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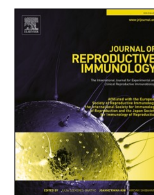

The serotonin reuptake inhibitor fluoxetine induces human fetal membrane sterile inflammation through p38 MAPK activation

Veronica A. Fabrizio [a,1,2], Christina V. Lindsay [b,1], Maya Wilcox [b,1], Suyeon Hong [b,c], Tatyana Lynn [b], Errol R. Norwitz [d], Kimberly A. Yonkers [b,c,e,3], Vikki M. Abrahams [b,*,3]

[a] Department of Pediatrics, Yale School of Medicine, New Haven, CT, United States
[b] Department of Obstetrics, Gynecology and Reproductive Sciences, Yale School of Medicine, New Haven, CT, United States
[c] Department of Psychiatry, Yale School of Medicine, New Haven, CT, United States
[d] Department of Obstetrics and Gynecology, Tufts University School of Medicine, Boston, MA, United States
[e] Departments of Psychiatry and Obstetrics & Gynecology, University of Massachusetts Chan Medical School, Worcester, MA, United States

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ABSTRACT

Serotonin Reuptake Inhibitors (SRIs) are often used as first line therapy for depression and other psychiatric disorders. SRI use during pregnancy is associated with preterm premature rupture of membranes (PPROM) and subsequent preterm birth. The objective of this study was to investigate the mechanism(s) responsible for SRI-associated PPROM. Putative mechanisms underlying PPROM include fetal membrane (FM) inflammation, increased apoptosis, and/or accelerated senescence, the later which may be reversed by statins. Human FM explants from normal term deliveries without labor, infection, or antidepressant use were treated with or without the SRI, fluoxetine (FLX), either alone or in the presence of a p38 MAPK inhibitor or the statins, simvastatin or rosuvastatin. FMs were also collected from women either unexposed or exposed to FLX during pregnancy. FLX significantly increased FM p38 MAPK activity and secretion of inflammatory IL-6. Inhibition of p38 MAPK reduced FM IL-6 secretion in response to FLX. Statins did not reduce the SRI-induced FM IL-6 production. FMs from women exposed to FLX during pregnancy expressed elevated levels of p38 MAPK activity compared to matched unexposed women. FMs exposed to FLX did not exhibit signs of increased apoptosis and/or accelerated senescence. These results indicate that the SRI, FLX, may induce sterile FM inflammation during pregnancy through activation of the p38 MAPK pathway, and in the absence of apoptosis and senescence. These findings may better inform clinicians and patients as they weigh the risks and benefits of SRI antidepressant treatment during pregnancy.

1. Introduction

Eight percent of pregnant individuals will suffer a major depressive episode (Vesga-Lopez et al., 2008); 14–23 % of pregnant women will experience some type of depressive disorder (Yonkers et al., 2009); and ~8–13 % of pregnant women will be prescribed antidepressants (Calderon-Margalit et al., 2009; Yonkers et al., 2009; Mitchell et al., 2011). Use of serotonin reuptake inhibitor (SRI) antidepressants in pregnancy has been associated with a variety of adverse outcomes, including fetal malformations, autism, small for gestational age, persistent pulmonary hypertension, and preterm delivery (Yonkers et al., 2014). While controversies exist regarding the association between adverse birth outcomes and some exposures, after carefully controlling for confounding factors, the literature consistently links SRIs with an increased risk of preterm birth (Yonkers et al., 2009; Roca et al., 2011; Yonkers et al., 2012; Ross et al., 2013; Huang et al., 2014; Eke et al., 2016; Sujan et al., 2017).

Preterm birth affects approximately 10 % of live pregnancies in the

* Correspondence to: Department of Obstetrics, Gynecology & Reproductive Sciences, Yale School of Medicine, 310 Cedar Street, LSOG 305C, New Haven, CT 06510, United States.

E-mail address: vikki.abrahams@yale.edu (V.M. Abrahams).

[1] These authors contributed equally.

[2] Present address: Reckitt|Mead Johnson, Evansville, IN, United States.

[3] This work was a collaboration between two principal investigators (KAY and VMA) who made equal contributions to this project.

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US (March, 2021) and is a major cause of neonatal morbidity and mortality. Preterm premature rupture of membranes (PPROM), defined as rupture of the fetal membranes (FMs) prior to 37 weeks' gestation, is the leading identifiable cause of preterm birth. PPROM occurs in 25–30 % of preterm births (Menon and Fortunato, 2007), and accounts for 18–20 % of all perinatal deaths in the US (Anon, 2007). In a recent study of nearly 3000 pregnant patients, when PPROM and preterm labor were separated into two groups, women who used serotonin reuptake inhibitors (SRIs) were over twice as likely to experience PPROM compared to women who did not (Yonkers et al., 2012). These analyses were adjusted for licit and illicit substance use, race/ethnicity, and infection. These findings are consistent with previous reports (Roca et al., 2011). Although it is recognized that SRIs can cross the placenta (Hendrick et al., 2003), little is known about how SRI exposure leads to PPROM or preterm birth.

