The Link Between Perinatal Depression and DNA Methylation of the Oxytocin Receptor Gene: A Study of Mothers and their Children

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Contributions

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Abstract

Background: There is mounting evidence to suggest that exposure to stress during critical periods of development may increase one's susceptibility to disease risk, including depression. Perinatal depression (PD) may not only be associated with adverse outcomes among the mothers experiencing it, but also for the children exposed to PD, via epigenetic programming. We sought to investigate how PD may be associated with certain epigenetic profiles among mother and child dyads. Specifically, we focused on the oxytocin receptor (OXTR) gene due to oxytocin's role in maternal behavior and stress regulation. It is hypothesized that increased OXTR methylation will be observed for those mothers who experience PD and among those children exposed to PD. **Methods:** Using longitudinal data from a cohort of mothers and their infants, we collected information about the mothers' depressive symptomology across 5 time points (twice during pregnancy and three times during the postpartum period). Depressive symptomology was assessed using the Edinburgh Postnatal Depression Scale (EPDS) and four groups were established based on EPDS cut-off scores: no PD, prenatal or postpartum depression only and persistent PD (depression both prenatally and postpartum). DNA methylation was assessed using saliva obtained from both mother and child at the last time point – 2.9 years postpartum, for which the OXTR gene was interrogated for methylation patterns. **Results:** Those mothers with persistent PD had significantly higher OXTR methylation overall compared to the other groups. Specifically, 9 of the 22 CpG sites interrogated were significantly higher for the persistent PD group. However, average OXTR methylation among those children who were exposed to their mothers' persistent PD were not significantly different when compared to the other groups, with none of the 22 sites investigated within the exon 3 region showing significantly different methylation patterns. **Discussion:** For mothers, only persistent PD was associated with significantly higher OXTR methylation suggesting that more transient episodes of depression may not impact the epigenome to the same degree. The children's methylation profiles were not consistently associated with their mothers' depression. Given the link found between persistent PD and OXTR methylation among mothers, the combined psychological and epigenetic risk may have implications for the oxytocin system as well as compromise maternal behavior which may result in negative outcomes for the developing child.

Résumé

Contexte: Il existe des preuves qui suggèrent que l'exposition au stress pendant les périodes critiques du développement peut augmenter la sensibilité au maladie, y compris la dépression. La dépression périnatale (DP) peut être associée à des effets indésirables non seulement chez les mères, mais aussi chez leurs enfants exposés à la PD, via la programmation épigénétique. Nous avons cherché à étudier comment la DP peut être associée à certains profils épigénétiques entre les dyades mère-enfant. Plus précisément, nous nous sommes concentrés sur le gène du récepteur de l'ocytocine (OXTR) en raison du rôle de l'ocytocine dans le comportement maternel et la régulation du stress. Notre hypothèse est que l'augmentation de la méthylation de l'OXTR sera observée pour les mères qui vivent DP ainsi que chez leurs enfants exposés à la DP. **Méthodes:** Pour obtenir des données longitudinales d'une cohorte de mères et de leurs enfants, nous avons recueilli des informations sur la symptomatologie dépressive des mères à 5 moments différents (2 fois pendant la grossesse et 3 fois au cours de la période post-partum). La symptomatologie dépressive a été évaluée en utilisant l'échelle de dépression postnatale d'Edinbourg (EDPE) et 4 groupes ont été créés selon les valeurs limites du EPDE: pas de DP, dépression prénatale ou post-partum seule et DP persistante (dépression à la fois prénatale et post-partum). La méthylation de l'ADN a été évaluée en utilisant la salive obtenue de la mère et de l'enfant au dernier point de mesure - 2,9 années post-partum – pour lequel les profils de méthylation de plusieurs sites CpG du OXTR ont été étudiés. **Résultats**: Les mères avec DP persistante montraient de façon significative une méthylation du OXTR plus élevé en moyenne par rapport aux autres groupes. Plus précisément, 9 des 22 sites CpG étudiés étaient significativement plus méthylés pour le groupe à DP persistante. Cependant, le degré de méthylation du OXTR chez les enfants qui ont été exposés à la DP persistante de leurs mères ne sont pas significativement différente par rapport aux autres groupes, avec aucun des 22 sites étudiés dans la région exon 3 ne montrant une différence de méthylation significative. **Discussion:** Pour les mères, seulement celles avec la DP persistante ont été associée à un taux de méthylation significativement plus élevée de OXTR suggérant que les épisodes plus transitoires de dépression pourraient ne pas influer l'épigénome de la même manière. Aucun profil de méthylation n'a été retrouvé de façon consistante chez les enfants exposés à la DP de leur mère. Compte-tenu de la corrélation établie entre un taux plus élevé de méthylation du OXTR et les mères souffrantes de DP persistante, le risque psychologique et épigénétique combiné pourrait avoir des implications pour le système

ocytocine et pourrait compromettre le comportement maternel ce qui pourrait entraîner des conséquences négatives pour l'enfant en développement.

Introduction

Perinatal depression (depression around the time of pregnancy) is a condition that not only affects the expectant mother, but can also have implications for the developing child. Perinatal depression (PD) is often associated with poorer maternal behavior in the postnatal period which may have long-term impacts on the child, potentially increasing his/her risk for subsequent psychopathology. Epigenetic mechanisms may be partly responsible for programming these long-lasting effects as they are a dynamic and ongoing biological process which reflect gene-environment interactions and are particularly receptive to environmental signals during critical periods early in development. In the current study, we assessed the methylation patterns in both mother and children by targeting relevant sites within the CpG island of the oxytocin receptor (OXTR) gene since the oxytocin system is implicated in stress regulation and maternal behavior. Specifically, at 2.9 years postpartum, we collected saliva from 221 mother and child dyads to investigate potential methylation patterns that may have emerged from exposure to PD at several time-points during pregnancy and postpartum. PD symptoms were assessed using the Edinburgh Postnatal Scale, whereby we examined OXTR methylation in relation to: 1) women who had no PD; 2) women who were prenatally depressed; 3) women who had postpartum depression; and 4) women who had persistent PD (prenatal and postpartum depression). The methylation patterns of their children were also investigated. This is the first study to examine OXTR methylation in relation to not only the mother and child pair, but also in relation to depressive symptoms experienced over the entire perinatal period.

Background

Early life stress (ELS) has been shown to have a substantial impact on neural, cognitive and affective functioning potentially exerting long-lasting effects on the brain and behavior (Auger & Auger, 2013; Barrett & St Pierre, 2011). Early life typically comprises significant periods of development ranging from the prenatal period to adolescence (Marsh, Gerber, & Peterson, 2008; Paus et al., 1999) while ELS can encompass events ranging from childhood maltreatment (including physical and/or sexual abuse) and parental neglect to famine, war and exposure to domestic violence and parental psychopathology (Lutz & Turecki, 2014). The enduring effects of ELS are likely due to the fact that early life is a critical period for brain development, making the nervous system particularly receptive to incoming signals from its environment thereby "programming" neural pathways in response to these signals (Douglas, 2011; Reynolds, Labad, Buss, Ghaemmaghami, & Raikkonen, 2013; Stiles & Jernigan, 2010). Specifically, ELS has been linked to structural changes to the limbic system (including the hippocampus, amygdala and anterior cingulate gyrus) and other circuitry systems such as the hypothalamic-pituitary-adrenal (HPA) axis (Buss et al., 2007; Gonzalez, 2013; Vela, 2014). Alterations to these systems are known to have implications for subsequent behavioural and psychological development, including mental illness, given their role in emotional regulation and stress reactivity.

Since early life can include the period in utero as well as the postpartum period, the maternal environment[1] has been implicated as one of the factors shaping early life experience. For this reason, perinatal depression has been the subject of emerging research in the field of ELS and vulnerability to disease. Perinatal depression (PD) refers to a major depressive episode

[1] In this context, the maternal environment refers to the uterine environment as well as maternal behavior and the various influences that may directly impact these factors.

occurring during pregnancy and/or up to 6 months after childbirth (American Psychiatric Association, 2013). PD occurs in 10-15% of pregnant women (Gavin et al., 2005) and is more likely to occur among those who lack social support or who suffer from marital dissatisfaction (Beck, 2001) as well as circumstances of financial strain (Tannous, Gigante, Fuchs, & Busnello, 2008). Since PD usually corresponds to less attuned maternal care in the postpartum period, including reduced maternal sensitivity, responsiveness and attachment (Baram et al., 2012; Campbell, Matestic, von Stauffenberg, Mohan, & Kirchner, 2007; Perry, Ettinger, Mendelson, & Le, 2011), the development of the infant could be compromised (Narayanan & Naerde, 2016; Sirvinskiene, Zemaitiene, Jusiene, & Markuniene, 2016). The maternal environment, both in utero and postpartum, is critical not only because it constitutes the infant's first experiences, but because the establishment of neural networks and pathways are very much influenced by the mother's nutritional intake, level of stress and, in the postpartum period, her caretaking of the infant (Szyf, Weaver, & Meaney, 2007).

Animal Studies

Although not directly comparable, animal studies have shed some light on how ELS and maternal care can affect offspring. Animal models of prenatal depression (e.g., gestational stress[2]) and ELS (e.g., maternal separation) as well as variations in maternal care, have shown that the effects of such experiences are long-lasting and may explain individual differences in stress reactivity later in life (Meaney, 2001). Several studies have demonstrated that pups born to mothers who have experienced gestational stress exhibit increased anxiety (as measured by the open-field exploration test) (F. A. Champagne & Meaney, 2006), a depressive-like phenotype (as measured by the forced swimming task) (Alonso, Arevalo, Afonso, & Rodriguez, 1991) and/or neurological changes such as decreased dendritic spine density (Murmu et al., 2006), compared

[2] Some examples of gestational stress include physical restraint, food scarcity and the introduction of an intruder.

to those whose mothers did not undergo stress during pregnancy (Babenko, Kovalchuk, & Metz, 2015).

Another commonly used paradigm in animal studies is maternal separation, whereby newborn pups are separated from their mothers (for 1 to 24-hour periods over several days) shortly after birth. Maternal separation is inevitably accompanied by food shortage, a lack of maternal stimulation and a reduction in body temperature. It appears that maternal separation not only results in a more hyperactive stress response and increased gene expression of corticotropin-releasing factors (CRF) in the short-term, but it can also be associated with reductions in maternal care among those female pups who later become mothers themselves (D. D. Francis, Diorio, Plotsky, & Meaney, 2002; Lovic, Gonzalez, & Fleming, 2001). Moreover, the effects of maternal separation can be seen up to 2 generations later (Franklin et al., 2010).

In terms of postnatal care, Meaney and colleagues have demonstrated that when pups receive poor maternal care (which is characterized as low licking and grooming behavior), they exhibit heightened stress reactivity and compromised immune systems (Caldji, Diorio, & Meaney, 2000). Although variations in maternal care and stress reactivity seem to be transmitted from one generation to the next via the neural systems[3] that regulate those behaviours (F. Champagne & Meaney, 2001), this effect can be reversed with cross-fostering, suggesting that postnatal maternal care can override inter-generational predispositions (D. Francis et al., 1999).

Human Studies

Maternal separation and experiments involving gestational stress are not possible in human studies for ethical reasons. However, naturally occurring stressful events can reveal

[3] The neural systems implicated in this particular study are hypothalamic corticotropic releasing hormone (CRH) levels and hippocampal glucocorticoid receptor mRNA expression (D. Francis, Diorio, Liu, & Meaney, 1999).

information about the long-term effects of stress, including prenatal maternal stress[4]. The work of Dr. Suzanne King documents how pregnant women and their later-to-be-born infants are impacted by natural disasters – in particular, the ice storm that affected about 3 million people in southern Quebec. The ice storm left many residents without power and heat for days and in some cases weeks, which caused some families to relocate to temporary shelters. For Project Ice Storm, 224 women who were pregnant or who became pregnant shortly after the ice storm were recruited during the winter of 1998. Researchers followed up with these mothers and their infants for several years following the natural disaster. At 2 years of age, lower scores on tests of language and cognitive ability were observed among those children whose mothers reported high objective[5] stress and were in their first to second trimester of pregnancy during the ice storm (King & Laplante, 2005). Similar cognitive and linguistic profiles were found for the same cohort about 3 years later when the children were 5 ½ years-old (Laplante, Brunet, Schmitz, Ciampi, & King, 2008), suggesting that exposure to objective stress while in utero may have long-term negative consequences in terms of the child's cognitive development. However, these same studies discovered that subjective stress which in this case, can be defined as the mother's perceived stress in response to the damage inflicted by the ice storm, did not have an effect on the children's cognitive development (King, Dancause, Turcotte-Tremblay, Veru, & Laplante, 2012).

