C until used for qRT-PCR.

Each primer set was tested for suitable amplification and melting curves by performing qRT-PCR with SYBR Green PCR Master Mix (ThermoFisher) and creating a standard curve using three dilutions of cDNA in triplicate for each gene with the thermal profile detailed in Table 2 and a ramp speed of 0.5°C/3 seconds on an AriaMx Real-time PCR machine (Agilent Technologies). Specifically, all primer sets were tested using 1:10, 1:5, and undiluted cDNA reverse transcribed from 1 μg placental RNA. Standard curves were created by plotting the log of the dilution series on the x-axis and the $C_T$ values for each sample on the y-axis. Percent

*Table 1*. Sequences and product length for qRT-PCR primers. Columns represent forward and reverse primer sequences and product length of cDNA amplified by the primers. Rows represent each gene of interest and the housekeeping gene.

| | Forward Primer (5'-3') | Reverse Primer (5'-3') | Product Length (base pairs) |
|---|---|---|---|
| *LAT1* | TACAGCGTAAAGGCTGCGAC | GTTGGACGCATCACCTTGTCC | 129 |
| *Ido1* | CCCACACTGAGC ACGGACGG | TTGCGGGGCA GCACCTTTCG | 131 |
| *FATP4* | TCTATGGGGCCACTGAATGC | AGGACAGGATGCGGCTATTG | 87 |
| *Acsl3* | TGCTGAGCTTGTGTGTCTTT | GATCCGATCCATGATTTCC | 160 |
| *Polr2a* | TCGAGCAGATCAGCAAGGTGT | TTCTCACTCAGCACCCGCAT | 148 |

*Table 2*. Thermal profile for all qRT-PCR reactions. Columns represent the number of cycles, temperature of the cycle, and duration of the cycle, while the rows represent cycle components.

| | Cycles | Temperature | Duration |
|---|---|---|---|
| Hot Start | 1 | 95°C | 10 min |
| Amplification | 40 | 95°C | 15 sec |
| | | 60°C | 1 min |
| Melt | 1 | 95°C | 15 sec |
| | | 60°C | 1 min |
| | | 95°C | 15 sec |

primer efficiency was calculated for each primer set using the equation $(10^{\frac{-1}{slope}} - 1)\times 100$ where the slope was the slope of the linear line of best fit on the standard curve. Primers were considered efficient when cDNA samples showed clean amplification curves, melting curves showed one distinct peak for all samples, and primer efficiency calculations were between 90-110% (Applied Biosystems). No-RT control samples were run for all primer sets using

undiluted cDNA reverse transcribed from 1 μg placental RNA to ensure no gDNA contamination in cDNA samples. Control reactions were also run for each primer set using all qRT-PCR reaction components except for the cDNA template (no template controls) to ensure no cDNA contamination of primers or SYBR Green.

**Quantitative Reverse Transcription Polymerase Chain Reaction**

qRT-PCR was carried out using SYBR Green PCR Master Mix (ThermoFisher) and primers for the 4 genes of interest and *Polr2a* in triplicate for each gene in each sample. All reactions were carried out using the thermal profile shown in Table 2 on the AriaMx Real-time PCR machine (Agilent Technologies). The average cycle threshold ($C_T$) value, the number of cycles at which a set threshold of amplification has occurred, for each gene in each animal was calculated from triplicate qRT-PCR reactions. $C_T$ values were excluded when their value differed more than one $C_T$ unit from the other two $C_T$ values obtained from the triplicate reaction. Expression levels of the four genes of interest were compared for treatment and strain effects using the $\Delta\Delta C_T$ method (Livak and Schmittgen, 2001). Specifically, $C_T$ values for the housekeeping gene *Polr2a* in each sample were subtracted from $C_T$ values for each gene of interest within that sample to yield a $\Delta C_T$ value. Gene expression $\Delta C_T$ values for all animals were compared to the control condition of each strain by subtracting the average $\Delta C_T$ of PBS animals to yield a $\Delta\Delta C_T$ value. Fold change of expression level, $2^{-\Delta\Delta CT}$, were calculated for use in statistical analyses.

To ensure consistency across the three PCR plates needed for experimental data, placental cDNA from the same animal was run as a control in triplicate on all three plates. Plates were only used when the average $C_T$ value of this control cDNA was no more than one $C_T$ value away from the average of the control cDNA $C_T$ values from the other plates. To further validate

reliability of results, amplification plots and melting curves were examined for each primer set on each plate, and the $C_T$ values of any obvious visual discrepancy on either plot were discarded from analysis.

**Statistics**

All placentae from a single dam were treated as one analytic unit and OVA conditions from each strain were compared back to PBS controls within a single strain. As a result, fold change values ($2^{-\Delta\Delta CT}$) were compared using Independent samples t-tests for each gene separately for each strain. The significance level was set at $p<0.05$.

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# RESULTS

## Primer Testing

In order to ensure that primer sets correctly amplified cDNA, each primer set was tested with three dilutions of placental cDNA, No-RT controls, and no template controls. No primer set showed any amplification in No-RT controls or no template controls, indicating a lack of gDNA in cDNA samples and lack of cDNA contamination in primer sets or qPCR reagents. Each primer set successfully produced smooth amplification plots (See Figure 8) and melting curves with a single peak suggesting high specificity without non-specific binding (See Figure 9). Standard curves for all primer sets showed that amplification was linear across dilutions, with $R^2$ values of 0.95 or higher in all cases (See Figure 10). Efficiency calculations were as follows: 94% for *LAT1*, 107% for *Ido1*, 95% for *FATP4*, 140% for *Acsl3*, and 106% for *Polr2a*. Given that the acceptable range for primer efficiency was set at 90-110%, all primers sets except for *Acsl3* were deemed to be efficient.

## Validation of Gene Expression Analysis

Because data collection for each primer set took place over three qRT-PCR plates, an identical placenta cDNA sample was run on each plate in triplicate to validate that values between the three plates were consistent. For all three plates, the average $C_T$ value obtained for this control cDNA sample were no more than one $C_T$ unit apart, confirming that the plates could be considered consistent and data could be combined for each primer set across the three plates. Importantly, amplification plots and melting curves for all three plates using each primer set,

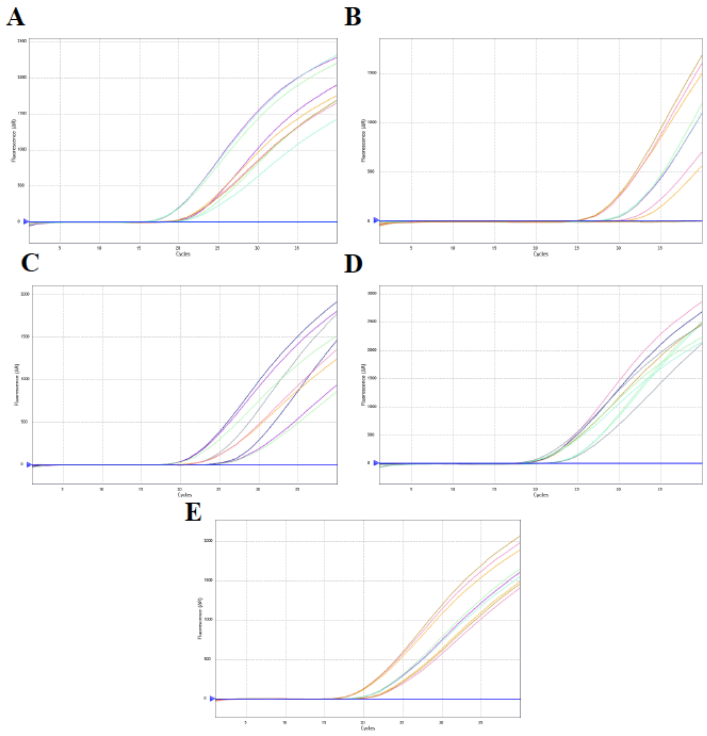


*Figure 8*. qRT-PCR amplification plots for primer testing using three different concentrations of cDNA amplified with each primer set, (A) *LAT1*, (B) *Ido1*, (C) *FATP4*, (D) *Acsl3*, and (E) *Polr2a*.