PPROM and subsequent preterm birth is thought to arise primarily from an insult in the form of either an infection or inflammation at the level of the fetal membranes (FMs), which then leads to an increase in the release of inflammatory cytokines, such as TNF-alpha (TNFα) and IL-1 beta (IL-1β). These cytokines in turn upregulate local mediators of membrane weakening, including prostaglandins and matrix metalloproteinases, and induce apoptosis (So et al., 1992; Arechavaleta-Velasco et al., 2002; Menon et al., 2002; Fortunato and Menon, 2003; Zaga-Clavellina et al., 2006; Li et al., 2007; Menon and Fortunato, 2007; Kumar et al., 2011). More recently, however, an alternative mechanism of cellular senescence was proposed as an underlying cause of PPROM (Dutta et al., 2016). Fetal membranes from women with PPROM and preterm birth, but not from women with spontaneous preterm birth in the absence of PPROM, had evidence of accelerated cellular senescence mediated by the stress kinase, phosphorylated (p)-p38 MAPK (Dutta et al., 2016). FM senescence is accompanied by the secretion of a unique pro-inflammatory signature known as a senescence-associated secretory phenotype (SASP) that contributes to membrane weakening (Menon, 2016; Menon et al., 2016). In addition, FM cellular aging is associated with elevated expression of the cell cycle inhibitors, p16 and p21; loss of the intermediate filament protein, Lamin B1; and reduced expression of ribosomal protein phosphorylated S6 (pS6) (Dutta et al., 2016; Menon, 2016; Menon et al., 2016; Gomez-Lopez et al., 2017). Thus, FM senescence or apoptosis may be underlying mechanisms for premature membrane weakening and rupture. In both pathways, the underlying mechanism appears to be inflammation.

While little is known about the biological effect of SRIs on human FMs, the commonly used SRI, fluoxetine (FLX), has been shown to upregulate p-p38 MAPK expression in hepatic cells (Mun et al., 2013). Since SRI exposure increases the risk of PPROM and preterm birth, the objective of the current study was to investigate the effect of FLX, a common and widely used SRI during pregnancy, on human FMs both *in vitro* and *in vivo*, and identify the mechanisms involved. Furthermore, since it is important to investigate solutions that may allow patients to continue taking SRIs during pregnancy, and recent studies reported that statins can reduce FM senescence and inflammation (Basraon et al., 2015; Ayad et al., 2018), we investigated whether statins could influence the impact of FLX on human FMs.

2. Materials and methods

2.1. Patient samples

Human FM tissue collection was approved by the Yale University's Human Research Protection Program (#0607001625). For all *in vitro* treatment experiments, human FMs were collected from uncomplicated term pregnancies (37–41 weeks' gestation) delivered by scheduled caesarean section, without evidence of labor or infection, and without maternal antidepressant use. For the analysis of FMs from women taking the SRI, FLX, human FMs were collected from uncomplicated term pregnancies (37–41 weeks' gestation) delivered by scheduled caesarean section, without evidence of labor or infection, but with exposure to FLX during their pregnancy. Each patient was paired with an unexposed control from women who did not have a history of psychiatric disorders and were not on any antidepressant or psychiatric medications during pregnancy. These too were collected from uncomplicated, term pregnancies (37–41 weeks' gestation) delivered by scheduled caesarean section, without evidence of labor or infection. FM tissues were collected at the time of delivery and were immediately washed, snap frozen and stored at −80 °C prior to analysis. Cases and controls were matched where possible for maternal age, gestational age, race/ethnicity and infant sex. Patient demographics are shown in Table 1. There were no significant differences in maternal or gestational ages.

2.2. Treatment of fetal membranes with fluoxetine

For all *in vitro* studies, we used an established FM explant system (Hoang et al., 2014; Cross et al., 2017; Tong et al., 2019; Miller et al., 2021). After washing with PBS supplemented with penicillin (100 U/ml) and streptomycin (100 μg/ml) (Life Technologies, Grand Island, NY), 6 mm FM explants were prepared, either keeping the chorion and amnion intact, or separating the chorion and amnion as described (Miller et al., 2021). The tissue explants were placed in 0.4 μm cell culture inserts, which were then placed in 24 well plates with 1 ml (500 μl in each chamber) of Dulbecco modified Eagle medium (DMEM; Life Technologies) with 10 % FBS overnight, prior to performing any experiments in serum-free media. The next day, the DMEM media was removed, the FMs were placed in serum-free OptiMeM (Life Technologies), and were treated with either media only (designated as no treatment (NT)) or FLX (1 nM) (Sigma Aldrich, St Louis, MI). After 48 hrs, cell-free supernatants and FM tissues were collected, tissues were snap frozen, and tissues and supernatants were stored at −80 °C. For some experiments, FMs were treated with NT or FLX in the presence of absence of either the high affinity and selective p38 MAPK inhibitor, SB203580 (1 μM; Selleckchem, Houston, TX), or the statins, simvastatin (200 ng/ml; Sigma Aldrich) or rosuvastatin (200 ng/ml; Sigma Aldrich) (Ayad et al., 2018). All treatments were performed either in duplicates or triplicates.

2.3. Analysis of fetal membrane supernatants for cytokines and chemokines

FM supernatants were measured for the following cytokines and chemokines by multiplex analysis (BioRad, Hercules, CA): G-CSF, GM-CSF, GRO-α, IL-1β, IL-6, IL-8, IL-10, IL-12, IL-17, IFN-γ, IP-10, MCP-1, MIP-1α, MIP-1β, RANTES, TNF-α and VEGF (Cross et al., 2017). Supernatants were also analyzed by ELISA for IL-1β, IL-6, IL-8 and G-CSF (R&D Systems, Minneapolis, MN).