Other research suggests that mothers who experience subjective stress (e.g., psychosocial stressors such as marital dissatisfaction, financial hardship and a lack of social support) during pregnancy, may put the developing fetus at a higher risk for negative neonatal outcomes (Talge et al., 2007). For example, prenatal subjective stress has been associated with a higher likelihood

[4] Prenatal maternal stress refers to an expectant mother's exposure to distress.
[5] Objective stress refers to stressors such as days without power and heat, injuries directly attributed to the ice storm, damage to the home due to the storm, loss of income, etc.

of preterm birth (Wadhwa, Sandman, & Garite, 2001), low birth weight (Hoffman & Hatch, 2000), alterations in certain brain structures in the infant (Rifkin-Graboi et al., 2013) and hormonal profiles in the infant that resemble those of the mother (e.g., higher cortisol levels) (Field, Diego, & Hernandez-Reif, 2006). Likewise, PD has also been associated with the same negative neonatal outcomes listed above (Jarde et al., 2016). In some cases, PD has been associated with obstetric complications such as preeclampsia (Kurki, Hiilesmaa, Raitasalo, Mattila, & Ylikorkala, 2000), placental defects (Jablensky, Morgan, Zubrick, Bower, & Yellachich, 2005) and spontaneous abortion (Nakano et al., 2004). More commonly, PD can contribute to poorer maternal care in the postnatal period as depressed mothers may have trouble bonding with their child, soothing them or responding to their needs (Campbell et al., 2007; Field, 2010). Due to the impact of PD on maternal care, combined with the potential long-term effects of PD on the developing fetus, PD has been identified as a risk factor for behavioral problems in children, including conduct disorders (Bowen et al., 2014) as well as learning difficulties and impaired emotional regulation (Pariante, 2014). Moreover, PD may contribute to poorer immune function (Kaminsky et al., 2012), increased sensitivity to stress later in life (Babenko et al., 2015), higher risk for depression (Pearson et al., 2013), and maladaptive social behaviours in the developing child (Rubin et al., 2016). Given the serious long-term implications of PD for both the mother and child, it would be worthwhile to better understand some of the biological mechanisms underlying PD and in doing so, potential biomarkers could be identified which could help confirm diagnoses and guide appropriate treatment for PD.

Although research to identify such mechanisms is still ongoing, there is growing attention to certain biomarkers and molecular mechanisms that may help to explain the neuroscience of depression and susceptibility to disease. The activity of the HPA axis has been proposed as one

possible mechanism due to its central role in regulating the stress response. In response to stress, the HPA axis carries out an integrative series of actions by releasing certain bio-molecules (e.g., corticotropin-releasing hormone and cortisol) into circulation (Nelson, 2005). It is hypothesized that exposure to chronic stress forces the HPA axis into overdrive and the long-term consequences of this are: impaired immune function, suppressed growth and in some cases, depression (Bao, Meynen, & Swaab, 2008; Sapolsky, 2004). In fact, an overactive HPA axis (dysregulated cortisol secretion) is a characteristic that is common to depression (Nemeroff et al., 1984; Palazidou, 2012) and has been seen in up to 80% of depressed patients (Heuser, Yassouridis, & Holsboer, 1994), as well as among PD patients (de Rezende et al., 2016; Gelman, Flores-Ramos, Lopez-Martinez, Fuentes, & Grajeda, 2015; Jolley, Elmore, Barnard, & Carr, 2007). That being said, the body is equipped with feedback mechanisms to balance various processes and one such feedback mechanism that acts to buffer the stress response is the oxytocin system.

Oxytocin

Oxytocin (OXT) is a neuropeptide and hormone that has been shown to dampen HPA axis over-activity by reducing excess cortisol in the system (Bisagno & Cadet, 2014). OXT has anxiolytic properties in part because it reduces stress, but also because it increases the salience of pro-social cues thereby facilitating trust, bonding, social support seeking, empathy, attachment, social cognition and positive affect (Heinrichs, von Dawans, & Domes, 2009; Seltzer, Ziegler, Connolly, Prososki, & Pollak, 2014). OXT also stimulates lactation as well as uterine contractions and is associated with maternal behavior (Carson, Guastella, Taylor, & McGregor, 2013). In fact, the evolutionary function of OXT in most animals is rooted in reproductive behaviors, from bond formation and mating to pregnancy/labor and parenting. In this way, it is

believed that OXT came to reduce anxiety and stress in order to effectively initiate and sustain the reproductive behaviors mentioned above (Pedersen, Chang, & Williams, 2014). With respect to maternal behavior, OXT enables mothers to be more attuned and sensitive to their infants' cues which may contribute to the development of secure attachment in their children (Feldman, Weller, Zagoory-Sharon, & Levine, 2007; S. Kim, Fonagy, Koos, Dorsett, & Strathearn, 2014).

In terms of systemic function, the effects of OXT are carried out via the nervous and endocrine systems (Charmandari, Tsigos, & Chrousos, 2005; Olff et al., 2013) whereby they are known to act locally (in the brain) as well as peripherally, through the bloodstream to reach distant targets. In fact, OXT along with vasopressin (AVP) are the only neuropeptides released by the pituitary gland that act distally (DNA Learning Centre, 2015). OXT is produced in the hypothalamus, but is stored and secreted by the posterior pituitary gland. Via certain pathways extending from the hypothalamus, OXT can be found in such brain areas as the hippocampus, nucleus accumbens, medial preoptic area (MPOA) and the amygdala (Stoop, 2012). Once OXT leaves the brain, it cannot reenter due to the blood brain barrier. Peripherally, OXT is synthesized in such organs as the placenta, uterus, liver and heart. Oxytocin receptors (OXTR) are also found in the brain and peripherally. The kidney, heart, ovaries, uterus and mammillary glands all express OXTR, whereas in the brain, OXTRs can be found in the substantia nigra and basal nucleus of the midbrain (Gimpl & Fahrenholz, 2001) as well as in the spinal trigeminal nucleus and inferior olivary nucleus of the brain stem (Freeman, Smith, Goodman, & Bales, 2016).

OXT Studies

There remains some inconsistency in findings about how circulating OXT levels are associated with psychological and social functioning (Garfield, Mathews, & Janusek, 2015) although the majority of studies have found that lower levels of plasma OXT are associated with

various psychopathologies, including depression (Scantamburlo et al., 2007), autism (Modahl et al., 1998) and anorexia nervosa (Maguire, O'Dell, Touyz, & Russell, 2013). As for other tissue types, OXT concentrations in the cerebrospinal fluid (CSF) of women who experienced child abuse and/or parental neglect were significantly lower compared to women who did not experience such abuse (Heim et al., 2009) while salivary OXT was found to be lower in 6 year-old children whose mothers were chronically depressed during the early postpartum period, compared to children whose mothers were not depressed (Apter-Levy, Feldman, Vakart, Ebstein, & Feldman, 2013). However, a recent meta-analysis revealed that there were no substantial differences in OXT levels (as measured in saliva, urine, CSF and plasma) between psychiatric patients[6] and healthy controls (Rutigliano et al., 2016). One explanation for these inconsistencies in findings could be due to the fact that OXT levels tend to vary throughout the day and according to where a woman is in her menstrual cycle (Acevedo-Rodriguez, Mani, & Handa, 2015; Huber, Veinante, & Stoop, 2005). Also, methodological issues such as whether samples are extracted or not heavily influence how circulating OXT is detected and measured (Bachner-Melman & Ebstein, 2014). Extraction is a purification process that involves separating out interfering substances present in the samples so that when assayed, the resulting quantities are a true reflection of the concentration of the organic compound in question (McCullough, Churchland, & Mendez, 2013). Taken together, measures of peripheral OXT (and hormones, in general) are highly variable, making it necessary to explore other aspects of the OXT system that are likely to influence OXT function, as well as other biomarkers that are relatively more stable and reliable (Auger & Auger, 2013).

[6] The range of psychiatric illnesses featured in the meta-analysis include bipolar disorder, eating disorders, autism spectrum disorder, major depressive disorder, obsessive compulsive disorder.

One such biomarker is genetics. Genetic variants or single nucleotide polymorphisms (SNPs) are fixed and they can reveal how genetic predispositions underlie certain behavioral phenotypes. However, a thorough meta-analysis of common SNPs on the OXT receptor (OXTR) gene revealed that not only were the findings from such studies inconsistent, but the effect sizes for these studies were small and thus insufficient to explain the outcome variables in question, which were a wide range of social behaviors (Bakermans-Kranenburg & van Ijzendoorn, 2013). In relation to OXTR variants and mood in a large sample of pregnant women, no substantial link was found (Connelly et al., 2014). Furthermore, genotype studies do not inform whether or not a particular gene is expressed. In other words, possessing a certain genetic variation does not guarantee that the variation will have an influence on phenotype since the gene (or a portion of the gene where the SNP is found) may not even be expressed. For this reason, studies that can infer gene expression would offer better insight into important mechanisms underlying gene-environment interactions.

Epigenetics

With the advancement of technology in recent years, it has become possible to investigate the biological pathways that link environmental stressors to the various behavioral and health outcomes described above. It is now evident that one of the mechanisms driving the environment's influence on biology and behavior is epigenetics. Epigenetics is a molecular process that influences gene expression without modifying the underlying genetic sequence and has been proposed as a mechanism that can account for lasting gene-environment interactions and their effects (Lester, Conradt, & Marsit, 2016). The process of epigenetics is dynamic in that external factors and environmental influences can regulate gene function (alter gene expression) by conveying information to the cell environment which then signal the cell's genetic machinery

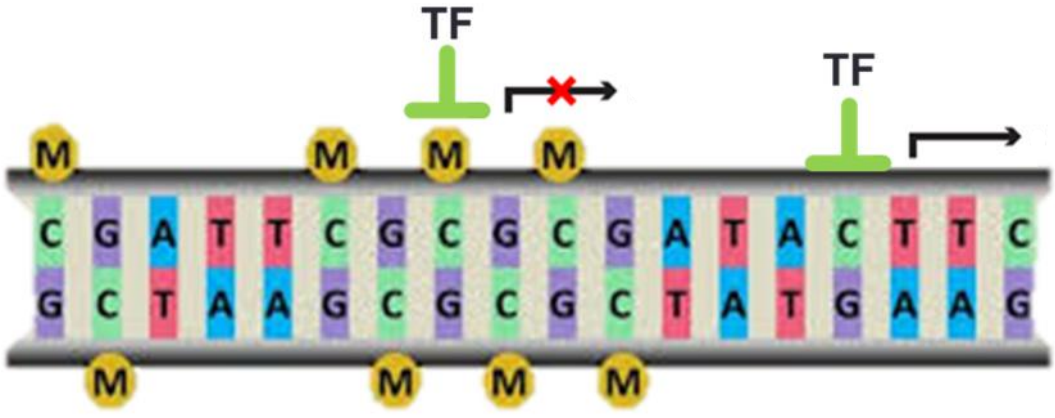
to respond accordingly. A range of environmental influences including nutrition, parenting, toxins and exposure to stress may modify gene expression (Franklin & Mansuy, 2010; T. Y. Zhang, Labonte, Wen, Turecki, & Meaney, 2013) by adapting the genome in anticipation of similar environmental triggers (Szyf, 2013). The evolutionary function of epigenetics is threefold: 1) to equip the genome with an adaptable feature to be able to respond to environmental demands; 2) to carry out cellular differentiation whereby only the genes that are necessary for a given cell's activity are expressed; and 3) to undergo genomic imprinting (selective inheritance) (Kumsta, Hummel, Chen, & Heinrichs, 2013; Weber & Schubeler, 2007).