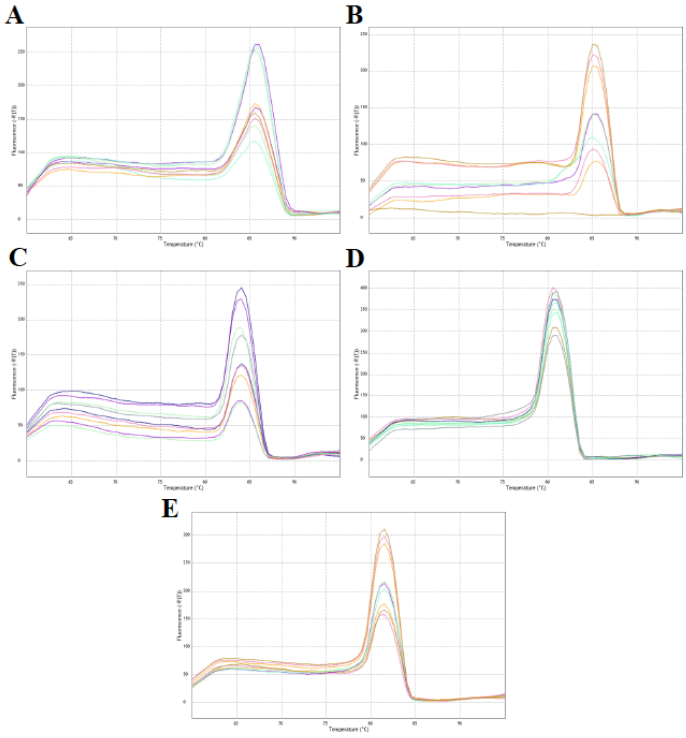


*Figure 9*. qRT-PCR melting curves for primer testing using three different concentrations of cDNA amplified with each primer set, (A) *LAT1*, (B) *Ido1*, (C) *FATP4*, (D) *Acsl3*, and (E) *Polr2a*.

*LAT1* (See Appendix A), *Ido1* (See Appendix B), *FATP4* (See Appendix C), *Acsl3* (See Appendix D), and *Polr2a* (See Appendix E) were consistent for all samples, further underscoring consistency and specificity across all three plates. For representative amplification plots and melting curves for all primer sets on experimental plate 1, see Figures 11 and 12, respectively.

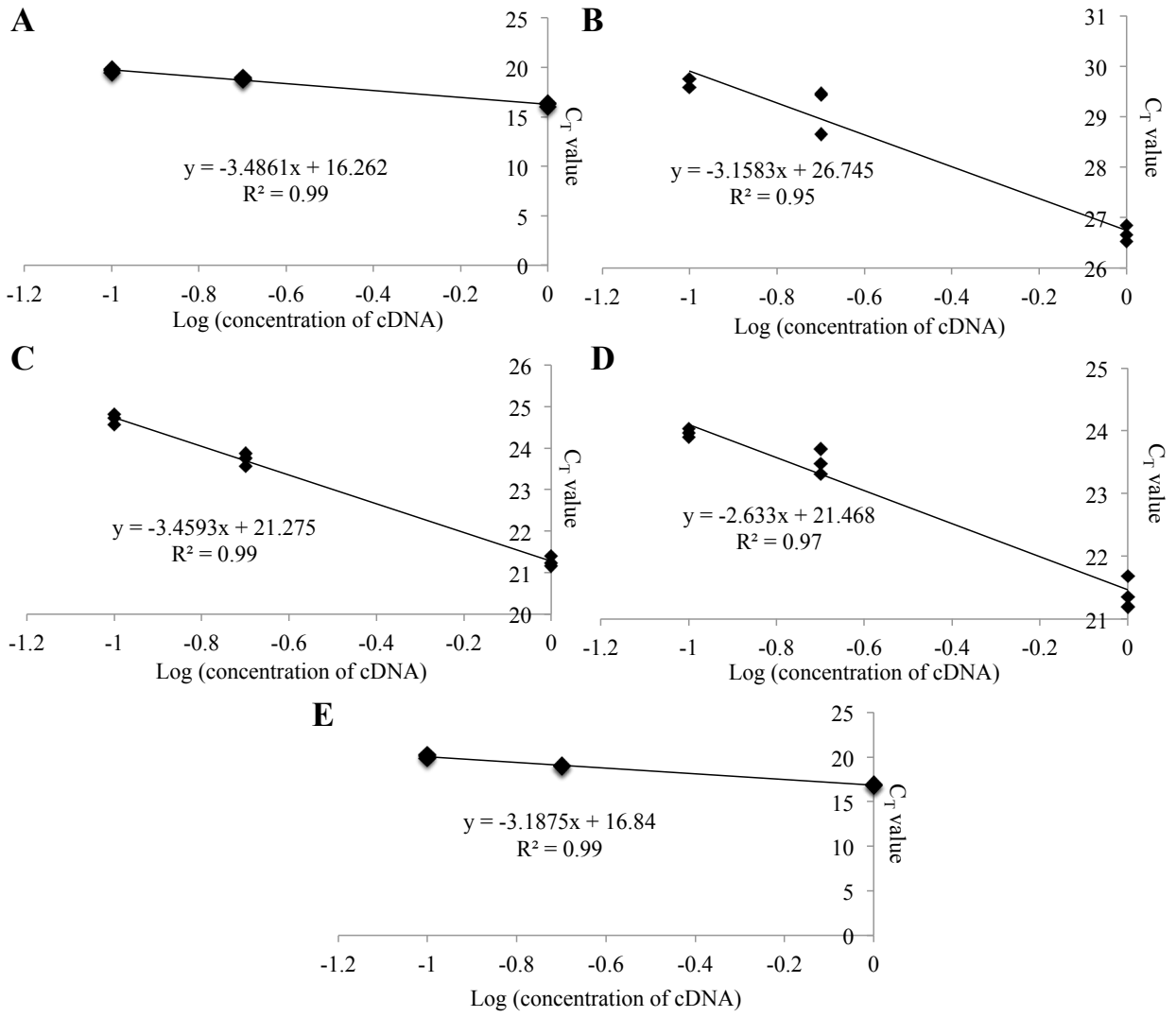


*Figure 10.* Standard curves generated to test primer efficiency of (A) *LAT1*, (B) *Ido1*, (C) *FATP*, (D) *Acsl3*, and (E) *Polr2a* primer sets, respectively. Primers were tested using 1:10, 1:5, and undiluted cDNA reverse transcribed from 1 µg placenta RNA.

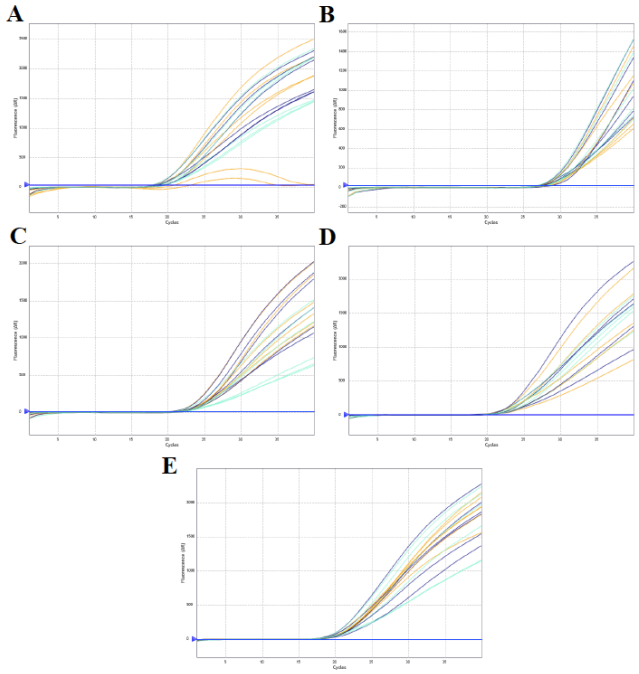


*Figure 11.* qRT-PCR amplification plots for all experimental placenta cDNA samples on plate 1 amplified with primer sets for (A) *LAT1*, (B) *Ido1*, (C) *FATP4*, (D) *Acsl3*, and (E) *Polr2a*. $C_T$ values from samples with amplification curves that did not match the group, such as the two lowest lines in A, were discarded and not used for analysis.