2.4. Analysis of fetal membrane tissues by Western blot

FM tissues were homogenized for protein and concentrations measured using the Pierce BCA protein assay (ThermoFisher Scientific, Waltham, MA). Membranes were probed with the following primary antibodies all from Cell Signaling Technology (Danvers, MA): Lamin B1 (#13435; 1:1000); p16 (#92803; 1:1000); p21 (#2946; 1:2000); phosphorylated (p)-p38 MAPK (#9211; 1:400); total (t)-p38 MAPK (#9212; 1:1000); p-S6 ribosomal protein (#4858; 1:1000); t-S6 ribosomal protein (#2217; 1:1000); p-ERK (#9101; 1:1000); t-ERK (#4695; 1:1000); p-p65 NFκB (#3033: 1:1000); t-p65 NFkB (#8242; 1:1000); p-JNK (#9251; 1:500). β-Actin (A2066; 1:10,000; Sigma Aldrich) was used as a loading control. Chemiluminescence was detected using an Amersham Imager 680 (General Electric, Boston, MA) and semi-quantitative densitometry was performed using Image Studio Lite (Li-Cor Biosciences, Lincoln, NE).

Levels of phosphorylated protein were normalized against the total amount of that specific protein. For non- phosphorylated proteins,

Table 1
Demographic and clinical characteristics of the matched cases and controls for fluoxetine exposure during pregnancy.

Sample Pair	Maternal Age	Maternal Race	Maternal Ethnicity	Gestational Age	Infant Sex	FLX dose	FLX duration
1	36	White	Not-hispanic or Latino	40 + 2	Female	20–40 mg/day	0–8 + 0 & 26 + 0 - delivery
1	36	White	Not-hispanic or Latino	39 + 6	Female	–	–
2	37	White	Not-hispanic or Latino	39 + 2	Female	10 mg/day	Entire Pregnancy
2	37	White	Not-hispanic or Latino	39 + 0	Female	–	–
3	39	Black or AA	Hispanic or Latino	39 + 2	Male	10–20 mg/day	Entire Pregnancy
3	34	Black or AA	Not-hispanic or Latino	38 + 2	Female	–	–
4	27	Asian	Not-hispanic or Latino	40 + 6	Male	10 mg/day	Entire Pregnancy
4	28	Asian	Not-hispanic or Latino	39 + 4	Male	–	–
5	40	White	Not-hispanic or Latino	39 + 0	Male	10 mg/day	20 + 2 - delivery
5	43	White	Not-hispanic or Latino	39 + 0	Male	–	–
6	34	White	Not-hispanic or Latino	39 + 1	Female	10 mg/day	Entire Pregnancy
6	36	White	Not-hispanic or Latino	39 + 0	Female	–	–
7	38	White	Not-hispanic or Latino	39 + 0	Male	20 mg/day	Entire Pregnancy
7	38	White	Not-hispanic or Latino	39 + 5	Male	–	–
8	34	White	Not-hispanic or Latino	40 + 5	Male	20 mg/day	Entire Pregnancy
8	31	White	Not-hispanic or Latino	40 + 4	Male	–	–
9	39	White	Not-hispanic or Latino	39 + 0	Male	15–30 mg/day	Entire Pregnancy
9	37	White	Not-hispanic or Latino	39 + 0	Male	–	–
10	38	White	Not-hispanic or Latino	38 + 0	Female	20 mg/day	24 + 4 - delivery
10	36	White	Not-hispanic or Latino	39 + 0	Male	–	–
11	26	White	Not-hispanic or Latino	37 + 2	Male	20 mg/day	Entire Pregnancy
11	26	White	Not-hispanic or Latino	39 + 0	Male	–	–
12	40	White	Not-hispanic or Latino	39 + 6	Female	10 mg/day	Entire Pregnancy
12	41	White	Not-hispanic or Latino	39 + 0	Female	–	–
13	29	White	Not-hispanic or Latino	39 + 0	Male	40 mg/day	Entire Pregnancy
13	29	White	Not-hispanic or Latino	39 + 0	Male	–	–

normalization was performed against the signal for β-actin.

2.5. *Measurement of fetal membrane apoptosis*

FM lysates were measured for caspase-3 activity using the Caspase-Glo assay (Promega, Madison, WI). Activity levels were measured using a D-20/20 luminometer (Turner Designs, Sunnyvale, CA) and were recorded as relative light units (RLU).

2.6. *Statistical analysis*

Each *in vitro* experiment represents an individual patient FM. All data are reported as mean ± standard error of the mean (SEM). Statistical significance ($p < 0.05$) was determined by performing, for normalized data, a paired t-test, or if not normally distributed, the Wilcoxon matched-pairs signed rank test, using Prism Software (Graphpad, Inc, La Jolla, CA).