Whether epigenetic modifications are inherited and maintained through cellular division is still under investigation. Rodent models have found that epigenetic profiles are indeed transferred from parent to offspring (F. A. Champagne, 2008; Rando, 2015) although other studies have failed to find such evidence (McRae et al., 2014). There are several known epigenetic mechanisms including: DNA methylation, histone modification, chromatin remodeling and microRNAs (Marsit, 2015). DNA methylation has been the most studied epigenetic modification to date and is typically associated with gene silencing (Dalton, Kolshus, & McLoughlin, 2014; Sun, Kennedy, & Nestler, 2013). DNA methylation can occur throughout the gene although methylation exerts the most influence on gene expression when it occurs in regulatory regions of the gene (i.e., promoter and/or enhancer regions) where transcription[7] is likely to take place (Strachan, 2011). Typically, methylation refers to the addition of a methyl group to a cytosine in the context of a CpG (cytosine-phosphate-guanine) site in the genome (A. P. Bird, 1986; Holliday, 1989; Razin & Cedar, 1993). The methyl group blocks transcription factors from binding to the gene, making the DNA inaccessible for transcription and thereby

[7] Transcription is the process of copying specific genetic coding sequences for the purposes of translating genetic information into its respective gene product or protein.

suppressing gene expression (Figure 1). Another function of DNA methylation involves the recruitment of specific enzymes that regulate and remodel chromatin structures (Strachan, 2011). As methylation is characterized as a covalent bond between a methyl group and a cytosine, this epigenetic mark is thought to be more stable than other epigenetic mechanisms (Murgatroyd & Spengler, 2011). CpG rich areas (or CpG islands) are regions of high CpG density, usually about > 200 basepairs (bp) in length and composed of (50%) cytosine/guanine nucleotides (UCSC Genome Bioinformatics, 2009a). CpG islands are usually found in the promoter region (transcription start sites) of a gene, which explains why methylation is more prevalent in these regions. As for the biomarker in question (oxytocin), there is one gene that codes for OXTR and it is found on chromosome 3. OXTR consists of 4 exons and 3 introns (see figure 2) and has a considerably large CpG island that spans 2319 bp (UCSC Genome Bioinformatics, 2009e, 2009g). OXTR (as opposed to the OXT gene itself) was targeted for our methylation analysis given that the actions of hormones are mediated by their receptors and can thus provide insight into physiological responses such as whether there are sufficient receptors to allow circulating OXT to bind, thereby stimulating its effect (Scitable by Nature Education, 2014).

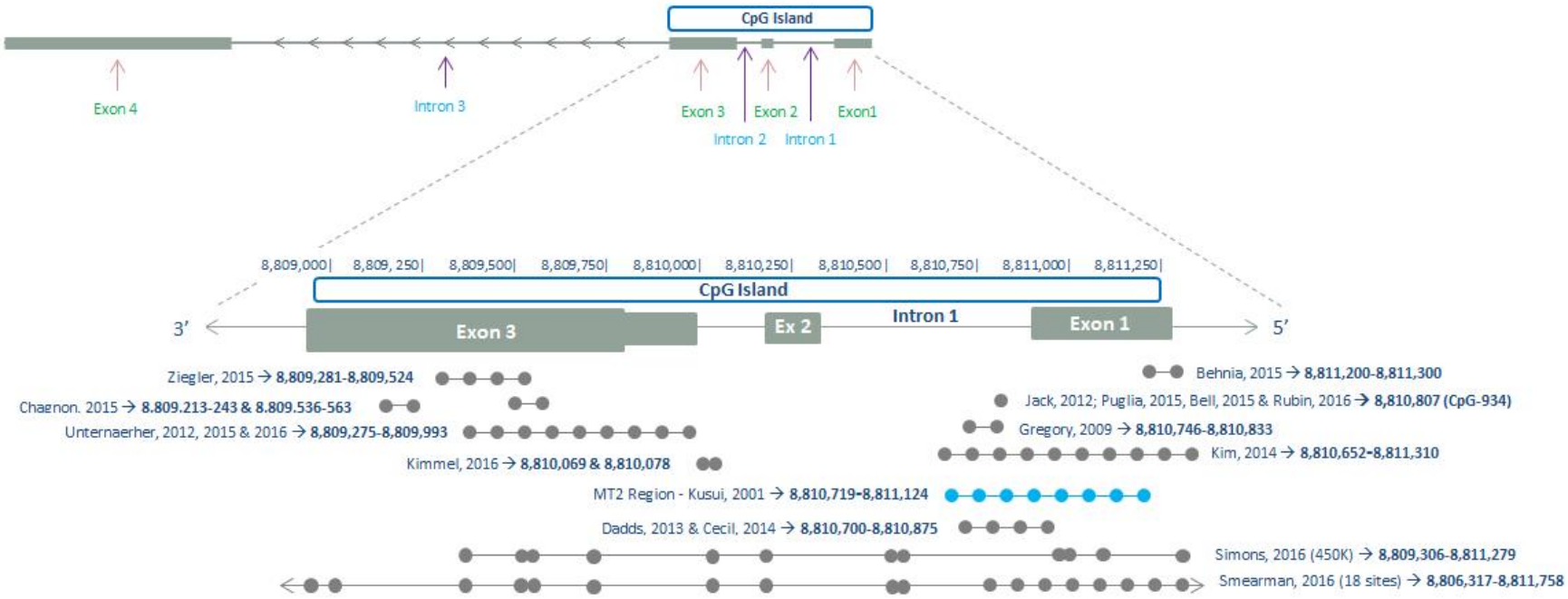
Figure 1: Methylation at CpG Sites



* *Methyl groups* M *block transcription factors* ⊥ *from binding to the gene.*

Epigenetic modifications are an ongoing process with certain genes or developmental periods being more prone to changes in gene expression than others (Auger & Auger, 2013; O'Connor, Monk, & Fitelson, 2014). For example, the embryonic and sexual maturity (puberty) phases are periods of rapid growth and brain development, which correspond to increased cellular differentiation, and consequently, higher rates of gene expression alterations (Marsit, 2015). However, it has been argued that the embryonic phase is most susceptible to epigenetic changes since it is at this stage when the fetus is most genetically vulnerable as cellular differentiation and gene expression are first being laid down and encoded (F. A. Champagne, 2008; Weaver et al., 2004). The fetus' genome is likely more vulnerable since it is also developing according to anticipated environmental cues and in order to do this, it must be somewhat plastic to be able to adapt to incoming signals. As a result, the maternal environment is particularly influential as it constitutes the fetus' first exposure to external signals on which the fetus is dependent to form and develop. It is also important to note that not only is the fetus' genome more sensitive to environmental cues during the prenatal and early postnatal periods, but the epigenetic changes that take place during this period are more likely to be sustained (long-lasting) and robust (Roth, 2013). The same principle can be applied to hormones also, in that being exposed to certain hormones during critical periods of development can induce lasting gene expression alterations compared to in adulthood, when the same exposure would elicit more transient effects (Auger & Auger, 2013). This evidence suggests that although epigenetic modifications may be more stable relative to hormones, during critical stages of growth, the nervous system is dynamic to the point that various influences can shape overall brain development and functioning.

Figure 2: Map of the OXTR Gene and OXTR Methylation Studies



OXTR Methylation Studies

To date, 20 studies have published data on OXTR methylation in humans of which the majority focus on the association between methylation of the OXTR gene and psychopathology; namely depression (Chagnon, Potvin, Hudon, & Preville, 2015; Reiner et al., 2015; Simons, Lei, Beach, Cutrona, & Philibert, 2016), anorexia nervosa (Y. R. Kim, Kim, Kim, & Treasure, 2014), autism (Gregory et al., 2009; Yuksel, Yuceturk, Karatas, Ozen, & Dogangun, 2016), social anxiety (Ziegler et al., 2015) and conduct problems (Cecil et al., 2014; Dadds et al., 2014). To our knowledge, although a few studies have examined OXTR methylation in relation to postpartum depression (Bell et al., 2015; Kimmel et al., 2016) and prenatal depression (Unternaehrer E, 2016), none have investigated OXTR methylation with respect to depression experienced over the entire perinatal period. The two studies assessing OXTR methylation and postpartum depression found that increased methylation of CpG site -934 (8,810,807) was only associated with the occurrence of postpartum depression if participants also possessed an A-allele for the rs53576 SNP (Bell et al., 2015) while the other study discovered that postpartum depression was linked to decreased methylation – but independent of genotype and at two different CpG sites: 8,810,078 and 8,810,069 (Kimmel et al., 2016). A third study examining prenatal depression and OXTR methylation within exon 3, found that depressive symptoms predicted decreased methylation (Unternaehrer E, 2016).

There are two studies that have investigated how OXTR methylation relates to depression in a women-only sample. The first study by Reiner et al. discovered that decreased methylation of the CpG sites contained in OXTR's exon 1 was related to a higher incidence of depression which was moderated by genotype such that possessing an A-allele for rs53576 was associated with higher methylation in depressed women (Reiner et al., 2015). The second study observed

increased methylation among those women who reported higher levels of anxiety and depression, but only at 1 of the 9 CpG sites interrogated (8,809,549) and only if carrying the AA genotype (for SNP rs53576) (Chagnon et al., 2015). Regardless of the CpG sites investigated in these two studies, it appears that possessing the A-allele for OXTR's SNP rs53576 is associated with higher methylation among depressed patients.

Two other studies assessed OXTR methylation and depression in African-American samples. The first study by Smearman et al. found that 3 CpG sites interacted with abuse to predict depression whereby higher methylation was observed for CpG site 8,810,980 and lower methylation was observed for CpG sites 8,806,317 and 8,811,004 (Smearman et al., 2016). The second study reported that adult adversity predicted higher OXTR methylation and that the effect of depression on methylation was mediated by distrust and pessimism, but only for 4 out of 12 sites investigated (8,810,980, 8,811,004, 8,811,092 and 8,811,279) (Simons et al., 2016).

As for OXTR methylation studies in children, two have been conducted and both examined OXTR methylation in relation to callous-unemotional (CU) traits. The first study included boys aged 4 to 16 years (Dadds et al., 2014) while the second was a longitudinal 13-year study that assessed cumulative environmental risk[8] at 4 time-points (birth, 7, 9 and 13 years of age) (Cecil et al., 2014). Both studies looked at the same regions of the OXTR gene, but not the same CpG sites. For example, the Dadds, 2014 study found increased methylation at only one site (8,810,833) among older boys (9-16 years old) with high levels of CU traits (Dadds et al., 2014) and although the Cecil, 2014 found a similar pattern with regards to higher methylation among older boys (13 years-old) with high levels of CU traits, this trend was observed at two different sites (8,809, 306 and 8,809, 501).

[8] Some examples of cumulative environmental risk include: being exposed to poor housing conditions, bullying, domestic violence, parent psychopathology, parent substance abuse, etc.

The relationship between OXTR methylation and psychopathology is unclear. The direction of methylation (e.g., hyper- vs. hypomethylation) seems to vary depending on CpG site. Furthermore, not all studies interrogate the same regions of the OXTR gene, let alone the same sites. In order to contribute to and validate these findings, it would be necessary to replicate previous studies by investigating the methylation patterns of the same CpG sites of the OXTR gene. It is also important to consider which sites may be most biologically relevant to the function of the OXT system, especially given that CpG sites vary in their influence on gene transcription and subsequent phenotypes (Byun et al., 2009; W. Zhang, Spector, Deloukas, Bell, & Engelhardt, 2015). Without doing so, the impact of methylation on downstream biological processes would remain speculative. Confirming how methylation at specific CpG sites (either individually or in combination) affects gene expression (mRNA levels) will reveal how functional certain CpG sites are.

The only study to date that has validated the biological implications of OXTR methylation is a study by Kusui and colleagues whereby they demonstrated that methylation of the promoter region of the OXTR gene suppressed transcription in human liver cells (Kusui et al., 2001). This finding suggests that methylation of a significant portion of the OXTR CpG island is associated with gene silencing, which is in line with other research demonstrating that methylation occurring in the gene body does not interfere with transcription to the same degree as if methylation occurs on a CpG island (A. Bird, 2002; A. P. Bird, 1986). There exist a few studies that have examined the relationship between OXTR methylation and its gene product or protein. One study observed hypermethylation of the promoter region of OXTR as well as higher quantities of OXTR protein in the fetal membranes of infants born prematurely, although gene expression profiles were unrelated to methylation (Behnia, 2015). A second study assessed

OXTR methylation in relation to circulating OXT levels in blood and found an inverse relationship, whereby higher methylation was associated with lower circulating OXT among adolescent males with conduct problems (Dadds et al., 2014). Another study discovered that higher OXTR methylation was related to reduced mRNA expression in autistic patients; however, these analyses were not conducted with the same patients. Rather mRNA samples were acquired from one cohort and the methylation data from a separate cohort; samples were matched for age and sex (Gregory et al., 2009). A final study examining methylation levels in schizophrenic patients failed to find a significant association between OXTR methylation and peripheral OXT levels overall. Conversely, when the sample of psychotic patients was divided by sex, female patients exhibited a positive correlation between methylation and OXT levels, whereas males exhibited a negative association (Rubin et al., 2016).