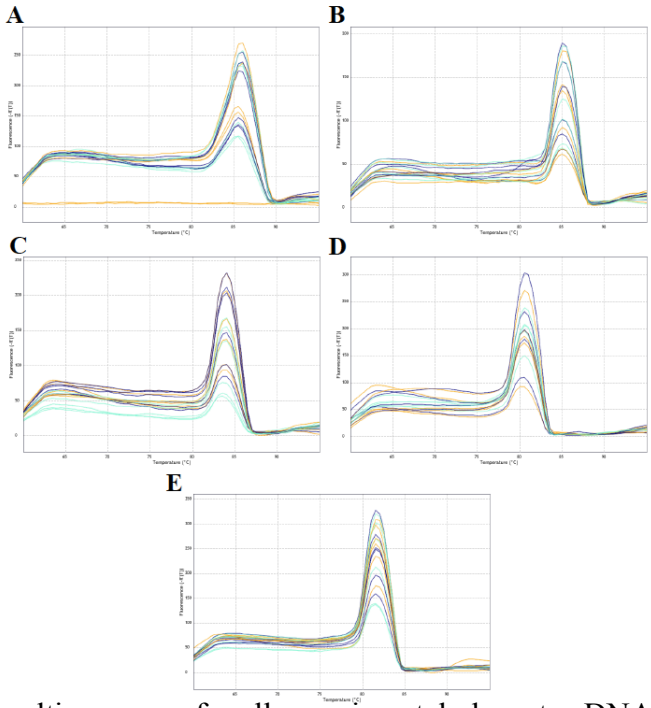


*Figure 12.* qRT-PCR melting curves for all experimental placenta cDNA samples on plate 1 amplified with primer sets for (A) *LAT1*, (B) *Ido1*, (C) *FATP4*, (D) *Acsl3*, and (E) *Polr2a*. $C_T$ values from samples with melting curves that did not match the group, such as the two lowest lines in A, were discarded and not used for analysis.

**Gene Expression Analysis**

To determine if OVA-exposed mice showed differences in placental gene expression, Independent samples t-tests were run separately for each gene in each strain. No differences were seen in placental expression of *LAT1* between OVA and PBS-exposed dams in either C57, $t(6) = -0.831$, $p = 0.438$ (See Figure 13A) or FVB mice, $t(6) = 0.783$, $p = 0.464$ (See Figure 14A). Interestingly, in C57 mice, *Ido1* was expressed significantly more in the placenta of OVA-exposed dams compared to PBS controls, $t(6) = 2.99$, $p = 0.024$ (See Figure 13B), with a 1.54-fold increase in OVA-exposed placenta samples compared to controls. Conversely, these differences in *Ido1* expression were not observed across treatment conditions in FVB mice, $t(6) = 1.58$, $p = 0.164$ (See Figure 14B). *FATP4* expression showed no significant differences for treatment in either C57, $t(6) = 1.82$, $p = 0.118$ (See Figure 13C) or FVB mice, $t(6) = 1.27$, $p = 0.250$ (See Figure 14C). Moreover, expression of *Acsl3* remained unchanged between OVA and PBS controls in both C57, $t(6) = 1.55$, $p = 0.170$ (See Figure 13D) and FVB mice, $t(6) = 1.49$, $p = 0.187$ (See Figure 14D).

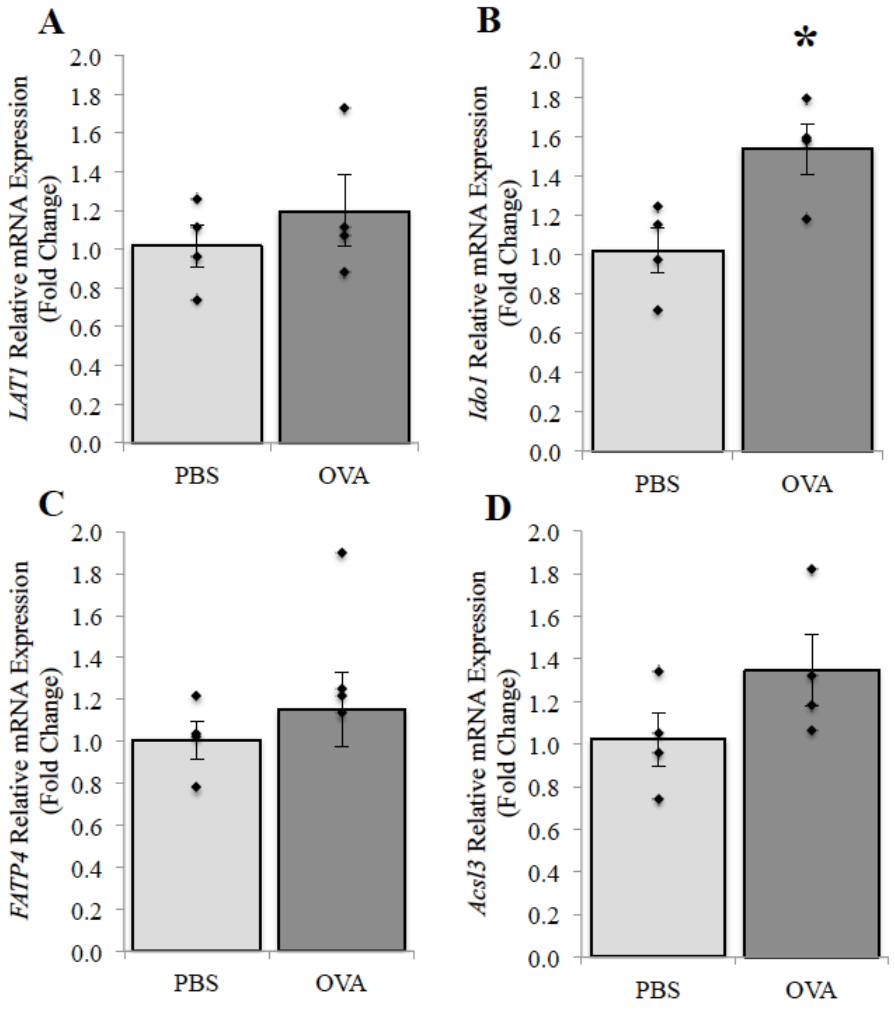


*Figure 13*. Fold change of placental mRNA levels of (A) *LAT1*, (B) *Ido1*, (C) *FATP4*, and (D) *Acsl3* in OVA and PBS-treated C57 mice. Fold change values represent $2^{-\Delta\Delta CT}$ values calculated for each animal normalized to *Polr2a* expression and the C57 PBS control group. Error bars represent standard error and diamonds represent expression levels from individual animals. **p*<0.05.

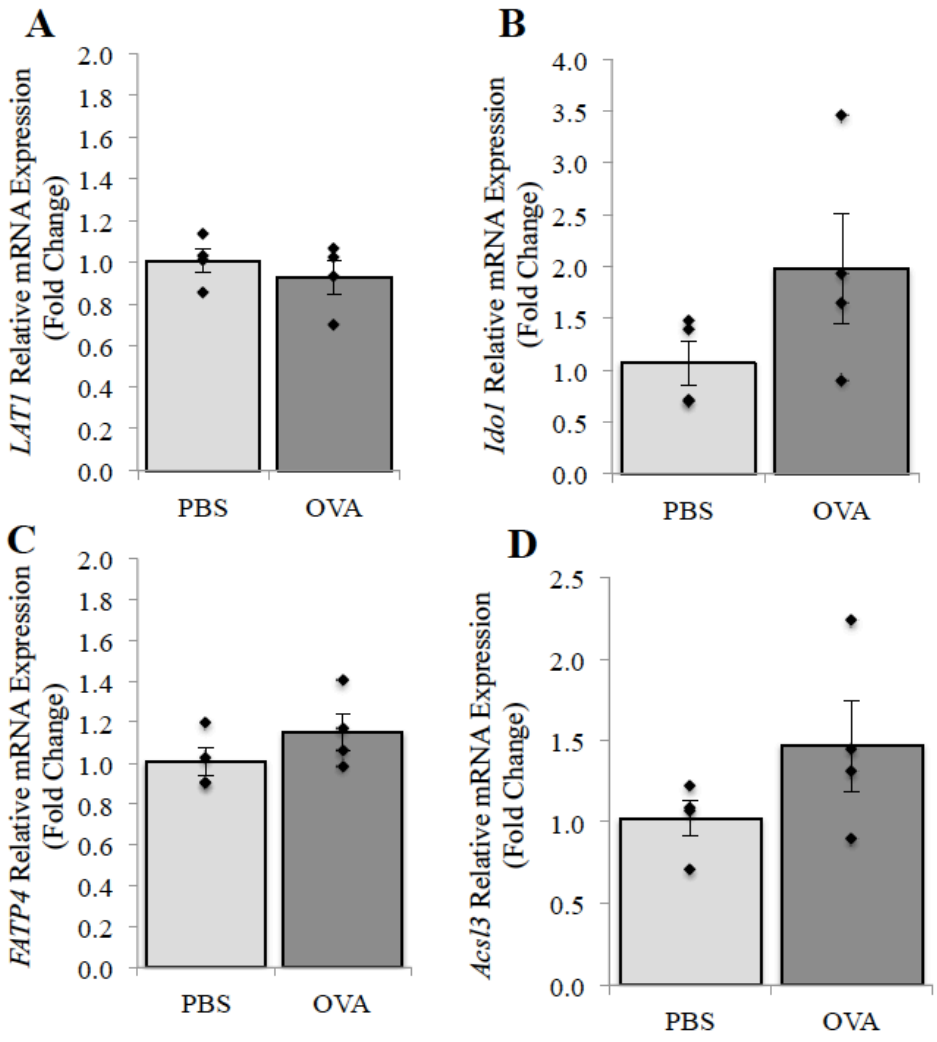


*Figure 14.* Fold change of placental mRNA levels of (A) *LAT1*, (B) *Ido1*, (C) *FATP4*, and (D) *Acsl3* in OVA and PBS-treated FVB mice. Fold change values represent $2^{-\Delta\Delta CT}$ values calculated for each animal normalized to *Polr2a* expression and the FVB PBS control group. Error bars represent standard error and diamonds represent expression levels from individual animals.