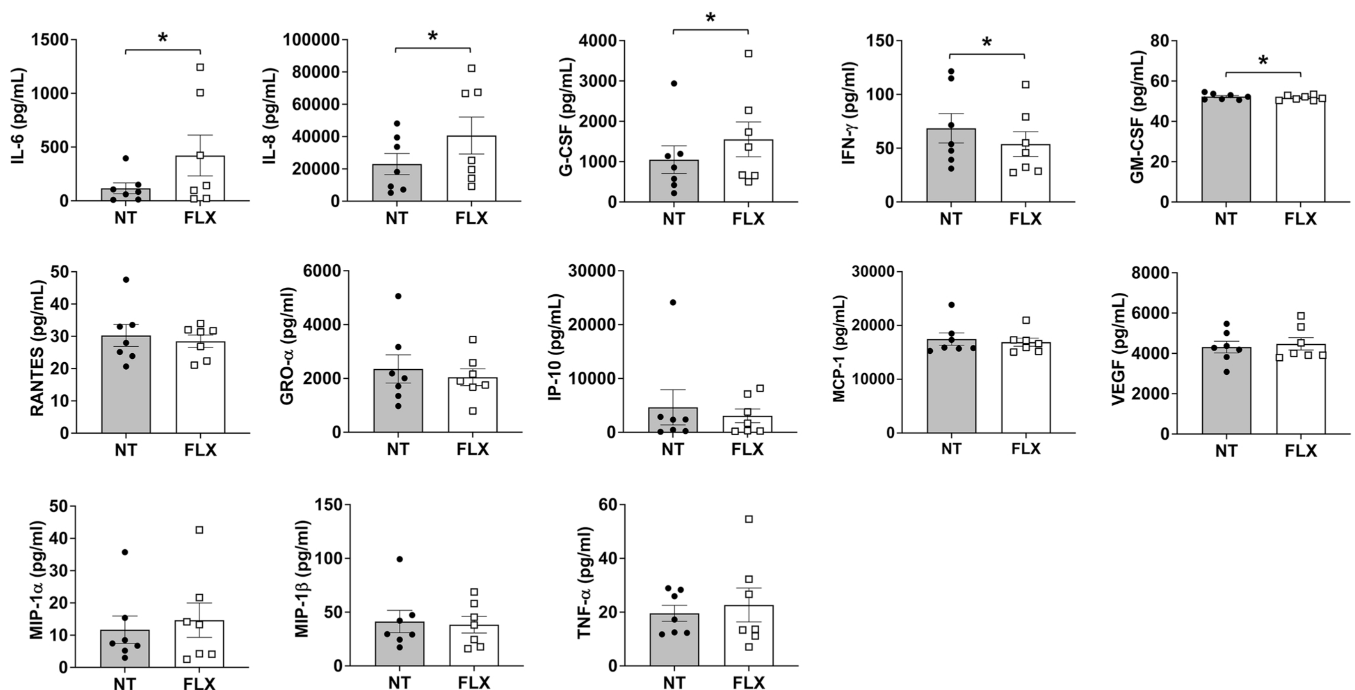

Fig. 1. Fluoxetine increases human fetal membrane inflammatory IL-6, IL-8 and G-CSF secretion. Intact human FM explants from 7 patients were treated with no treatment (NT) or fluoxetine (FLX, 1 nM). After 48hrs, cell-free supernatants were collected and analyzed by multiplex analysis ($p < 0.05$).

3. Results

3.1. Fluoxetine increases human fetal membrane inflammatory IL-6, IL-8 and G-CSF secretion

To investigate the effect of FLX on human FM inflammation, multiplex analysis was used to assess an array of cytokines and chemokines. As shown in Fig. 1, FM secretion of IL-6, IL-8, and G-CSF were significantly increased following FLX exposure by 3.5 ± 1.1-fold, 1.9 ± 0.3-fold, and 1.6 ± 0.2-fold, respectively, when compared to the no treatment (NT) media control ($p < 0.05$). FLX exposure also slightly but significantly reduced the secretion of IFN-γ and GM-CSF ($p < 0.05$) (Fig. 1). FM secretion of RANTES, GRO-α, IP-10, MCP-1, VEGF, MIP-1α, MIP-1β, and TNF-α were not significantly different under NT and FLX conditions (Fig. 1). IL-1β, IL-10, IL-12, and IL-17 were under the detection limit of the assay (data not shown). By ELISA, IL-1β secretion was detectable at similarly low levels under both NT and FLX conditions (NT: 0.91 ± 0.5 pg/ml *vs.* FLX: 0.76 ± 0.4 pg/ml).

3.2. Fluoxetine increases chorion, but not amnion, IL-6, IL-8, and G-CSF secretion

To determine which FM compartment was responsible for generating the inflammatory cytokine/chemokine response after exposure to FLX, isolated chorion and amnion tissues were treated separately, and the supernatants evaluated by ELISA. As shown in Fig. 2, FLX significantly increased chorion secretion of IL-6 by 2.1 ± 0.5 fold, IL-8 by 1.7 ± 0.2 fold, and G-CSF by 2.9 ± 0.7 fold compared to the NT control ($p < 0.05$). In contrast, FLX treatment did not significantly change the levels of IL-6, IL-8 and G-CSF secreted by the amnion (Fig. 2). Under all conditions, the chorion secreted significantly higher levels of IL-6, IL-8 and G-CSF than the amnion under both NT and FLX conditions ($p < 0.05$) (Fig. 2).