Research Questions

Despite the different CpG sites that are interrogated across various studies, the overall trend seems to be that hypermethylation is associated with psychopathology, including depression and to complement that, several studies can confirm that lower circulating OXT is common among those experiencing depressive symptoms (Eapen et al., 2014; Skrundz, Bolten, Nast, Hellhammer, & Meinlschmidt, 2011). Given this evidence, it can be expected that hypermethylation of the OXTR gene may result in lower levels of circulating OXT (or less opportunities for OXT to bind) via suppression of OXTR gene expression. Therefore, the goal of the current study is to assess methylation patterns of functionally relevant CpG sites[9] in both mother and child with respect to PD. Specifically, I will aim to answer the following questions: 1) For mothers, will the experience of PD (either prenatally, postpartum or both) be associated

[9] "Functionally relevant" in this case refers to those CpG sites that are found within a CpG island, are located in a protein-coding region and where transcription factors bind. See the methods section for the exact gene coordinates.

with hypermethylation of the OXTR gene? 2) In the case of children, will exposure to their mother's PD be associated with hypermethylation of the OXTR gene? 3) Are the OXTR methylation patterns between mother and child similar? The answers to these questions will be the first of its kind to reveal how PD may be associated OXTR methylation in not only the mothers who are experiencing PD, but among the children who are exposed to PD also. Moreover, the results from this study would help us better understand how differential methylation patterns may emerge depending on the timing of depression – either prenatally, postpartum or both (persistent PD), since there is a lack of research demonstrating which developmental periods are more epigenetically responsive to the effects of environmental stressors, including exposure to PD.

Methods

Participants

A total of 381 eligible[10] women who were in the early stages of pregnancy were recruited for a larger study on perinatal mental health, oxytocin, and mother-infant relationships. The majority of these women (n = 341) were approached to participate in our study while awaiting their prenatal appointments at a tertiary care hospital (n = 244) or at a birthing center (n = 97); these women made up the community sample. An additional sample of women (n = 40) were recruited from the perinatal mental health (PMH) unit of the hospital's psychiatry department, in order to include participants who had more severe levels of depressive symptomatology than the community sample; these women made up the clinical sample (see Figure 3 for a detailed description of recruitment and drop-out rates).

[10] Eligibility criteria include being 18 years of age or older, fluent in either French or English and pregnant with a singleton baby.

Given the longitudinal design of the study and participant loss due to attrition, the final sample consisted of 218 mothers and their children for whom we had complete psychosocial and biological data. Data collection began in July 2009 and ceased September 2014. Drop outs (n = 196) and the final sample (n = 221)[11] did not differ on measures of income level and depressive symptomology at any of the time points, although they did differ with regards to education level (x^2 = 16.75 (1); $p < 0.001$) and immigrant status (x^2 = 11.97 (1); $p = 0.001$), with the group lost to follow up comprised of more immigrants (49.7% vs. 33.0%) and a higher proportion of less educated individuals (50% with less than a bachelor's degree vs. 30.3%) compared to the final sample (Table 1).

At recruitment the average age of the mothers was 35.52 years (SD=4.4) and for approximately a quarter of the women (26.8%), this was their first pregnancy. The mean age of children at follow up was 2.9 years (SD=0.43) and an equal number of boys (50.5%) and girls (49.5%) participated in the study. Two-thirds of the sample were married, university-educated, had a household income upwards of $65,000 and were Canadian-born (Table 1).

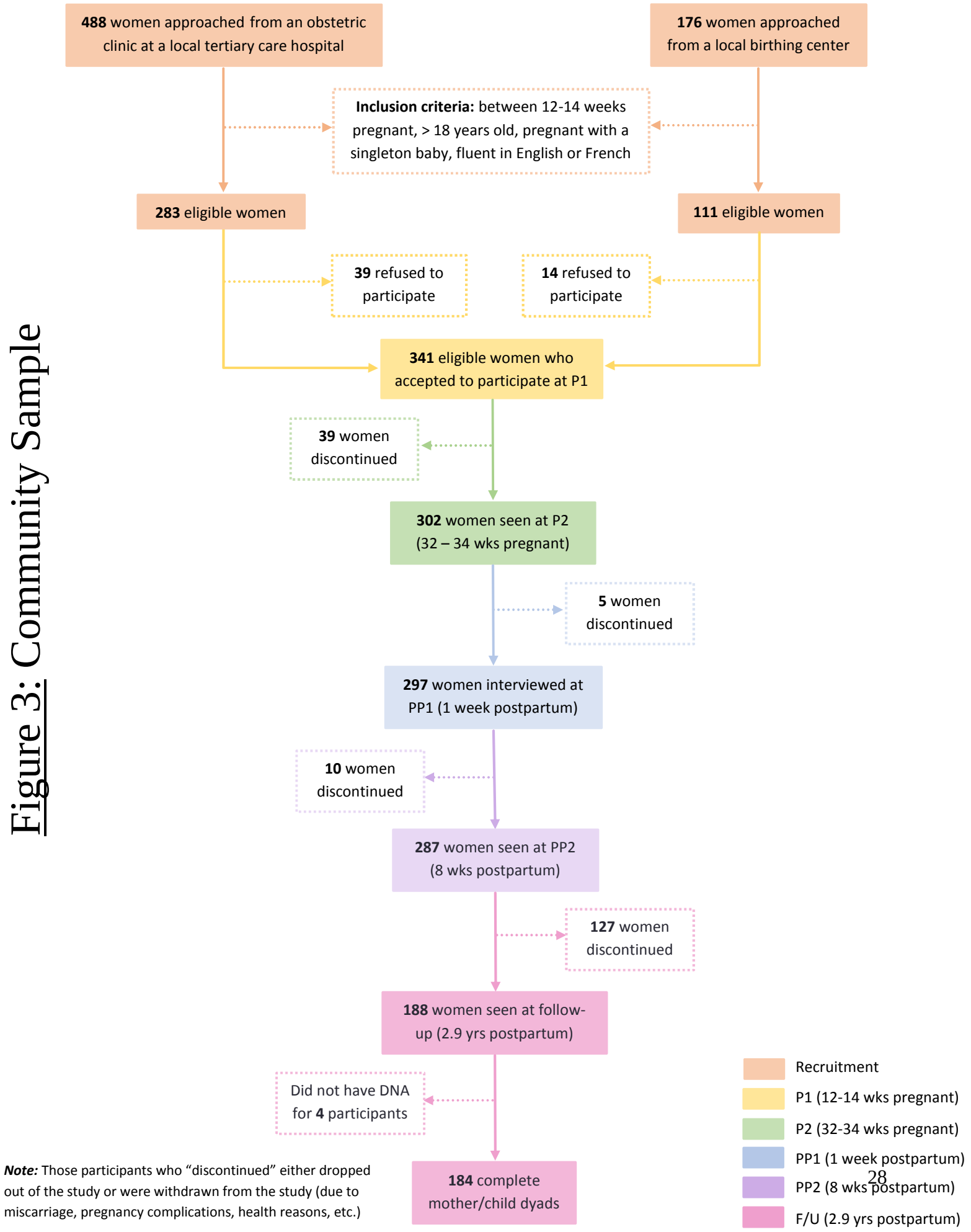
[11] N is 221 instead of 218 because we have complete data for 221 moms (218 refers to having complete data for 218 moms AND children).

Table 1: Demographics

	n (%)
Marital Status	
- Single/Divorced	20 (9.2)
- Living with partner	46 (21.0)
- Married	153 (69.9)
Income Bracket	
- Less than $25,000	17 (7.9)
- $25 - $64,999	61 (28.4)
- $65 - $104,999	68 (31.6)
- $105,000 or higher	69 (32.1)
Education Level	
- High school or less	20 (9)
- DEP/Trade school/CEGEP*	47 (21.3)
- Bachelor's Degree and Higher	154 (69.7)
Immigrant Status	
- Born in Canada	148 (67)
- Not Born in Canada	73 (33)

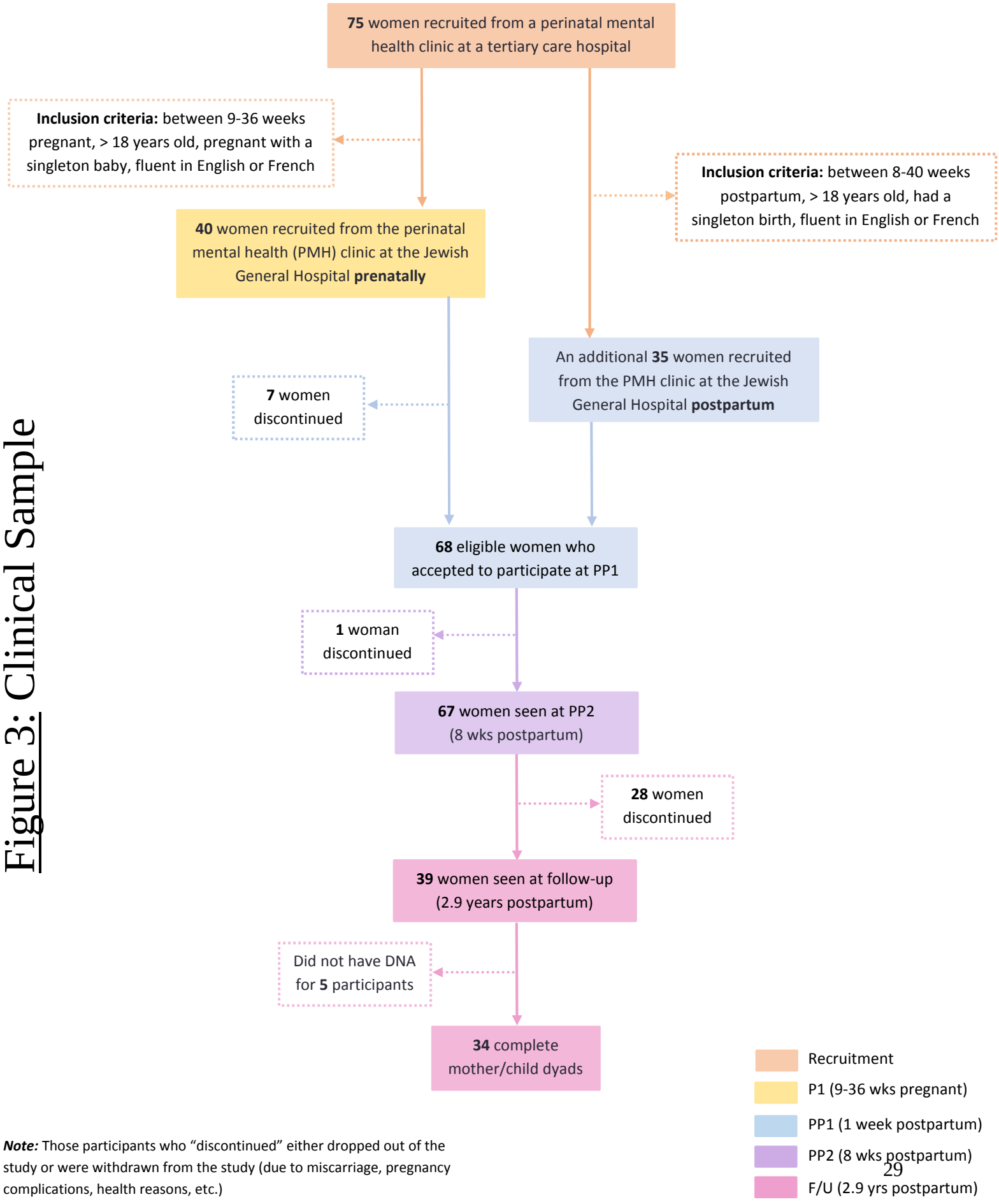
** DEP = diploma of vocational studies*
CEGEP = collège d'enseignement général et professionnel

Figure 3: Community Sample



Note: Those participants who "discontinued" either dropped out of the study or were withdrawn from the study (due to miscarriage, pregnancy complications, health reasons, etc.)

Figure 3: Clinical Sample



Note: Those participants who "discontinued" either dropped out of the study or were withdrawn from the study (due to miscarriage, pregnancy complications, health reasons, etc.)

Measures

Each mother's psychosocial and demographic information was collected throughout the study while biological data pertaining to DNA methylation was only collected from mother and child at the final time point – 2.9 years postpartum (Table 2). Demographic information included questions pertaining to each participant's age, parity, marital status, income level, occupation, immigrant status, level of education, religious affiliation and language(s) spoken at home.