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# DISCUSSION

## Overview

Although the etiology behind ASD remains largely unknown, epidemiological studies suggest that activation of a mother's immune system during pregnancy can substantially increase the risk of having a child with ASD (Atladóttir et al., 2010; Atladóttir, Henriksen, Schendel, & Parner, 2012; Zerbo et al., 2015). While most MIA research and animal models focus on bacterial or viral infection during pregnancy, a recent study suggests that onset allergies and asthma during pregnancy may increase the risk of having a child with ASD (Croen et al., 2005). Recently, Schwartzer et al. established a mouse model of MAA demonstrating a causal link between onset allergic asthma during pregnancy and autism-like behavioral deficits in offspring (2015). These behavioral changes were further demonstrated to be strain specific, with C57 MAA offspring displaying significantly reduced sociability compared to controls and FVB MAA offspring exhibiting significantly increased sociability (Schwartzer et al., 2016).

While species atypical social behavior has been demonstrated to result from MAA in two strains, the mechanisms underlying MAA remain unknown. Researchers often focus on the direct effects of maternal inflammation on offspring development, however it is still contentious whether maternal cytokines elevated by MIA are able to cross the placental barrier (Estes & McAllister, 2016). As such, alternative explanations, such as changes in gene expression in the placenta that may regulate key neurodevelopmental pathways, are under investigation in several MIA models. The current study marks the first attempt to elucidate mechanistic changes in the

placenta induced by MAA in both C57 and FVB mice, and sought to explore whether placental gene expression was altered in two distinct neurodevelopmental pathways: the TRP pathway and the LC-PUFA pathway. By examining a transporter and metabolism gene in each of these pathways, *LAT1* and *Ido1* for TRP and *FATP4* and *Acsl3* for LC-PUFA respectively, this study explored key players in highly regulated classes of genes (Jansson et al., 2009; Burton et al., 2016) that may be altered by MAA. Of the four genes investigated, *Ido1* was the only gene that showed significantly increased expression in C57 mice alone, while expression of all other genes in C57 and FVB mice remained unchanged. Taken together, these data suggest that TRP metabolism may contribute to imparting the strain-specific behavioral phenotype observed in the MAA mouse model.

**Tryptophan Pathway**

**Overview.** Within the placenta, TRP is imported from maternal circulation and converted to either KYN by the enzyme IDO1 or to 5-HT by the enzyme TPH1 (Goeden et al., 2016). The current study showed that MAA induces significantly increased expression of *Ido1* in the placenta of C57 mice alone, indicating that there is likely a higher conversion of TRP to KYN due to increased availability of the IDO1 enzyme in these C57 MAA placentae. This increase in *Ido1* expression is likely caused by maternal inflammation due to induced allergic asthma. In fact, LPS has been demonstrated to increase IDO1 activity in the periphery and brain of non-pregnant mice (Lestage, Verrier, Palin, & Dantzer, 2002), and specifically, IL-1, IL-2, IFN-γ, and TNF-α have all been shown to activate IDO in non-pregnant mice (Myint & Kim, 2003). These findings are consistent with the increased *Ido1* expression seen in PolyI:C placentae (Goeden et al., 2016), suggesting that maternal infection and MAA may operate through similar biological mechanisms.

**Kynurenine.** KYN plays an important role in suppressing the mother's immune system during pregnancy, necessitating its increased expression after immune insult (Badawy, 2015). Specifically, KYN and the KYN metabolites 3-Hydroxykynurenine (3-HK), 3-Hydroxyanthranilic acid (3-HAA), and quinolinic acid (QA) prevent T-cell proliferation, likely by promoting apoptosis of T helper type 1 (Th1) cells (Terness et al., 2002; Fallarino et al., 2002). Accordingly, the upregulation of *Ido1* mRNA as seen in C57 MAA placentae likely represents a direct immunological response to maternal inflammation following allergic asthma stimulation. When stimulated, the maternal immune response includes increased T-helper cell activation which may lead to fetal rejection (Badawy, 2015). As a result, increases in *Ido1* expression may act to suppress the maternal immune system by increasing synthesis of KYN metabolites that suppress the T-helper cell activation, thereby acting to prevent fetal rejection.

Regardless of this seemingly benevolent purpose, increased KYN concentrations in the placenta as a result of increased *Ido1* expression may have secondary effects on fetal neurodevelopment that could be potentially detrimental, as the three KYN metabolites that suppress the maternal immune system, 3-HK, 3-HAA, and QA, are also neurotoxic (Dantzer, O'Conner, Lawson, & Kelley, 2011). Increased KYN levels generated within the placenta likely result in an increase of KYN and these neurotoxic KYN metabolites within the fetal brain. This insult is further provoked by the cell-type specific degradation of KYN within the brain (For overview of KYN metabolism relevant to MIA, See Figure 15). For example in microglia, the resident immune cells of the brain, KYN is broken down into QA, which is neurotoxic as a result of its agonist activity on N-Methyl-D-aspartate (NMDA) receptors and its ability to generate radical oxygen species, and 3-HK, another neurotoxic species. In contrast, astrocytes degrade KYN into kynurenic acid, which has antagonist effects on the NMDA receptor, while neurons

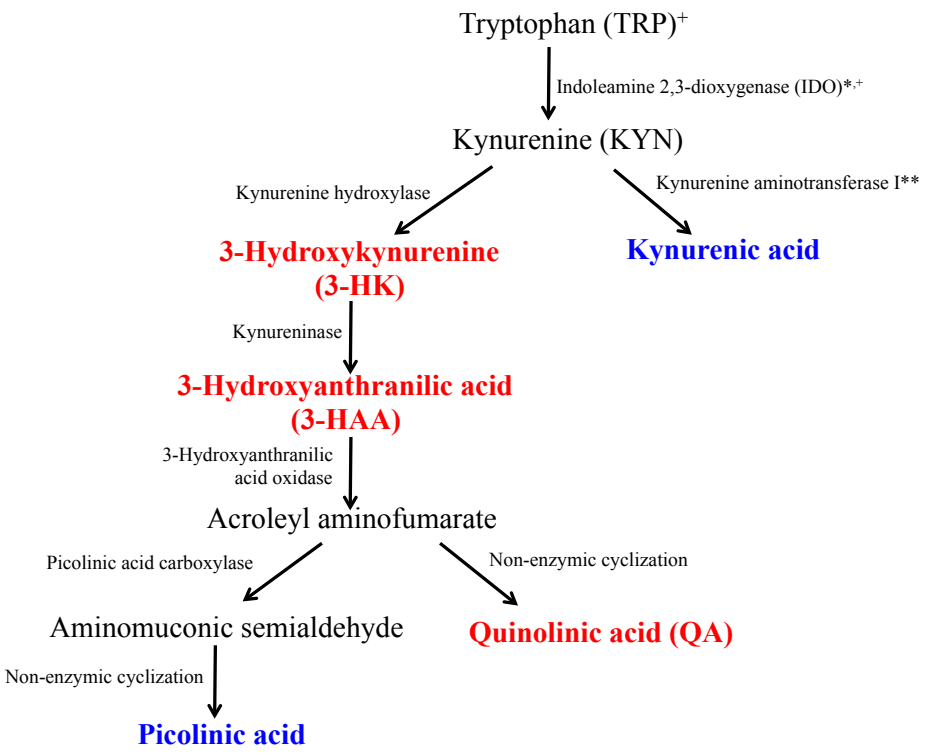


*Figure 15.* Overview of tryptophan metabolism via the kynurenine pathway, highlighting key metabolites in neurons (picolinic acid), microglia (quinolinic acid), and astrocytes (kynurenic acid). Pathways are simplified to emphasize important metabolites for neurodevelopment. Metabolites are in larger font and enzymes facilitating conversion are in smaller font along the arrows. Blue metabolites are neuroprotective while red metabolites are neurotoxic. *, increased by inflammation; **, decreased by inflammation; $^{+}$, known to be increased in maternal immune activation placentae. Adapted from Dantzer et al., 2011 & Badway, 2015.