3.3. Fluoxetine increases human fetal membrane p38-MAPK activity but does not induce senescence or apoptosis

Since FM apoptosis (Menon et al., 2002; Fortunato and Menon, 2003; Luo et al., 2010) and p-38 MAPK-mediated senescence (Dutta et al., 2016) were described as possible mechanisms underlying PPROM, these pathways were studied in FM explants exposed to FLX. To investigate senescence, FM expression of key markers were evaluated by Western Blot. As shown in Fig. 3A, while there was a 1.5.± 0.2 fold increase in p-p38/t-p38 MAPK expression in FMs treated with FLX when compared to the NT control ($p < 0.05$), there was no significant difference in the expression levels of Lamin B1, p16 and p-S6/t-S6 (Fig. 3A). p21 expression was undetectable (data not shown). To then further investigate the possibility that FLX was inducing p38 MAPK activity as part of an inflammatory FM response in the absence of senescence, other inflammatory signaling pathways were also examined. Treatment of FMs explants with FLX had no significant effect on the expression levels of p-p65/t-p65 NFκB or p-ERK/t-ERK (Fig. 3A), and p-JNK was undetectable under all conditions (data not shown). To investigate whether FLX was inducing FM apoptosis, levels of caspase-3 activity was measured. As shown in Fig. 3B, FLX had no significant effect on the levels of active caspase-3 compared to the NT control.

3.4. Fluoxetine exposure during pregnancy correlates with elevated fetal membrane p38 MAPK activity

Having found that FM explants exposed to FLX *in vitro* exhibited elevated p38 MAPK activation, we sought to validate this *in vivo* using clinical samples (Table 1). For this study we chose to examine FMs from women unexposed or exposed to FLX during pregnancy that were collected at term from uncomplicated pregnancies in the absence of labor so that we would be able to uncouple the effects of the SRI medication from inflammatory signals of parturition or PPROM/preterm birth. As shown in Fig. 3C, FMs from women exposed to FLX during pregnancy expressed significantly higher levels of p-p38/t-p38 MAPK when compared to FMs from unexposed controls ($p < 0.05$).

3.5. p38MAPK regulates fluoxetine-induced fetal membrane IL-6 secretion

Since we found that p-p38 MAPK was significantly increased in FMs treated with FLX *in vitro*, we sought to test whether this was responsible for mediating the SRI-induced FM inflammation using the inhibitor SB203580. As shown in Fig. 4, FLX significantly increased FM chorionic secretion of IL-6 by 1.4 ± 0.2 fold ($p < 0.05$) and the presence of SB203580 significantly inhibited this FLX-induced IL-6 secretion by 45.8 ± 8.9 % ($p < 0.05$). In contrast to our earlier findings, while FLX increased FM chorionic secretion of IL-8 and G-CSF by 1.3 ± 0.2 and 3.9 ± 1.7 fold, respectively, significance was not reached. Further, the presence of SB203580 did not significantly alter FM IL-8 and G-CSF levels (Fig. 4).

3.6. Statins do not prevent fluoxetine-induced fetal membrane inflammation

The statins, simvastatin and rosuvastatin, have been shown to reduce human FM p38 MAPK-associated senescence and SASPs in response to cigarette smoke extract (CSE)-induced oxidative stress (Ayad et al., 2018), and simvastatin was reported to reduce lipopolysaccharide (LPS)-induced FM inflammation (Basraon et al., 2015). Therefore, we sought to determine if these statins could prevent FLX-induced FM inflammation. As shown in Fig. 5 FLX significantly increased FM chorionic secretion of IL-6 by 2.8 ± 1.5 fold ($p < 0.05$). Neither simvastatin

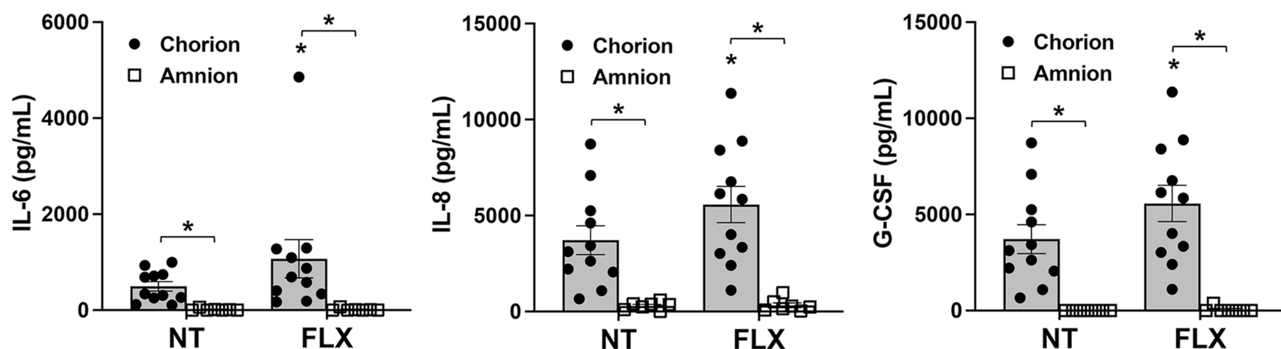

Fig. 2. Fluoxetine increases chorion, but not amnion, IL-6, IL-8 and G-CSF secretion. FM explants separated into the chorion and amnion compartments from 7 to 11 patients and were treated with no treatment (NT) or fluoxetine (FLX, 1 nM) for 48hrs. Cell-free supernatants were collected and analyzed by ELISA for IL-6, IL-8 and G-CSF. $p < 0.05$ relative to the NT control unless otherwise indicated.