Perinatal depression (PD) was measured using the Edinburgh Postnatal Depression Scale (EPDS) at 12-14 weeks and 32-34 weeks gestation, 1 week and 8 weeks postpartum as well as 2.9 years postpartum. The EPDS is used among pregnant and postpartum women to assess perinatal depressive symptomology and has been validated cross-culturally (Cox, Holden, & Sagovsky, 1987; Zubaran, Schumacher, Roxo, & Foresti, 2010). It is a self-report 10-item scale of depressive symptoms experienced during the past week and has a positive predictive value (diagnosis confirmed by clinical interviews) ranging from 22% to 82% (Shrestha, Pradhan, Tran, Gualano, & Fisher, 2016). Each item is rated on a Likert scale (0 = no, not at all to 3 = yes, most of the time) whereby scores can range from 0 to 30. Typically, the EPDS is evaluated using a cut-off score as an indicator of clinically significant depressive symptomology; for our study, we used a conservative cut-off score of 12 or higher. Sample questions include: "I have been so unhappy that I have been crying" and "I have been anxious or worried for no good reason".

Some items from the Antenatal Risk Questionnaire (ANRQ) were included in our analyses. The ANRQ is typically used as a screening tool for postpartum depression in that it measures cumulative psychosocial risk (Austin, Colton, Priest, Reilly, & Hadzi-Pavlovic, 2013). It assesses social support, recent stressful life events, as well as a history of mental illness, emotional abuse and sexual or physical abuse. Some ANRQ items require a yes/no response

(e.g., Were you emotionally abused when you were growing up?) while others incorporate a Likert scale (e.g., Do you feel you have people you can depend on for support with your baby? 1 = very much to 5 = not at all). Previous studies have found that being the victim of abuse has been significantly associated with differential methylation (Smearman et al., 2016; Suderman et al., 2014). Additionally, having a supportive partner is a major risk factor for PD (Nilaweera, Doran, & Fisher, 2014; Ratcliff, Sharapova, Suardi, & Borel, 2015). Therefore, we included those ANRQ items that addressed having had a history of emotional, physical and/or sexual abuse as well as the item that asked about having an emotionally supportive partner. The decision to include these ANRQ items was based on the fact that these psychosocial risk factors could be potential confounding variables in our analyses.

Data on oxytocin receptor (OXTR) DNA methylation of mother and child were acquired via cells collected from saliva. Percent methylation data was generated for a total of 182 CpG sites on the OXTR gene although only 22 sites were included in the final analysis based on 3 criteria: 1) coverage of 20 or more reads[12]; 2) sufficient methylation variation across participants (SD of ≥ 2%) when averaged; and 3) biologically relevant (e.g., located on a CpG island as well as within an exonic region that contains transcription factor binding sites). Specifically, the region of interest is found on exon 3 of OXTR and corresponds to chr3: 8,809,306 – 8,809,501 of UCSC's hg19 assembly (UCSC Genome Bioinformatics, 2009g).

[12] Including sites that generate a modest number of reads provides less protection against sequencing errors; according to a methods paper on whole genome bisulfite sequencing , 5 to 30 reads is recommended with 10 or more reads not necessarily providing added benefit (Ziller, Hansen, Meissner, & Aryee, 2015).

Table 2: Measures by Time-Point

MEASURES	Community Sample					Clinical Sample			
	Prenatal 1 (12-14 wks)	Prenatal 2 (32-34 wks)	Postpartum (1 week)	Postpartum (8 wks)	Follow-up (~2.9 years)	Prenatal (9-36 wks)	Postpartum (1 week)	Postpartum (8 wks)	Follow-up (~2.9 years)
Background Information	X	X		X	X	X	X	X	X
Antenatal Risk Questionnaire (ANRQ)	X					X			
Edinburgh Postnatal Depression Scale (EPDS)	X	X	X	X	X	X	X	X	X
DNA Methylation (saliva)					X				X

Procedures

For the community sample, data were collected at two points during pregnancy (12-14 weeks and 32-34 weeks gestation) and 3 times during the postpartum period (1 week and 8 weeks postpartum as well as 2.9 years postpartum) while for the clinical sample, data were collected only once prenatally and 3 times postnatally (Table 2). Given the small pool of clinical women (i.e., the PMH clinic sees about 100 women per year - both pregnant and postpartum), we did not restrict our recruitment of clinical participants to only those women who were in the initial stages of pregnancy. Consequently, we relaxed our criteria on stage of pregnancy, thereby establishing one prenatal time point that covers the last 2 trimesters of pregnancy.

The administration of questionnaires for the initial prenatal visits took place at the three recruitment sites (tertiary care hospital, birthing center or PMH clinic) while participants were awaiting their prenatal appointments. At one week postpartum, questionnaires were administered over the phone. For the 8-week postpartum data collection, researchers visited participants at their residence to administer the questionnaires and to observe mother-infant interactions while the final postpartum visits (2.9 years post-birth) took place at the local tertiary care hospital – specifically in the department of psychiatry.

DNA Collection

The Oragene™ DNA (OG-250 and OG-500) kits were used for the collection of saliva of children and mothers respectively; the former kit was preferred for children in order to facilitate the non-invasive recovery of buccal cells and saliva from children who are not able to spit. Mothers and their children were asked to refrain from eating or drinking 30 minutes prior to the collection of saliva to reduce the possibility of sample contamination. For the OG-250 kit, preliminary tests were conducted to determine how many swabs were necessary to obtain a sufficient amount of cells from which to extract DNA. DNA quantity assessments revealed that 6 swabs would be satisfactory for obtaining saliva and buccal cells from children, so researchers were instructed to collect cells by swabbing the inside of the child's mouth 6 times (using a fresh swab each time). The OG-500 kit comes with a tube into which mothers spit until the recommended quantity of 2 ml of saliva is obtained. Both kits also include a solution containing specific enzymes that prevent DNA degradation, allowing the stable storage of saliva samples at room temperature for an indefinite period of time.

DNA Extraction

Extraction of DNA was performed using Oragene's prepIT-CD2 kit in accordance with the manufacturer's instructions (Program of Genomic and Nutrition Laboratory, 2009). Briefly, samples were treated with Oragene's DNA purifier, then incubated at 4°C for 10 minutes, followed by centrifugation at 12,000 rpm for 20 minutes after which the supernatant was separated from contaminants, treated with 100% ethanol as well as 70% ethanol with a centrifugation step in between (20 minutes at 12,000 rpm) and finally incubated at 55°C for 60 minutes once eluted in a water-based buffer. Given the smaller amount of saliva and resulting DNA obtained from children, children's samples were eluted to a volume of 400 ul while those

of the mothers were eluted to a volume of 1200 µl. Concentration and quality assessments were performed using NanoDrop spectrophotometer after which samples were stored in a -80ºC freezer until DNA extraction of all samples was complete. The concentration of samples ranged from 11.9 to 462.2 ng/µl (average = 109.25 ng/µl) while the 260/280 ratio[13] ranged from 1.2 to 2.28 (average = 1.86) which falls within the recommended 1.8 range of acceptable purity (Thermo Fisher Scientific, 2011).

Bisulfite Conversion

Following extraction, 20µl of DNA from each participant underwent sodium bisulfite treatment using the EZ DNA Methylation-Gold Kit™ by Zymo and treatment of the DNA was done in accordance with the manufacturer's instructions (96-well plate format; catalogue #: D5007). This kit has been used in several studies for which methylation data is generated from saliva (Cao-Lei et al., 2014; Conradt et al., 2016; Vukojevic et al., 2014). Furthermore, tests in our lab demonstrated that the bisulfite kit by Zymo resulted in better quality output DNA, likely due to the minimal presence of fragmented DNA after conversion. Bisulphite treatment converts un-methylated cytosines to uracils (leaving methylated cytosines intact) in order to protect methylation patterns from being erased during polymerase chain reaction (PCR) amplification as well as to distinguish methylated cytosines from non-methylated cytosines during sequencing and data analysis (Strachan, 2011). In the process, double-stranded DNA is converted to single-stranded DNA. It is important to note, however, that standard bisulfite treatment does not distinguish between methylated cytosines and hydroxyl-methylated cytosines (Huang, 2010), thus methylation results may reflect a combination of both types of methylation.

[13] 260/230 refers to the ratio of nucleic acid to protein/contaminants and is an indication of DNA purity

Primer Design & Testing

Primers for the OXTR gene were designed and tested before undergoing PCR amplification. In all, 8 primer pairs were designed to cover the OXTR CpG island. These primers were designed based on sequences obtained from the GRCh37/hg19 assembly of UCSC's Genome Browser (Kent WJ, 2002). The software, MethPrimer (Li & Dahiya, 2002) was used to design primers based on bisulfite-converted DNA sequences and parameter checks for the primers were assessed using IDT's OligoAnalyzer tool 3.1 (IDT - Integrated DNA Technologies, 2015). Specifications for primer selection were as follows: the melting temperature must be in the range of 50-60°C and the primer length must be a minimum of 19bp and a maximum of 30bp. Optimal annealing temperatures for each primer were established using gradient PCR (ProFlex™ 96-well PCR System) and target amplicons were visualized on an 1.5% agarose gel to ensure the accuracy of amplicon length. The size of OXTR amplicons ranged from 251 bp to 500 bp. For a complete list of forward and reverse primer sequences, see Appendix I.

PCR Amplification

Bisulfite converted DNA was amplified using the following master mix: 8 µl of Kapa HiFi Uracil + ^{TM} (by Kapa Biosystems), 2 µl of template DNA, 1 µl forward primer, 1 µl reverse primer and 3 µl distilled H_2O to make a final reaction volume of 15 µl. Kapa HiFi Uracil + is a polymerase that is compatible with bisulfite converted DNA as it contains dUTPs that enable the amplification of uracil-containing DNA (Kapa Biosystems, 2013). PCR amplification and thermo-cycling conditions were done in accordance with the manufacturer's instructions and were as follows: initial denaturation, 95°C for 2-5 minutes (1 cycle); denaturation, 98°C for 20 seconds (25 cycles); annealing step, 57°C-61°C for 15 seconds (25 cycles); extension, 72°C for 30 seconds (25 cycles); final extension, 72°C for 1 minute (1 cycle); and hold, 4°C for an infinite

period of time. After the first round of amplification with all 8 primer pairs, amplicons underwent an additional 15 cycles of amplification to bind Fluidigm™'s common sequence primers (CS1-forward primer and CS2-reverse primer). The purpose of these primers is to attach primer-specific adaptor tags to each amplicon; these tags are compatible with Illumina's universal indexes (barcodes) allowing the identification of each individual participant during the sequencing step. The same PCR conditions described above were applied to incorporate the CS1/CS2 primers during amplification, for 15 cycles (instead of 25) and with the annealing temperature set 2°C higher as these adaptors elongate the primers and therefore require a higher melting temperature. After samples underwent a total of 40 cycles of amplification, all amplicons were pooled by subject (5µl x 8 amplicons = 40µl per subject) resulting in a total of 3,536 reactions (442 participants x 8 amplicons).

Purification & Sequencing

AmPure™ magnetic bead technology was then used to separate contaminants and PCR products (including primer dimers) from DNA by binding magnetically-charged DNA molecules using micro-magnets so that rigorous washing steps can be performed. Several rounds of purification are necessary to remove fragments that are both smaller and larger than the target fragments. The first round of purification was applied at a concentration of 0.8x (75 µl pooled DNA: 60 µl AmPure beads) and was performed after samples were amplified with CS1/CS2 primers. After this purification step, samples underwent 10 additional cycles of amplification to bind Illumina's universal indexes and consequently, 2 more rounds of purification were required to eliminate any remaining PCR products. The first round of purification was set to a total concentration of 0.9x (0.5x + 0.4x) as a "lifting" step to eliminate large fragments and the second round of purification was set to a concentration of 0.8x to eliminate small fragments. Prior to

sequencing, purified samples were quantitated using Tape Station™ (Agilent Technologies). Precise concentrations were obtained and the pooled samples were diluted to 2nmol for optimal cluster density (~700-800K/mm^2) on Illumina's®MiSeq for which runs were done in duplicate. PhiX was incorporated into the sequencing run as a calibration control which functions to provide an estimation of error rates. The MiSeq platform was chosen as it offers high-density coverage for targeted gene sequencing while generating up to 15 million reads per run.

Ethical Considerations

Ethical approval to conduct the current study was obtained from the research ethics committees of the birthing center and tertiary care hospital where recruitment of the participants took place. Participants gave written informed consent for themselves, and for their children at the 2.9 year visit. At each visit, participants were compensated $25. For the final visit which took place at the hospital, parking fees were reimbursed if participants arrived by car; otherwise, taxi or public transportation fares were reimbursed.