degrade KYN into picolinic acid. Both kynurenic acid and picolinic acid have been shown to be neuroprotective, however episodes of increased inflammation suppress kynurenic acid production by increasing factors that inhibit kynurenine aminotransferase I, the enzyme that produces kynurenic acid from KYN (Dantzer et al., 2011). As a result, there is a disproportionate amount of neurotoxic to neuroprotective KYN metabolites produced during times of inflammation, and this increase in neurotoxic metabolites is due, in part, to inflammation-induced increases in IDO1 (Myint & Kim, 2003). Considering the increased need for 3-HK, 3-HAA, and QA to suppress T-helper cell activation after immune insult and the

skewed production of these same neurotoxic KYN metabolites in the brain as a result of inflammation, it is plausible that the increase in *Ido1* expression in C57 MAA placentae mediates a shift toward the overproduction of neurotoxic KYN metabolites in the fetal brain. This may have downstream effects on fetal brain development that could account for the autistic-like social behavioral deficits seen in C57 MAA offspring. Future studies should address this possibility by directly measuring levels of TRP, KYN, 3-HK, 3-HAA, and QA within the placenta and fetal brain to better understand the changes in TRP metabolism induced by MAA and the potential implications for fetal neurodevelopment. While the current study cannot determine the specific distribution of KYN metabolites in the fetal brain, the observed increase in *Ido1* expression in C57 MAA placentae indicate adaptive changes to the maternal immune environment which could have downstream consequences for the developing fetus.

**Serotonin.** 5-HT, or serotonin, is critical for neuronal development, migration, and differentiation during gestation and acts as an essential neurotransmitter in adulthood (Brummelte et al., 2017). There is overwhelming evidence that individuals with ASD exhibit misregulation of serotonin (Schain and Freedman, 1961; Muller et al., 2016), positioning it as a potential pathway of interest in mouse models that induce autism-like behavioral deficits. Because serotonin levels are not measured in the current study, there are two distinct possibilities concerning the effects of *Ido1* upregulation on serotonin levels, highlighting an important limitation of this study as the lack of analysis of *Tph1* expression, a gene that codes for an enzyme that converts TRP to 5-HT.

One possibility is that the upregulation of *Ido1* may result in a decrease in serotonin in the placenta and fetal brain. Specifically, if TRP levels entering the placenta and *Tph1* expression stay constant in the placenta following MAA exposure, the increased expression of *Ido1* would

likely indicate a decrease in TRP substrate availability, resulting in decreased conversion of TRP to 5-HT and lower exogenous serotonin levels to be exported to the fetal brain. In support of this, studies have shown that SERT activity is increased by IL-1β and TNF-α (Zhu, Blakely, & Hewlett, 2006), both of which are increased in C57 dams four hours after OVA exposure (Schwartzer et al., 2016). Further, SERT protein levels are significantly increased in MAA offspring compared to controls (Schwartzer et al., 2015), confirming that altered serotonergic neurotransmission is induced by MAA. Increased SERT would likely result in a decrease in serotonin availability by increasing serotonin reuptake at the synapse. In this case, the MAA phenotype could be explained by an increase in KYN and decrease in 5-HT available to the developing fetal brain. However, SERT protein levels to date have only been measured in adult MAA offspring and it remains unknown whether this increase in SERT expression is present during fetal development or if the increase in SERT emerges postpartum in an attempt to compensate for an overabundance of 5-HT at an earlier developmental time point. Additionally, Badawy, Namboodiri, & Moffett propose that a depletion of TRP during pregnancy is unlikely even in times of inflammation, as the fetal demand for TRP utilization in protein synthesis, $NAD^+$ synthesis, and growth and development among other processes is quite high (2016).

Alternatively, increased *Ido1* expression may be paralleled by increased TRP levels and increased enzyme expression in the serotonin pathway, including *Tph1*. This proposed increase in TPH1 would result in more rapid metabolism rates of TRP to be converted both to KYN and 5-HT. Inflammation stimulated by LPS has been shown to increase TRP levels and serotonin metabolism in the brain (O'Connor et al., 2009; Dunn, Wang, & Ango, 1999), and although these studies were not done in pregnant mice, data from MIA PolyI:C mice offer further support. Importantly, dams injected with PolyI:C showed significantly increased TRP levels and *Tph1*

expression in the placenta 24 hours after injection, while *Tph1* expression was significantly decreased 48 hours after injection. KYN and 5-HT levels were altered in the fetal brain, with KYN levels significantly increased in the fetal hindbrain 48 hours after injection and KYN and 5-HT levels both significantly elevated in the fetal forebrain 48 hours after injection (Goeden et al., 2016). If the mechanisms underlying MAA are similar to PolyI:C, this could indicate that rather than a depletion of 5-HT causing neurodevelopmental deficits, it could be the excess of both KYN and 5-HT that negatively impact the developing fetus.

In order to determine which hypothesis is correct, future work must examine changes in *Tph1* gene expression and directly measure TRP levels in the placenta following OVA exposure. In addition to measuring changes in the placenta, these studies should be extended to the fetal brain to identify how altered TRP metabolism may result in downstream changes in KYN and 5-HT in the developing fetal brain after maternal OVA exposure. Although the current study cannot draw conclusions about 5-HT levels in the placenta or fetal brain following MAA, the significant increase in *Ido1* expression in the placenta support the overwhelming evidence that altered TRP and 5-HT may play a substantial role in the development of autism-like behavioral deficits in mice.

**Transporter expression.** The change in *Ido1* expression in C57 MAA placentae without a change in *LAT1* indicates that if TRP levels entering the placenta are increased it is likely due to a mechanism other than increased transporter expression. For example, TRP levels might be increased in maternal circulation, leading more TRP to be imported into the placenta without necessitating increased transporter expression. Alternatively, it is possible that *LAT1* protein levels are actually increased, although not because of differences in transcription. Importantly, *LAT1* is not selective only for TRP, but also transports other large non-polar amino acids into the

placenta (Kudo & Boyd, 2002), including tyrosine and glycine, amino acids important for other neurochemical signaling pathways. It is possible that a change in *LAT1* expression would cause a more drastic phenotype by influencing other metabolic pathways. While *Ido1* expression has been previously profiled in PolyI:C placentae, this study marks the first investigation of transporter gene expression analysis in any MIA placenta, and overall the findings indicate that changes in the KYN/5-HT pathway are likely due to changes in TRP metabolism and not increased transport of TRP into the placenta via *LAT1*.

**Strain differences.** Intriguingly, in contrast to the increased *Ido1* expression in C57 MAA placentae, FVB mice showed no significant increase in expression of placental *Ido1*. Considering the divergence in behavioral phenotypes seen in MAA pups of C57s and FVBs (Schwartzer et al., 2016), this difference in *Ido1* expression is likely due to strain differences and strain specific susceptibilities to MAA. The altered susceptibility to MAA may be at least in part due to strain-specific basal TRP and TRP degrading enzyme levels. Specifically, the C57 strain is known to have the highest levels of Trp 2,3-dioxygenase (TDO), another enzyme responsible for converting TRP to KYN (Badawy, 2015), which accounts for the low baseline level of TRP found in C57s. Even within the strain, females are believed to have increased TDO activity compared to males, making female C57 mice extremely susceptible to the effects of altered TRP levels (Badawy, 2015).

Importantly, differences in *Ido1* expression between C57 and FVB placenta samples may be explained by strain-specific peripheral serum cytokine elevation in response to OVA exposure. Specifically, C57 and FVB dams exposed to OVA both have significantly increased levels of IFN-γ four hours after allergic asthma induction, however only C57 mice exhibit increases in IL-1β, IL-2, and TNF-α (Schwartzer et al., 2016). As previously discussed, IL-1,

IL-2, TNF-α, and IFN-γ all increase IDO activity (Myint & Kim, 2003), so considering that increased levels of *Ido1* mRNA were only observed in C57 MAA placentae, it is plausible that elevations of IL-1β, IL-2, and/or TNF-α are required to elicit altered TRP metabolism in response to maternal inflammation from allergic asthma. This may potentially explain the dichotomy of social behavioral deficits seen between C57 and FVB OVA offspring in which C57s exhibited significantly decreased socialization while FVBs exhibit significantly increased socialization compared to controls.