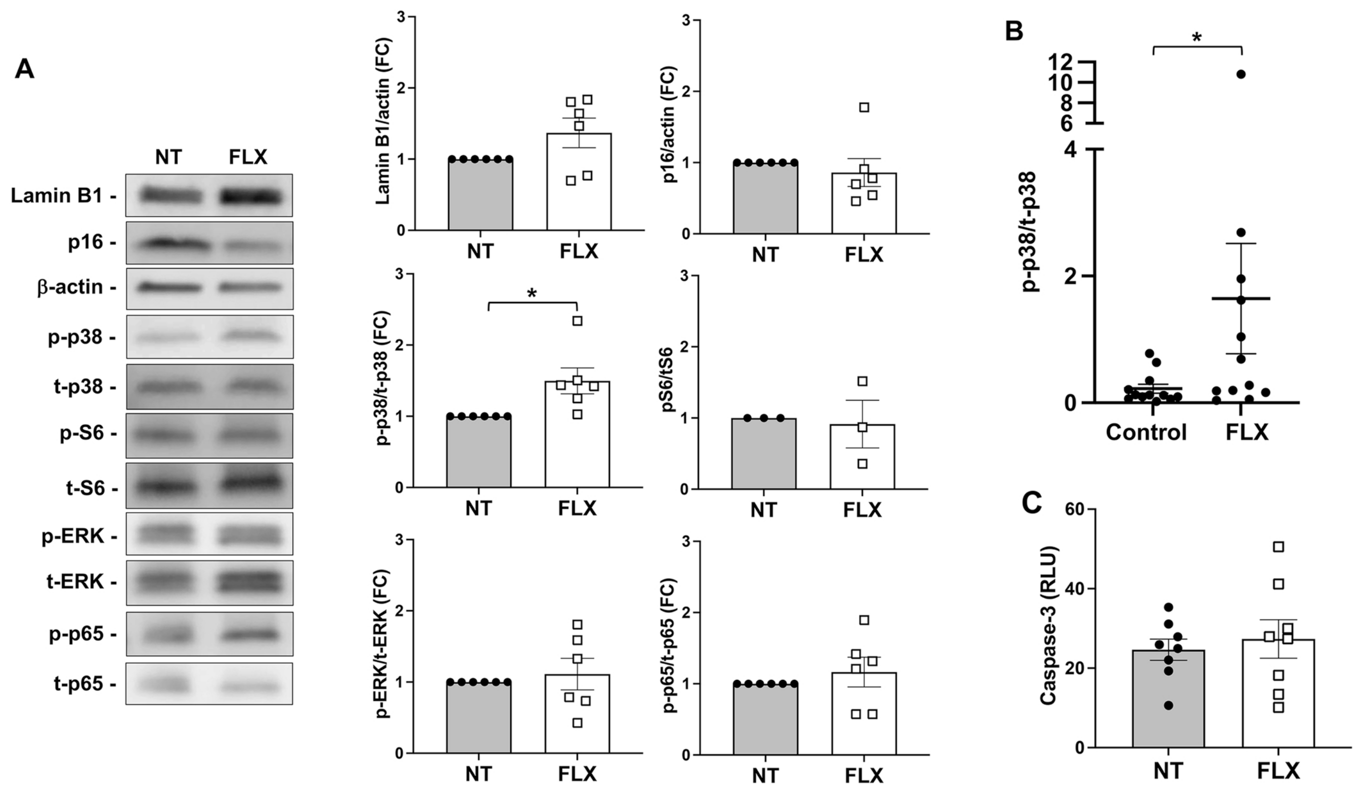

Fig. 3. Fluoxetine increases human fetal membrane p38-MAPK activity but does not induce senescence or apoptosis. (A) FM chorion explants from 3 to 6 patients were treated with no treatment (NT) or fluoxetine (FLX, 1 nM) for 48hrs after which tissues were homogenized for protein and Western blot performed. Images are from representative blots. Bar charts show quantification of protein expression as determined by densitometry ($p < 0.05$; FC= fold change). (B) FM explants from 8 patients and were treated with NT or FLX for 48hrs after which tissues were homogenized for protein and caspase-3 activity measured and recorded as relative light units (RLU). (C) FM tissue collected at the time of delivery from women either unexposed (control; n = 13) or exposed to FLX during pregnancy (n = 13) were homogenized for protein and Western blot performed for p-p38 MAPK and t-p38 MAPK expression. Chart shows the ratio of p-p38/t-p38 MAPK as determined by densitometry. Each dot/square represents a patient ($p < 0.05$).