Data analysis

In order to obtain a full range of scores on the EPDS, the data from both the community and clinical samples were combined and analyzed as one group given that similar data were collected for both groups. All statistical analyses were conducted with SPSS software (IBM SPSS Statistics 19). The sample was classified into 4 groups for the purposes of comparison: 1) women who did not score above the EPDS cut-off either prenatal or postnatally **(no PD)**, 2) women who scored above the EPDS cut-off at least once prenatally **(prenatal depression only)**, 3) women who scored above the EPDS cut-off at least once postnatally **(postpartum depression only)**, and 4) women who scored above the cut-off at least once prenatally as well as at least once postnatally **(persistent PD)**. The purpose of formulating 4 groups (1 healthy control group

vs. 3 depressive groups) was to detect whether psychosocial and methylation data would differ based on acute forms of depression (prenatal depression only or postpartum depression only) versus a more chronic form of depression (e.g., persistent PD). Since contextual or situational factors such as having had a difficult pregnancy or adjusting poorly to motherhood, could respectively be contributing to depressive symptoms in the prenatal or postpartum periods, the inclusion of a "persistent PD" group may be a more accurate representation of underlying pathology and thus, more likely to reveal differential methylation patterns.

Certain demographic categories were also collapsed for the purposes of comparison whereby 2 groups were made for parity (1 child or 2 children or more), household income (less than $65,000 or more than $65,000), education level (less than a bachelor's degree and holding bachelor's degree or higher) and marital status (married/common-law or single/divorced). Sample characteristics were determined using descriptive statistics while overall differences between groups were assessed using the independent samples T-test as well as the chi-square test.

For the methylation data, target sequencing results produced from MiSeq were exported as FastQ data after which bio-informaticians aligned sequencing reads and converted sequencing data as % methylation and % coverage for individual CpG sites. MiSeq runs were done in duplicate and the methylation data generated from these runs were merged to create an average methylation score for each site per participant. Given that CpG sites do not tend to act independently of the other, but rather operate in networks, all 22 CpG sites were averaged to create one OXTR methylation score for each participant. Subsequently, univariate analyses of variance (ANOVA) were conducted with EPDS group[14] entered as the independent variable (or factor) and % methylation entered as the dependent variable. The possibility of running an

[14] "EPDS group" refers to the 4 groups established based on depressive symptomology described above.

analysis of covariance (ANCOVA) was based on the premise that breastfeeding and antidepressant medication could be confounding the mothers' OXTR methylation. Given OXT's role in lactation (Feldman, 2012) as well as the effect of antidepressant medication on DNA methylation (Gentile & Fusco, 2016), we assessed whether these factors could explain differences in OXTR methylation. Similar preliminary tests were done with regards to the children's methylation and the covariate of gender, since some literature suggests that sex-differences could contribute to variations in the OXT system (Dumais & Veenema, 2016; Shamay-Tsoory & Abu-Akel, 2016). Bonferonni post-hoc analyses were then conducted to highlight how each of the EPDS groups compared to one another in terms of overall methylation. Before assessing which individual CpG sites could be driving overall methylation patterns, we first applied the generalized linear model – repeated measures design, to detect main effects and interactions. Methylation was "repeatedly" measured 22 times since the region of interest is composed of 22 CpG sites that tend to correlate with one another and thus would be unlikely to be contributing to overall methylation independently of the other. Once main effects and possible interactions were detected, the multivariate analyses of variance (MANOVA) was used to identify differentially methylated CpG sites after which Bonferroni post-hoc analyses revealed specific comparisons between the EPDS groups with regards to methylation for each CpG site.

The methylation analyses involving mothers included a total of 221 samples while for that of the children, 218 samples were included. Finally, given that we have methylation data for the mother and child pair, we conducted paired-samples t-tests to investigate how the methylation patterns between mother and child compare – regardless of EPDS group.

Results

Demographic and Psychosocial Comparisons

The majority of the sample did not have PD in that 59.2% of women did not score above the EPDS cut-off for any of the time-points investigated. Only a small proportion of the sample (7.8%) reported persistent PD (experienced depression both prenatally and postpartum – see Table 3). Of the 17 women who had persistent PD, 9 were from the clinical sample and 8 were from the community. Prenatal and postpartum depression was exhibited in 12.4% and 20.6% of the sample respectively. When comparing the 4 groups in terms of demographic variables, there were no differences, except for marital status. Specifically, the 4 groups did not differ in age, parity, immigrant status, income or education level. However, women who had postpartum depression (75.6%) were least likely to be partnered (married or common-law). Despite this finding, women with persistent PD (64.7%) were the least likely to report having a partner that was emotionally supportive. In comparison, of those women who had no PD, 94.5% reported having an emotionally supportive partner. As for having had a history of abuse, 41.2% of women with persistent PD reported having been the victim of emotional abuse at some point in their lives and 29.4% had been physically and/or sexually abused. This is contrary to what was found in the no PD group, who reported the lowest levels of emotional abuse (4.7%) and physical/sexual abuse (10.2%; Table 3).

Table 3: Demographic and Psychosocial Comparisons

Total Sample = 221	No Perinatal Depression, n (%) 129 (59.2)	Prenatal Depression Only, n (%) 27 (12.4)	Postpartum Depression Only, n (%) 45 (20.6)	Persistent Perinatal Depression, n (%) 17 (7.8)	ANOVA or Chi-Square
Age (years)	35.58	35.55	35.32	35.54	F (3,214) = 0.39, p = 0.990
Parity (2 or more kids)	97 (75.8)	18 (66.7)	30 (66.7)	14 (82.4)	x^2 (3) = 2.73, p = 0.435
Born in Canada	84 (65.1)	18 (66.7)	35 (77.8)	10 (58.8)	x^2 (3) = 3.09, p = 0.378
Household Income (< $65K)	39 (31.5)	11 (40.7)	22 (48.9)	5 (31.3)	x^2 (3) = 4.75, p = 0.191
Education (bachelor's degree or higher)	97 (75.2)	18 (66.7)	28 (62.2)	9 (52.9)	x^2 (3) = 5.42, p = 0.144
Partnered	122 (96.1)	25 (92.6)	**34 (75.6)**	16 (94.1)	x^2 (3) = 17.72, p = 0.001*
Partner Emotionally Supportive	120 (94.5)	22 (81.5)	33 (73.3)	**11 (64.7)**	x^2 (6) = 22.01, p = 0.001*
Emotional Abuse	6 (4.7)	3 (11.1)	9 (20)	**7 (41.2)**	x^2 (3) = 23.51, p < 0.001*
Physical/Sexual Abuse	13 (10.2)	3 (11.1)	**12 (26.7)**	**5 (29.4)**	x^2 (3) = 9.90, p < 0.05*

** Percentages calculated "within" EPDS group*

CpG Site Correlations

To ensure that all 22 CpG sites included in the analyses were indeed acting interdependently and could therefore, be combined to make one methylation score per participant, we ran a correlation matrix to assess the degree of relation between sites. The correlations (r) for the mothers' CpG sites ranged from 0.557 to 0.849 (n = 219) with all comparisons reaching statistical significance at the $p < 0.001$ level. The children's CpG sites were less related to each other with correlations ranging from r = -0.044 to 0.649 (n = 210/211). Specifically, out of 441 (21 x 21) comparisons, 82 did not reach statistical significance when set at the $p < 0.05$ level. Given that the majority of sites were nonetheless correlated with one

another, we proceeded with the next step of analyses which was to assess how overall methylation differed based on EPDS group.

OXTR Methylation - Mothers

Despite there being unequal sample sizes across EPDS group (Table 4), the Levene's Test of Equality of Error Variances was not violated, suggesting that the error variance across the dependent variable (methylation) was equal across the EPDS groups ($F_{(3,213)} = 1.412$, $p = 0.240$). Approximately three-quarters (76.5%) of the women in our sample were not breastfeeding at the time of saliva collection and a minority (8.5%) were taking antidepressant medication. T-tests confirmed that there were no differences in overall methylation, nor was there differential methylation of individual CpG sites with regards to these two cofounding variables. Therefore, neither breastfeeding nor medication were included as covariates in our analyses. Similarly, we evaluated whether certain psychosocial risk factors, such as having had a history of abuse and having an emotionally supportive partner, could explain some of the variation in OXTR methylation. However these factors did not result in any differences in methylation patterns either; therefore, it was not necessary to control for any of these variables.

Overall OXTR methylation was lowest for those mothers who were suffering from postpartum depression only (8.5%), followed by mothers who reported no PD (8.8%) and then those mothers who had prenatal depression only (8.9%). The highest methylation was seen for those mothers who reported persistent PD (11.9% - Table 4). Results from the ANOVA confirmed that overall OXTR methylation was significantly different between EPDS groups ($F_{(3,213)} = 3.732$, $p < 0.05$). Post-hoc analyses revealed that the persistent PD group had methylation levels that were significantly higher than the postpartum depression only ($p < 0.05$) and no PD groups ($p = 0.01$), but not necessarily the prenatal depression only group ($p = 0.06$).

Figure 4: Overall Methylation - Mothers

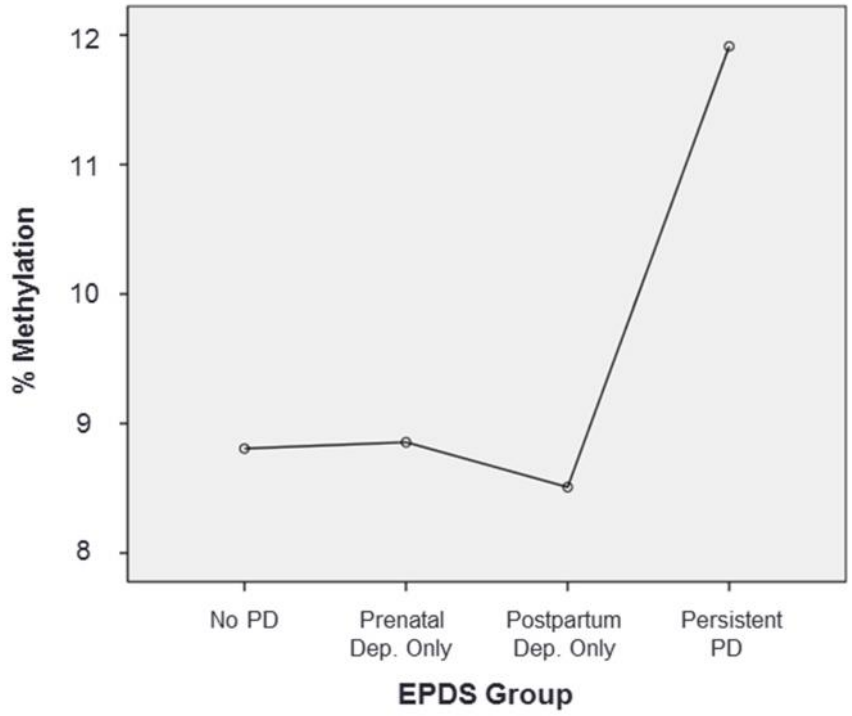


Table 4: ANOVA – Mothers

EPDS Group	N	Mean Methylation (%)	Standard Error	ANOVA
No PD	128	8.80	0.35	*f* (3, 213) = 3.73, *p* < 0.05*
Prenatal Depression Only	27	8.86	0.68	
Postpartum Depression Only	45	8.54	0.40	
Persistent PD	17	11.91	1.17	

To investigate whether there was an effect of EPDS group on the methylation levels of individual CpG sites, a repeated measures design was implemented. However, the sphericity assumption was violated (x^2 (230) = 1031.1, $p < 0.001$); therefore, we used more conservative F-ratios by referring to Greenhouse-Geisser corrected values. We observed significant main effects of OXTR methylation ($F_{(13,2705)} = 247.38$, $p < 0.001$) and EPDS group ($F_{(3,212)} = 2.92$, $p < 0.05$), but no significant interaction between OXTR methylation and EPDS group ($F_{(38,2705)} = 1.117$, *p*

= 0.287). Figure 5 shows that methylation patterns for individual CpG sites were similar to each other, whereby methylation was relatively consistent across the no PD, perinatal and postpartum depression groups; however, for the persistent PD group, methylation was significantly higher. In particular, results from the MANOVA indicated that 9 out of the 22 CpG sites showed significant differential methylation when compared across EPDS groups (the *P*-values for these sites have already been adjusted and corrected for multiple testing). Post-hoc analyses revealed that the most robust differences were found between the persistent PD group and the no PD or postpartum depression group (Table 5).