These findings place special interest on IL-1β, IL-2, and TNF-α as potential mediators of MAA and raises the question of whether these cytokines are necessary to induce increased *Ido1* expression and the MAA phenotype seen in C57 pups. Future research should inject FVB dams with IL-1β, IL-2, and TNF-α at the time of OVA exposure and measure resulting *Ido1* expression in the placenta along with behavioral deficits in pups. If *Ido1* expression is significantly increased and the social behavioral deficits of FVB MAA offspring more closely resemble the decreased socialization phenotype observed in C57 MAA offspring, then it could be concluded that the injected cytokines are necessary to induce this C57-like MAA phenotype. This should be done for each cytokine individually and for all three simultaneously to determine if IL-1β, IL-2, and TNF-α work together to increase *Ido1* expression and promote reduced social interactions, or whether it is primarily one of these three cytokines driving the response.

**LC-PUFA Pathway**

LC-PUFAs, and specifically omega-3 PUFAs, are critical for neurodevelopment and maintenance of adult neurons throughout life (Schuchardt et al., 2010). Omega-3 deficiency is associated with serotonergic misregulation and (Schuchardt et al., 2010) deficiency of omega-3 during pregnancy has been linked to risk of ASD development in offspring of both humans and

non-human primates (Neuringer et al., 1986; Schultz et al., 2006; Schuchardt et al., 2010). Moreover, omega-3 is thought to be involved in the pathogenesis of MIA because the behavioral deficits seen in offspring of PolyI:C injected mice can be rescued by DHA supplementation during pregnancy (Weiser et al., 2016). Although neither of the LC-PUFA genes tested in C57 or FVB placentae showed differences in expression levels between the MAA and control conditions, the possibility that LC-PUFAs are not involved in MAA seems somewhat unlikely given the previous work implicating LC-PUFAs in MIA. Importantly, the two genes tested in the current study, *FATP4* and *Acsl3*, have not previously been tested in PolyI:C or MAA mice, raising the question of whether other genes in the LC-PUFA pathway may be more susceptible to maternal inflammation.

It has been noted that in humans with ASD, the omega-3 to omega-6 ratio is often lower than in typically developed individuals, and that it is omega-3s, rather than all LC-PUFAs, that are critical in affecting ASD. While the genes studied here were the most selective for LC-PUFAs that are known, they are not exclusive to omega-3s but are thought to be used for transport and metabolism of many long-chain fatty acids (Duttaroy, 2009). There does exist an additional placental transporter, plasma membrane fatty acid binding protein (FABPpm), which is believed to be more selective for long chain fatty acids (Knipp, Audus, & Soares, 1999), however it has yet to be sequenced and therefore is not able to be examined via qRT-PCR. With the current available genes, it remains unclear whether the import or metabolism of omega-3 in the placenta is crucial for MAA. It could also be the case that less omega-3 or more omega-6 than normal is transported through *FATP4* or metabolized with *Acsl3* in the MAA placentae, but the expression of these genes remains constant. Although it is unknown whether MIA alters LC-PUFA availability, a change in the omega-3 to omega-6 ratio could reflect changes in LC-PUFA

levels in maternal circulation that may be passed to the developing fetus without altering gene expression. Again, it is a possibility that changes in translation or post-translational modifications alter *FATP4* and *Acsl3* protein levels, and that these proteins themselves are still critical to MAA, raising the need for future studies to analyze protein levels of *FATP4* and *Acsl3* in MAA placentae.

**Future Directions**

While the increased expression of *Ido1* in C57 MAA placentae is promising, future work is required to fully uncover the mechanisms underlying MAA. Moving forward, studies should address changes in gene transcription on a larger scale. Performing an RNA-sequencing (RNA-seq) experiment on MAA and control placentae would more powerfully uncover changes in gene expression throughout the genome and screen for candidate genes that may be involved in imparting the MAA phenotype. As a direct follow up on the current study, future research should examine the protein levels of all four targeted genes, *LAT1*, *Ido1*, *FATP4*, and *Acsl3*, by performing Western Blot analysis with placentae from MAA and control dams. This may be especially important for *LAT1* and *FATP4*, as transporters are known to be some of the most highly regulated proteins (Jansson et al., 2009), but not necessarily via transcriptional regulation. That is, these transporters could still play a role in MAA but could be altered post-transcriptionally.

To explain the mechanisms behind increased transcription of *Ido1*, future studies should examine epigenetic markers that may account for its increased transcription, such as increased histone acetylation or DNA methylation in the *Ido1* promoter. Moreover, *Ido1* expression and protein levels should be examined in the fetal brain to assess whether this altered expression extends directly to the fetal brain or whether changes in the output of KYN and 5-HT from the

placenta are the sole source of changes in the fetal brain. Future studies should also consider examining placentae at earlier gestational time points to determine whether *Ido1* is consistently upregulated after OVA exposure or whether changes in gene expression occur during specific critical windows across development. This may be especially important considering that *Ido1* is most highly expressed in the placenta during middle gestation (Badawy, 2015) and that the developing fetus relies on exogenous 5-HT production from the placenta during early stages of pregnancy while later during pregnancy it largely produces endogenous 5-HT for itself (Bonnin et al., 2011).

To directly follow up on the LC-PUFA pathway, primers for *Acsl3* should be redesigned and optimized. The primer set for *Acsl3* used in this study showed a primer efficiency of 140%, well outside the acceptable range of 90-110%, and because of this it is possible that real effects of treatment on *Acsl3* expression could not be identified due to sub-optimal primers. Moving forward, levels of omega-3 and omega-6 should be directly measured in maternal blood, placentae, and fetal brains to better elucidate the role that omega-3 and omega-6 play in MAA across fetal development. As an alternative direction, future experiments could take a different approach to the LC-PUFA pathway by injecting pregnant C57 dams with DHA, a type of omega-3 LC-PUFA, immediately following allergic asthma inductions. If offspring are found to have no behavioral deficits, as in pups of PolyI:C dams given DHA supplementation during pregnancy (Weiser et al., 2016), and show normal levels of placental *Ido1* expression, this could point to a simple prophylactic measure that could be used by pregnant women experiencing onset allergies and asthma during pregnancy to reduce the risk of having a child with a neurodevelopmental disorder.

## Conclusion

MAA has emerged in both human epidemiology and mouse models as a potent risk factor for having a child with ASD. This study marks the first attempt to classify biological changes during pregnancy that may contribute to the MAA phenotype. Here it is shown that in the placenta of C57 mice, MAA induces significantly increased expression of *Ido1*, a gene that codes for an enzyme that converts TRP to KYN, following repeated allergic asthma inductions. This is consistent with findings in PolyI:C mice (Goeden et al., 2016), and suggests that MAA may mechanistically impart its phenotype in a similar way as classical maternal infections. Overall, these findings further support the widely accepted literature that alterations in TRP metabolism and 5-HT signaling are associated with ASD, and strengthens the connection between the MAA model and risk for ASD development. Taken together, these findings have the potential to lead to the development of prophylactic measures to prevent ASD development in high-risk pregnancies.