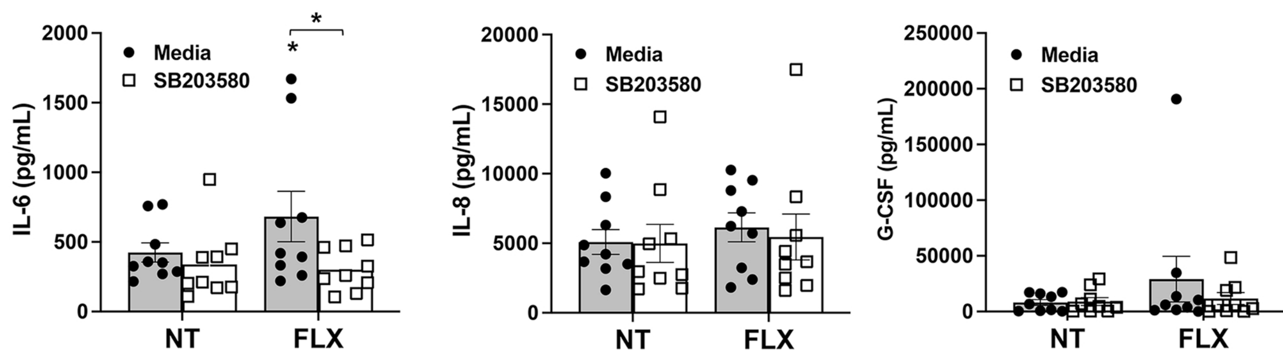

Fig. 4. p38MAPK regulates fluoxetine-induced chorionic IL-6 secretion. FM chorion explants from 9 patients were treated with no treatment (NT) or fluoxetine (FLX, 1 nM) in the presence of either media or the p38 MAPK inhibitor, SB203580. After 48 hr cell-free supernatants were collected and analyzed by ELISA for IL-6, IL-8 and G-CSF. $p < 0.05$ relative to the NT control unless otherwise indicated.

nor rosuvastatin reduced FLX-induced chorionic secretion of IL-6. However, simvastatin did significantly elevate FM chorionic secretion of IL-6 ($p < 0.05$) (Fig. 5). In contrast to our earlier findings, while FLX increased FM chorionic secretion of IL-8 and G-CSF by 1.5 ± 0.3 and 1.6 ± 0.4 fold, respectively, significance again was not reached. Rosuvastatin did not alter these levels, but simvastatin raised G-CSF levels under FLX conditions ($p < 0.05$) (Fig. 5).

4. Discussion

Antidepressant use in pregnancy is associated with an increased risk of PPROM and preterm birth (Roca et al., 2011; Yonkers et al., 2012; Huang et al., 2014). Despite antidepressants being the most commonly class of prescription medication used during pregnancy, and the widespread concerns about their use in pregnancy, few studies have attempted to understand the impact of SRIs on pregnancy, biologically or mechanistically. Mood and anxiety disorders have a peak age of onset in women during their reproductive years (Hasin et al., 2005). Pregnant women with a psychiatric illness requiring treatment are often faced with the difficult decision of stopping medication and risking relapse, or continuing medication and potentially increasing the risk of adverse outcomes for themselves and/or their fetus (Cohen et al., 2006). Indeed,

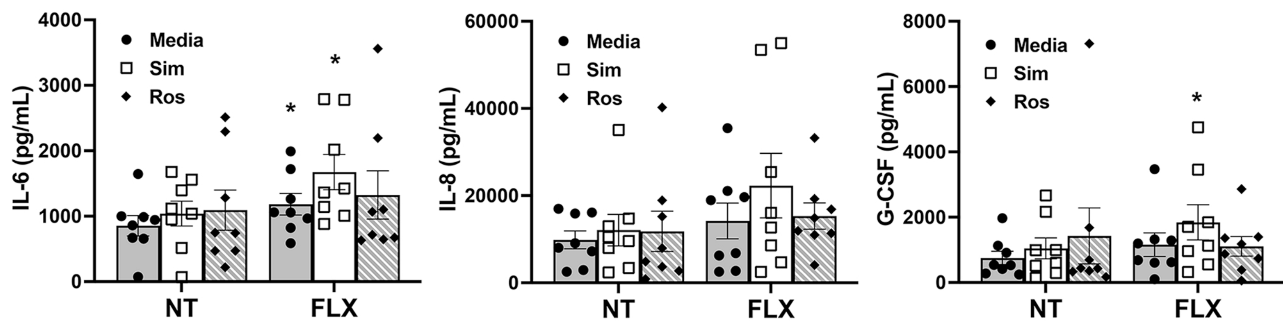

Fig. 5. Statins do not prevent fluoxetine-induced fetal membrane inflammation. FM chorion explants from 7 patients were treated with no treatment (NT) or fluoxetine (FLX, 1 nM) in the presence of either media, simvastatin (Sim) or rosuvastatin (Ros). After 48 hr cell-free supernatants were collected and analyzed by ELISA for IL-6, IL-8 and G-CSF. $p < 0.05$ relative to the NT/media control.

one of the most common reasons that women call the teratogen information service is for information about SRI use during pregnancy (Einarson et al., 2012). There is, therefore, an urgent need for translational and clinical research to help patients and providers faced with these difficult decisions. Given that some women need antidepressant treatment during pregnancy (Cohen et al., 2006), it is critical to know how these medications impact gestational tissues such as the FMs and whether we can prevent these adverse effects.

Although PPROM and preterm birth is common in the setting of infection, a significant proportion of such pregnancies have no clinical or histological evidence of infection (Anon, 2007). Rather inflammatory intermediaries may cause the tissue injury that promote membrane weakening and rupture. Recent studies found that PPROM is associated with accelerated cellular senescence and a unique inflammatory signature, known as a senescence-associated secretory phenotype (SASP), while other studies have correlated PPROM with increased fetal membrane apoptosis (Menon et al., 2002; Fortunato and Menon, 2003; Luo et al., 2010; Dutta et al., 2016; Menon, 2016; Menon et al., 2016). Thus, even in the absence of infection, FM senescence, apoptosis and associated inflammation may be an underlying mechanism of PPROM; and similar processes may underlie PPROM in women exposed to SRIs. In this current study we found that the commonly use SRI, FLX, induced FM sterile inflammation through activation of the p38 MAPK pathway. Although there are other SRIs, such as sertraline and citalopram, we focused on FLX, as one of the more common and widely used SRI during pregnancy.