Figure 5: Methylation of Individual CpG Sites – Mothers

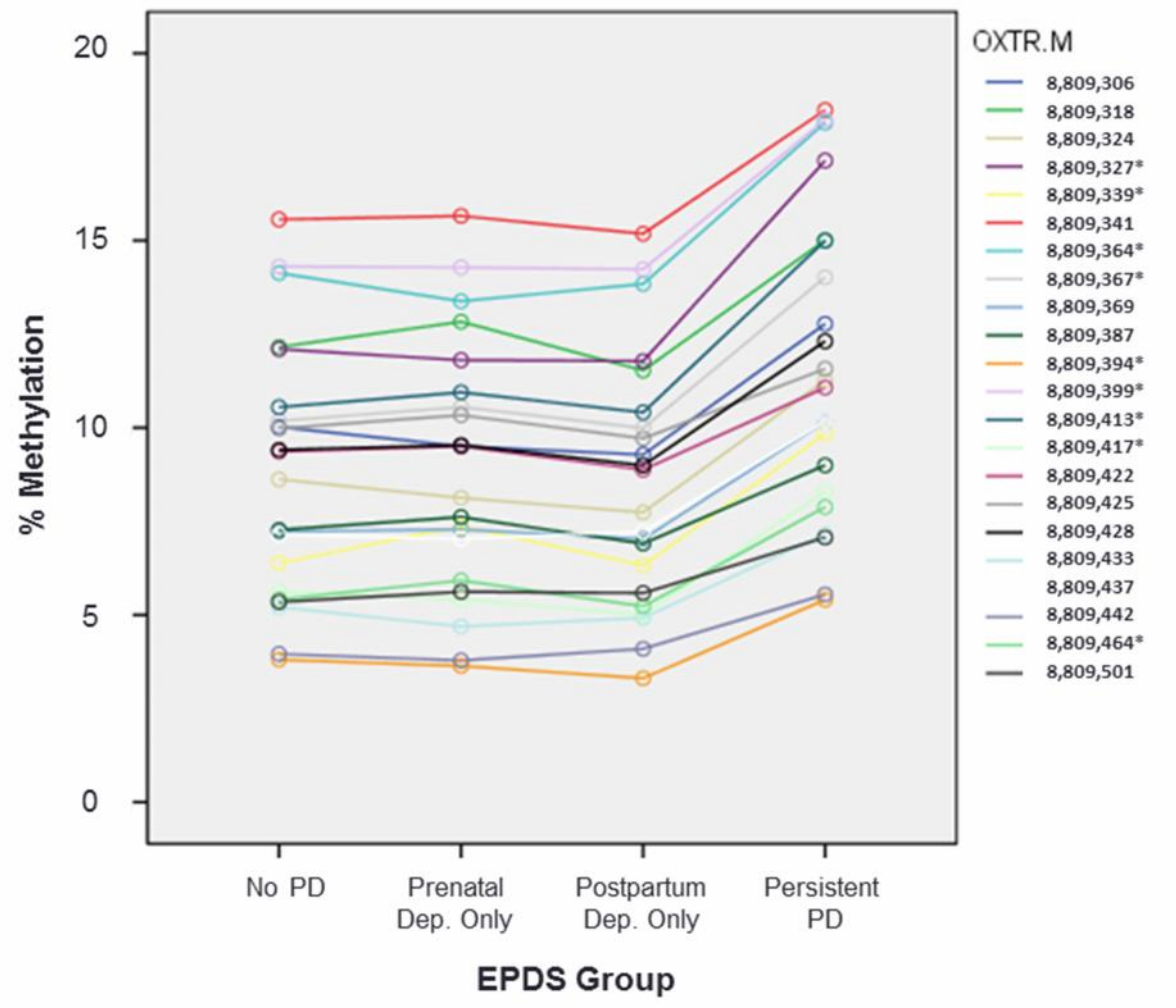


Table 5: Follow-up Analyses - Individual CpG Sites

CpG Site	MANOVA	POST-HOC Analysis Reference Category: Persistent PD	
8,809,327	$F_{(3,212)} = 4.91$, $p < 0.005$**	**No PD**	***p* < 0.005****
		Prenatal Dep. Only	***p* < 0.01***
		Postpartum Dep. Only	***p* < 0.005****
8,809,339	$F_{(3,212)} = 4.08$, $p = 0.01$*	**No PD**	***p* < 0.01***
		Prenatal Dep. Only	$p = 0.280$
		Postpartum Dep. Only	***p* < 0.05***
8,809,364	$F_{(3,212)} = 3.13$, $p < 0.05$*	**No PD**	***p* < 0.05***
		Prenatal Dep. Only	***p* < 0.05***
		Postpartum Dep. Only	***p* < 0.05***
8,809,367	$F_{(3,212)} = 3.25$, $p < 0.05$*	**No PD**	***p* < 0.05***
		Prenatal Dep. Only	$p = 0.135$
		Postpartum Dep. Only	***p* < 0.05***
8,809,394	$F_{(3,213)} = 4.88$, $p < 0.005$**	**No PD**	***p* < 0.005****
		Prenatal Dep. Only	***p* < 0.05***
		Postpartum Dep. Only	***p* < 0.005****
8,809,399	$F_{(3,213)} = 3.07$, $p < 0.05$*	**No PD**	***p* < 0.05***
		Prenatal Dep. Only	$p = 0.084$
		Postpartum Dep. Only	***p* < 0.05***
8,809,413	$F_{(3,212)} = 3.79$, $p < 0.05$*	**No PD**	***p* < 0.01***
		Prenatal Dep. Only	$p = 0.075$
		Postpartum Dep. Only	***p* < 0.05***
8,809,417	$F_{(3,212)} = 2.97$, $p < 0.05$*	**No PD**	$p = 0.055$
		Prenatal Dep. Only	$p = 0.109$
		Postpartum Dep. Only	***p* < 0.05***
8,809,464	$F_{(3,212)} = 2.65$, $p = 0.05$*	**No PD**	***p* < 0.05***
		Prenatal Dep. Only	$p = 0.452$
		Postpartum Dep. Only	$p = 0.061$

** P-values for all analyses have been adjusted by Bonferroni correction*

OXTR Methylation - Children

The sample was composed equally of girls (49.3%) and boys (50.7%). Results from t-tests revealed no detectable differences in OXTR methylation that can be attributed to gender; therefore, we did not control for gender in our analyses. Although there were unequal sample sizes across the groups that made up the independent variable (EPDS group - Table 6), homogeneity of variance can be assumed based on the Levene's test ($F_{(3,203)} = 0.942$, $p = 0.421$).

The range of overall OXTR methylation across EPDS groups was between 6.97 and 7.43%. Although the lowest levels of methylation were seen in those children who were not exposed to PD, while the highest levels were seen in those children who were exposed to

persistent PD (relatively similar methylation levels were seen among the prenatal and postpartum depression groups), this difference was not substantial enough to be statistically significant (Table 6) and post-hoc analyses did not reveal significant differential methylation between any of the EPDS groups either.

Figure 6: Overall Methylation - Children

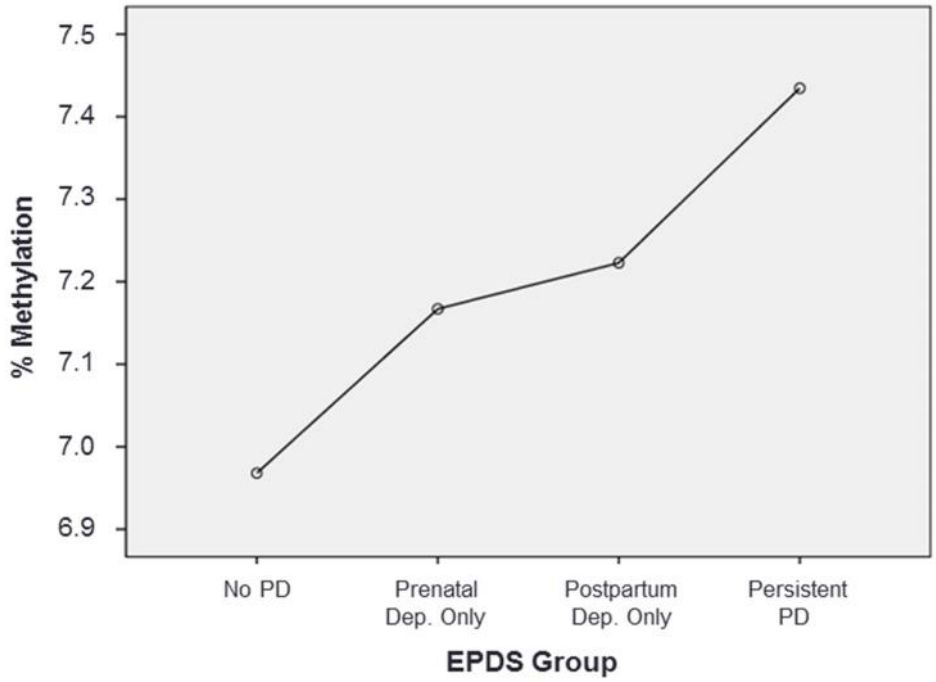


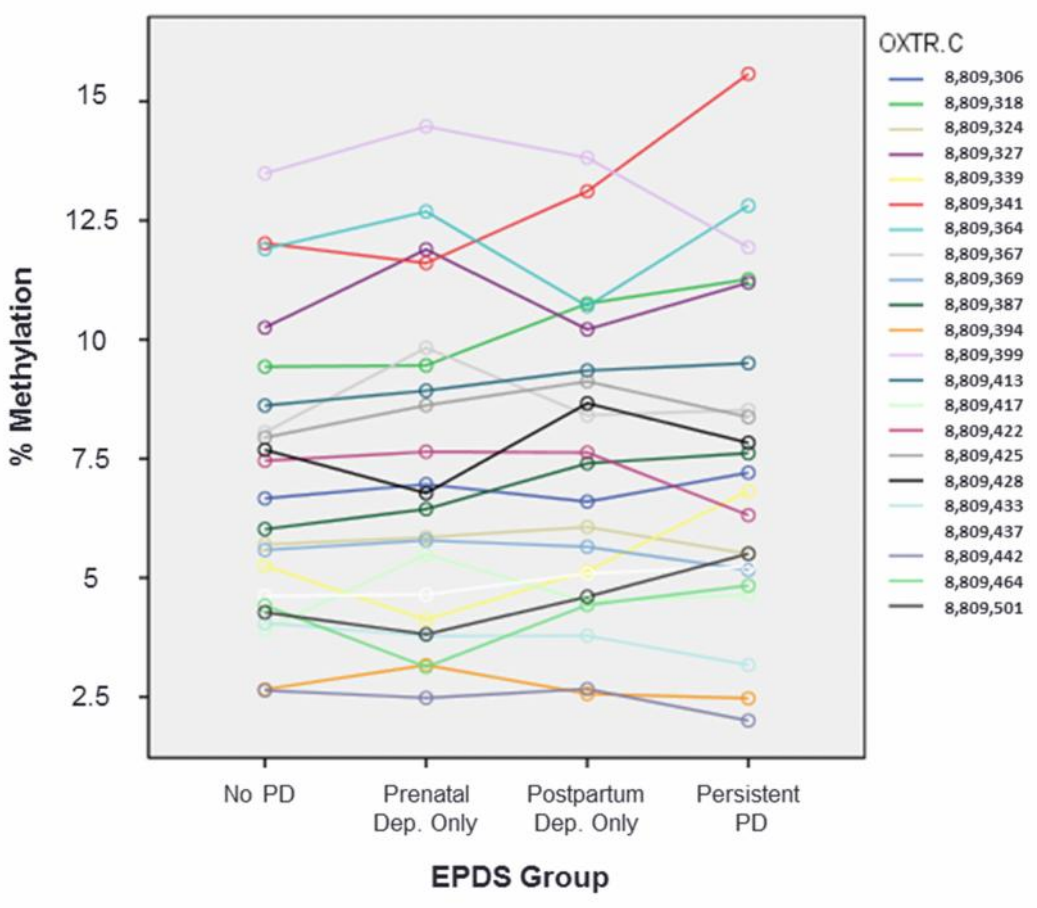
Table 6: ANOVA – Children

EPDS Group	N	Mean Methylation (%)	Standard Error	ANOVA
No PD	121	6.97	0.24	*f* (3, 203) = 0.25, *p* = 0.861
Prenatal Depression Only	27	7.16	0.39	
Postpartum Depression Only	44	7.23	0.36	
Persistent PD	15	7.43	0.41	

In attempt to identify individual CpG sites that could reveal differences in methylation across EPDS groups, we first tested the model for main effects and an interaction. Since the

sphericity assumption was violated (x^2 (230) = 1230.4, $p < 0.001$); we again referred to Greenhouse-Geisser corrected values as they are more conservative. We observed a significant main effect of OXTR methylation ($F_{(13,2570)} = 77.80$, $p < 0.001$), but no significant main effect of EPDS group ($F_{(3,202)} = 0.264$, $p = 0.851$), nor an interaction effect between OXTR methylation and EPDS group ($F_{(38,2570)} = 0.957$, $p = 0.546$). Figure 7 demonstrates that there is no consistent pattern in terms of the direction of methylation for various CpG sites as it relates to each of the EPDS groups. Furthermore, results from the MANOVA along with post-hoc analyses confirmed that when the methylation patterns of each CpG site were assessed independently of the other, none were significantly different when compared across the EPDS groups.