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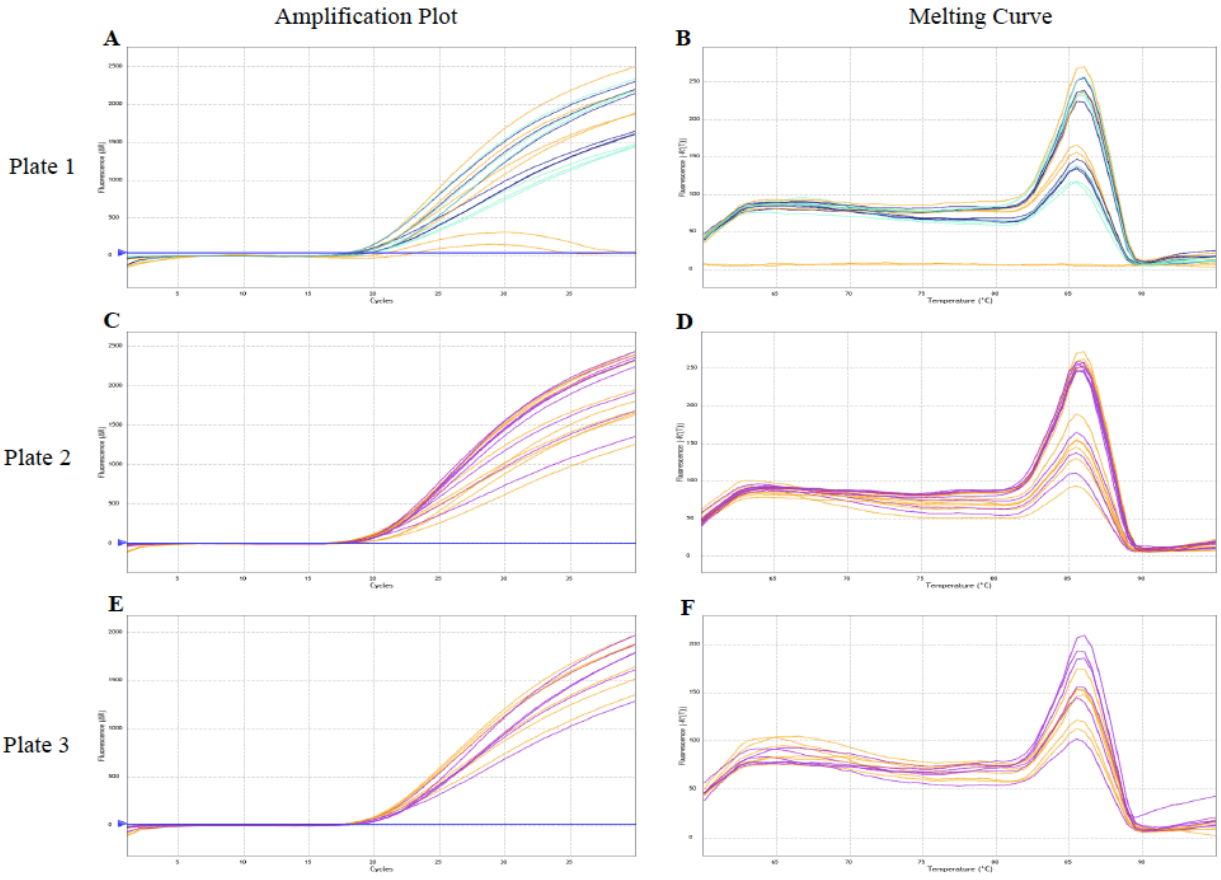
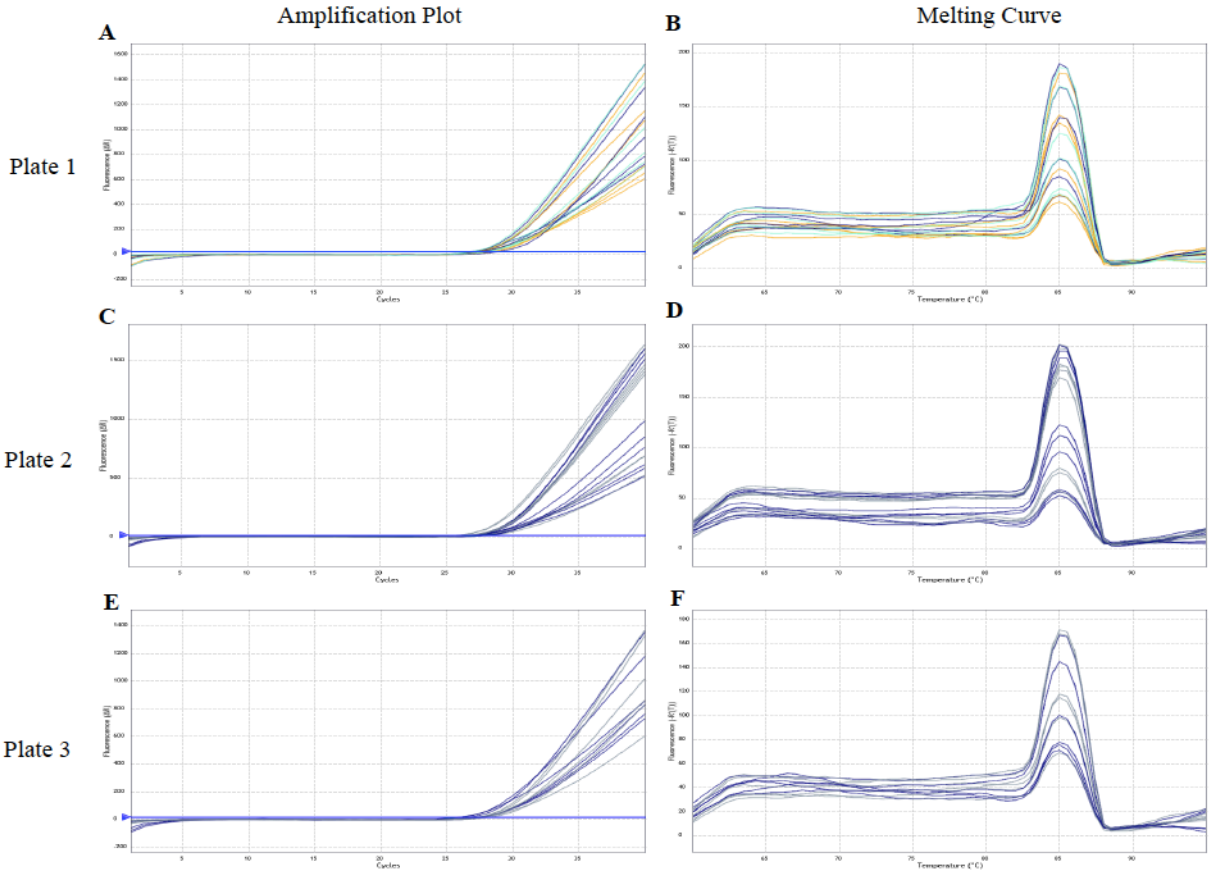
# APPENDIX A



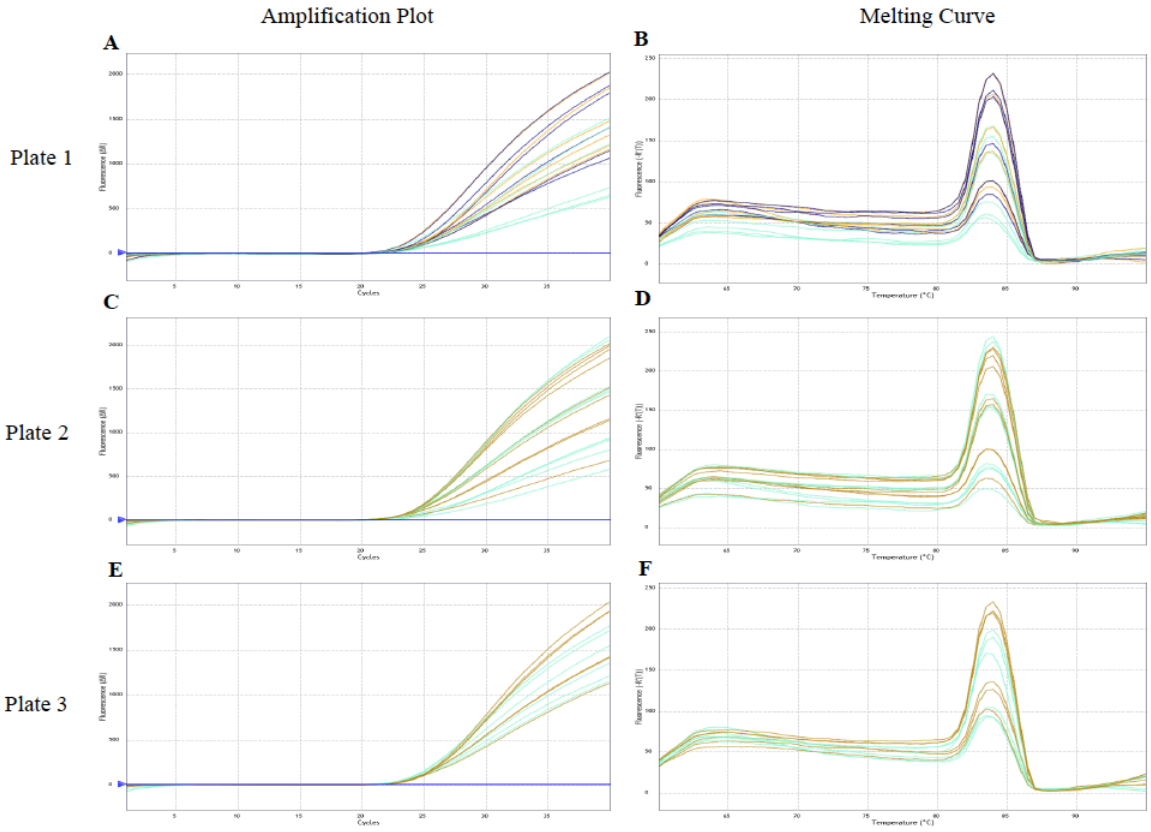

*Appendix A*. qRT-PCR (A, C, E) amplification plots and (B, D, F) melting curves for each experimental placenta sample amplified with primers for *LAT1* on experimental (A, B) plate 1, (C, D) plate 2, and (E, F) plate 3. $C_T$ values from samples with melting curves that did not match the group, such as the two lowest lines in A, were discarded and not used for analysis.