Using an established *in vitro* system (Hoang et al., 2014; Cross et al., 2017; Tong et al., 2019; Miller et al., 2021) combined with multiplex analysis, we found that the SRI, FLX, induced a FM inflammatory response characterized by elevated IL-6, IL-8, and G-CSF. Elevated IL-6 and IL-8 have both been strongly associated with PPROM and preterm birth (Mitchell et al., 1991; Hagberg et al., 2005). Validation studies using ELISA also demonstrated that the chorion, rather than the amnion, was the FM compartment responsible for responding to FLX exposure by secreting elevated levels of these inflammatory factors. This is consistent with what has been reported in other treatment models (Miller et al., 2021). While the inflammatory mediators, TNF-α and IL-1β are known drivers of FM apoptosis and weakening (So et al., 1992; Arechavaleta-Velasco et al., 2002; Menon et al., 2002; Fortunato and Menon, 2003; Zaga-Clavellina et al., 2006; Li et al., 2007; Menon and Fortunato, 2007; Kumar et al., 2011), FLX did not increase their production in our model system. Furthermore, FLX had no effect on FM apoptosis, indicating that this pathological pathway may not be induced in FMs by SRIs. While we were not able identify other studies that examined the effect of SRIs on FMs, our findings contrast with studies using placental explants and placental trophoblast cells. One study reported that FLX did not affect placental IL-6 production (Clementelli et al., 2021), while two other studies reported reduced trophoblast cell proliferation/viability and elevated apoptosis in response to SRIs, all at significantly higher concentrations than we used in our studies (Correia-Branco et al., 2019; Nabekura et al., 2022).

Since the FM inflammatory signature produced by FMs after exposure to FLX better represented a senescence-associated secretory phenotype (SASP) (Menon, 2016; Menon et al., 2016), we looked to see whether there was evidence of accelerated cellular senescence using p-p38 MAPK, a mediator of cellular senescence in human FMs (Dutta et al., 2016). Exposure of FMs to FLX did indeed increase p38 MAPK activation suggesting that FM senescence may be induced. However, expression patterns of other markers of senescence that were tested (Lamin B1, p16, p21 and p-S6/t-S6) were unchanged by FLX exposure and, thus, not consistent with the current literature (Dutta et al., 2016; Menon, 2016; Menon et al., 2016; Gomez-Lopez et al., 2017). We validated our observation of elevated p38 MAPK activity in FLX-treated FMs *in vitro*, using clinical samples. To this end, we examined p38 MAPK activity in FMs collected at delivery from uncomplicated pregnancies without labor to ensure that our data was not confounded by the inflammatory signals associated with parturition. Similar to our *in vitro* findings, FMs from women exposed to FLX during pregnancy expressed higher levels of p38 MAPK activity when compared to matched unexposed controls. Together, our findings suggest that FLX induces FM p38 MAPK activation and subsequent sterile inflammation in the absence of apoptosis and senescence. To examine this further, the role of p38-MAPK in mediating SRI-induced inflammation was tested using a specific inhibitor. Inhibition of p38 MAPK activity only reduced FM IL-6 secretion in response to FLX. Further, while IL-6 secretion was consistently found to be significantly elevated in FMs in response to FLX, the secretion of IL-8 and G-CSF in this set of experiments although trended towards being elevated with FLX treatment, did not reach significance, and we believe this to be due to greater spread in our untreated controls and patient variability.

Although there is currently no general way to prevent PPROM and preterm birth, there is a need to investigate novel therapeutic targets. Furthermore, investigating ways to reduce the risk of PPROM and preterm birth in women taking SRIs can allow patients to continue their antidepressant treatment during pregnancy. *In vitro*, the statins, simvastatin and rosuvastatin, have been shown to reduce human FM p38 MAPK-associated senescence and SASPs in response to CSE-induced oxidative stress (Ayad et al., 2018) and simvastatin has been shown to reduce LPS-induced FM inflammation (Basraon et al., 2015). Simvastatin also reduced inflammation and preterm birth in a mouse model of LPS-induced preterm birth (Gonzalez et al., 2014; Boyle et al., 2019). Since we found that FLX induced FM IL-6 secretion *via* p38 MAPK activation, and given recent clinical studies that have challenged safety concerns about statin use in pregnancy (Winterfeld et al., 2013; Costantine et al., 2016), we tested these statins in our *in vitro* system. However, unlike the studies using LPS or CSE, FLX-induced FM IL-6 was not reduced by either simvastatin or rosuvastatin and thus, these medications may not be useful at preventing adverse outcomes in pregnant

women taking SRIs.

In summary, exposure to the SRI antidepressant FLX during pregnancy may be associated with elevated FM p38MAPK activation and sterile inflammation, in particular IL-6 production. This may lead to an increased risk of PPROM and subsequent preterm birth. These findings may help clinicians and patients decide the best antidepressant to use in pregnancy in order to optimize their psychiatric treatment.

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