# APPENDIX B



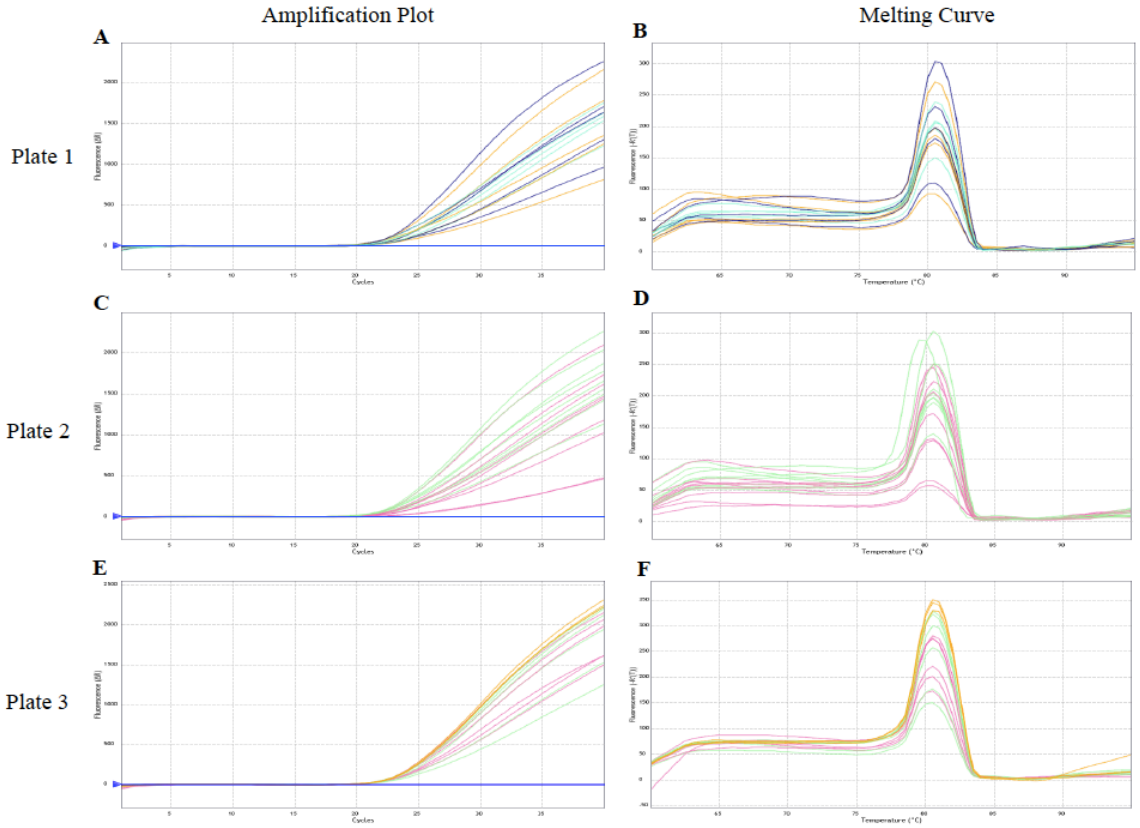

*Appendix B*. qRT-PCR (A, C, E) amplification plots and (B, D, F) melting curves for each experimental placenta sample amplified with primers for *Ido1* on experimental (A, B) plate 1, (C, D) plate 2, and (E, F) plate 3.

# APPENDIX C



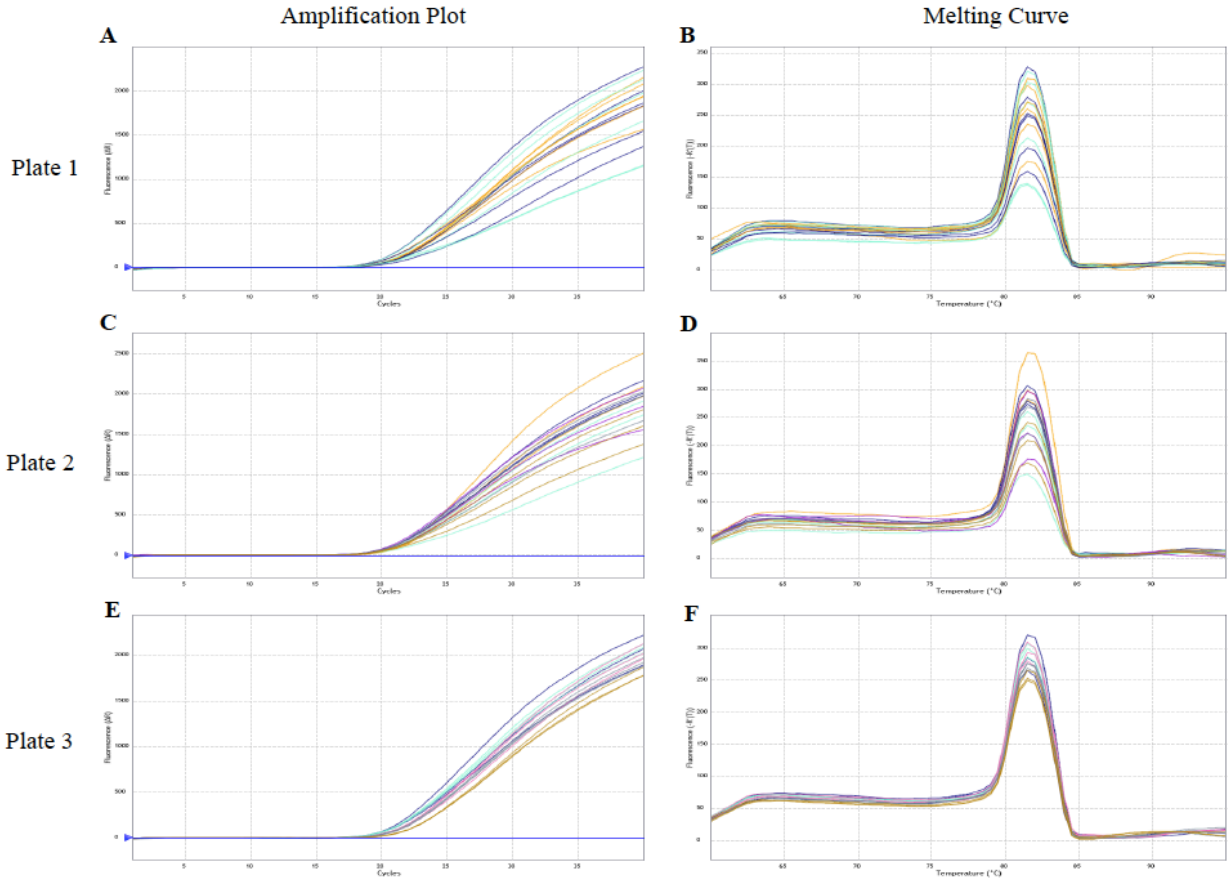

*Appendix C*. qRT-PCR (A, C, E) amplification plots and (B, D, F) melting curves for each experimental placenta sample amplified with primers for *FATP4* on experimental (A, B) plate 1, (C, D) plate 2, and (E, F) plate 3.

# APPENDIX D




*Appendix D*. qRT-PCR (A, C, E) amplification plots and (B, D, F) melting curves for each experimental placenta sample amplified with primers for *Acsl3* on experimental (A, B) plate 1, (C, D) plate 2, and (E, F) plate 3.

# APPENDIX E




*Appendix E*. qRT-PCR (A, C, E) amplification plots and (B, D, F) melting curves for each experimental placenta sample amplified with primers for *Polr2a* on experimental (A, B) plate 1, (C, D) plate 2, and (E, F) plate 3.

Pages Omitted

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