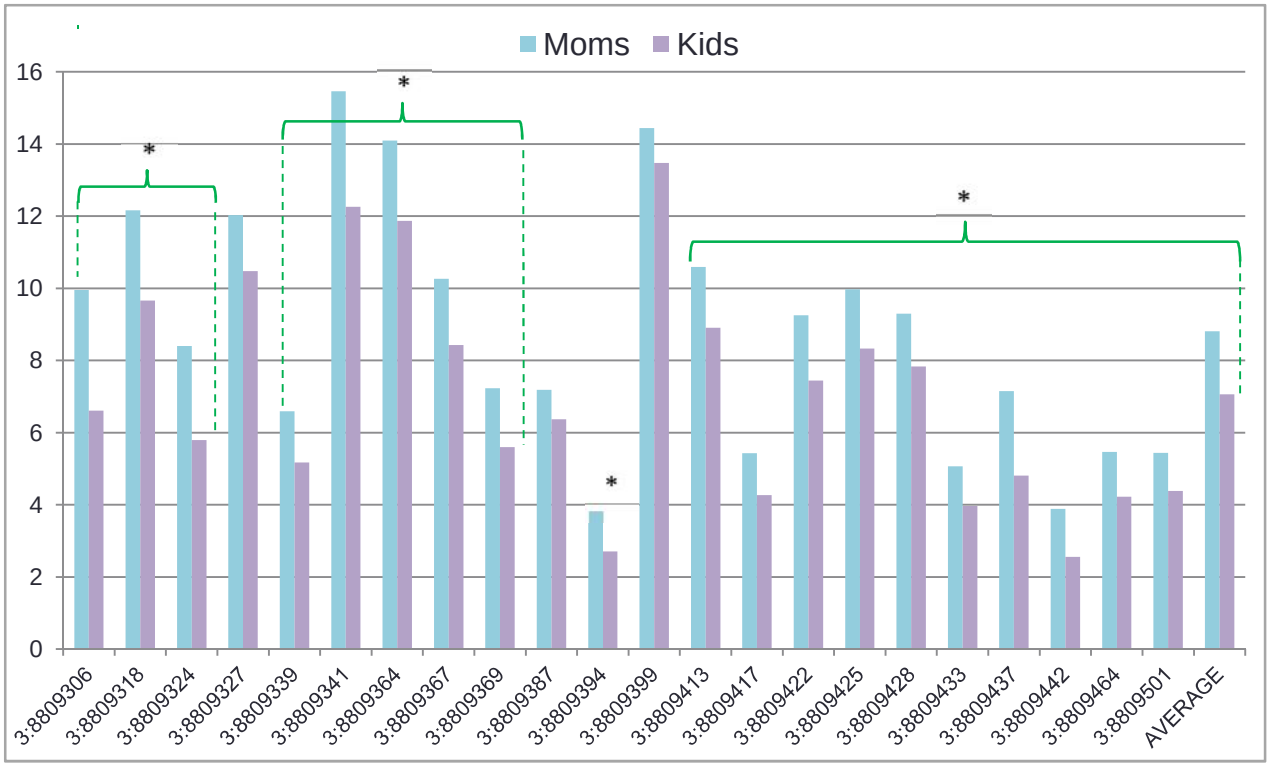
Figure 7: Methylation of Individual CpG Sites - Children



OXTR Methylation Patterns in Mothers and Children

Of the 22 CpG sites interrogated along with an average methylation score (all 22 sites combined), there were substantial differences in levels of OXTR methylation between mother and child when measured using the paired-samples t-test. Specifically, 19 of the 22 sites including OXTR methylation overall, were significantly different between the mother and child pair, with the mothers' methylation being consistently higher for all of the sites investigated (Figure 8). However, the direction of OXTR methylation across each of the 22 CpG sites shows a similar pattern for mothers and children whereby some sites are generally more methylated than others, regardless of whether the methylation reflects that of the mothers or children.

Figure 8: Paired Samples T-test - Methylation of CpG Sites – Mother vs. Children



** Statistically significant at the p < 0.05 level; range of comparisons: n = 207 – 209*

Discussion

Interpretation of Findings - Mothers

Our study on PD and OXTR methylation demonstrates that persistent PD (experiencing depressive symptoms both prenatally and postpartum) is associated with significantly higher methylation on a region of the OXTR gene that is likely to have downstream effects on gene expression and circulating OXT. Furthermore, there were no significant differences in OXTR methylation between those mothers who reported no PD whatsoever and those who were only depressed prenatally or only depressed postnatally. It is possible that more transient episodes of depression, as in the case of prenatal or postpartum depression only, may not be associated with changes in OXTR methylation whereas depression of a longer duration may elicit a more substantial insult to the OXT system. Chronic depression along with other chronic diseases are likely to deplete energy reserves in that they can usurp immune function and jeopardize metabolic processes (Sapolsky, 2004), so it may not be surprising that epigenetic changes may ensue as a result. However, there is some evidence suggesting that acute and temporary stress is sufficient to elicit changes in OXTR methylation as well (Unternaehrer et al., 2012). In light of this, there is a need to investigate which factors may be regulating more stable methylation marks and which factors may result in more dynamic methylation changes.

It is challenging to compare our findings with that of other studies since other studies look at slightly different variations of the behavioral phenotype investigated here. For instance, if we just consider the 5 studies that assess OXTR methylation and depression in women only, our findings are consistent with 2 out of the 5 studies, whereby increased OXTR methylation[15] has been linked to postpartum depression (Bell et al., 2015) as well as depression in older women

[15] The Bell, et al. study interrogated CpG site 8,810,807 while the Chagnon, et al. study examined mean methylation of OXTR's exon 3 region.

(Chagnon et al., 2015), but only when stratified by genotype. For the other three OXTR methylation studies, decreased methylation was observed in relation to: postpartum depression for 2 sites found within OXTR's intron 2 region (Kimmel et al., 2016); depression in Caucasian women for sites within exon 1 (Reiner et al., 2015) and prenatal depression with respect to CpG sites localized within exon 3 (Unternaehrer E, 2016).

The reason for the inconsistencies in findings could primarily be due to the fact that different regions of the OXTR gene are being interrogated, making the results non-comparable. Of course, given the genetic technology available at the time as well as available funding, studies are confined to the specifications of each platform. For example, Illumina's (genome-wide) 450k platform is programmed to sequence methylation data for 12 distinct probes which are distributed across the OXTR gene (Simons et al., 2016). Whereas Sequenom's Epityper and Illumina's MiSeq, which are both targeted gene approaches, permit the researcher to target their region(s) of interest with more specificity and flexibility at a higher resolution. The disadvantage of the latter platforms, however, is that there is no standardized approach to choosing CpG sites or gene regions. Consequently, researchers are obliged to design primers/probes based on arbitrary criteria that have not necessarily been validated elsewhere. Additionally, the CpG island of the OXTR gene is considerably large (2319 bp) compared to the islands of other related genes[16], such as the vasopressin receptor gene (1331 bp), the serotonin transporter gene (799 bp) and the estrogen receptor gene (949 bp) (UCSC Genome Bioinformatics, 2009b, 2009c, 2009f), making it difficult to sequence the whole island with relative ease and accuracy. Despite the methodological and technical issues in measuring OXTR methylation, it may be futile to measure methylation at individual CpG sites since single CpG sites are unlikely to drive

[16] However, one exception is the glucocorticoid receptor gene which is 3,000 bp in length (UCSC Genome Bioinformatics, 2009d).

phenotype differences. Rather, overall OXTR methylation at key regions or in clusters may reveal more robust information about the relationship between methylation and phenotype (Labonte et al., 2012; Szyf & Bick, 2013).

Interpretation of Findings - Children

As for the children in our sample, we did not find an association between overall OXTR methylation and exposure to their mothers' PD, despite there being a trend showing that methylation steadily increases (from no PD → prenatal depression → postpartum depression → persistent PD). The reason why post-hoc tests did not capture a significant effect of EPDS group on OXTR methylation between the 2 most extreme groups (those children who were not exposed to PD versus those who were exposed to persistent PD), is likely because the range of differential methylation was not that pronounced, suggesting that there was insufficient variation to detect any real differences.

There are several other possibilities that could explain why we did not see an effect of PD on the children's methylation profiles. For one, methodological factors including the age at which we assessed OXTR methylation in children as well as the candidate gene approach we applied, could account for the null findings. The genome is vast and complex, with genes unlikely to act independently of the other, but rather inclined to operate in conjunction with each other. Similarly, the stress system involves the coordinated actions of several biological components which are likely to be regulated by a circuit of different genes and biological mechanisms. Therefore, it is possible that other genes (not investigated here) could have been implicated in the children's exposure to PD.

Also, perhaps a mother's PD is likely to have an impact on her child's epigenome during prenatal or early postpartum periods, rather than almost 3 years postpartum. For example, the

fetal programming theory posits that exposure to stress while in utero, can prime the fetus for subsequent disease risk since this phase of development is most prone to environmental signals (Ellison, 2010). Conversely, there is the early life stress (ELS) argument which claims that the psychological and physiological impacts of ELS are long-lasting and sustained years later (Babenko et al., 2015; Silberman, Acosta, & Zorrilla Zubilete, 2016; Szyf, 2013). If this is the case and if PD indeed qualifies as a substantial early life stressor for the developing fetus/infant, then we should have seen respective alterations in methylation 2.9 years later, providing that the OXTR gene is relevant in this context. However, no such methylation pattern emerged in our dataset, lending us to believe that other factors are at play.

More research is needed on this topic because on the one hand, the third trimester of pregnancy is associated with significant neural proliferation including myelination as well as a doubling in whole brain volume, suggesting that the latter stages of pregnancy are more critical for development (Lodygensky, Vasung, Sizonenko, & Huppi, 2010; Ouyang et al., 2015). But on the other hand, since stress hormones are downregulated (receptors become less sensitive) as pregnancy progresses, stressful events experienced earlier in pregnancy, compared to later in pregnancy, are more likely to impact the fetus (Sandman, Davis, Buss, & Glynn, 2012). Therefore, it appears that brain development is the most expansive at later stages of pregnancy, but the fetus is more responsive to stressful signals earlier in pregnancy. In light of these arguments, there is a need to assess how methylation may be altered in response to various environmental signals throughout early stages of development and whether this responsiveness is directly related to brain development.

Other factors that could be influencing children's methylation patterns are maternal behavior and parental care overall. The question of maternal behavior is an essential one since

numerous studies have found that the quality of early life care may moderate and even override the epigenetic impact of prenatal stress on the fetus (Bergman, Sarkar, Glover, & O'Connor, 2010; Weaver et al., 2004). More specifically, maternal sensitivity and responsiveness may buffer the child to the potential maladaptive effects of PD (DiCorcia & Tronick, 2011). This question is worth investigating with regards to the OXTR gene since the only human study to date to have looked at the relationship between the quality of maternal behavior and OXTR methylation, has been done retrospectively – in adults (Unternaehrer et al., 2015).

We used a repeated measures design to measure the methylation of inter-related CpG sites. There were significant main effects of OXTR methylation in both mothers and children, suggesting that differential methylation across EPDS groups was observed for various CpG sites, independent of the other sites included in the model. Nonetheless, interaction effects were not detected in either sample – in mothers nor children, which confirms that differences in OXTR methylation are not attributed to EPDS group. Of the 9 sites that were found to be differentially methylated according to EPDS group among the mothers, none had been previously investigated. The only exception would be those studies conducted by Unternaerher and colleagues where the same region of exon 3 is investigated; however, these studies looked at overall methylation and did not include information about individually methylated CpG sites within the region of interest (Unternaehrer E, 2016; Unternaehrer et al., 2012; Unternaehrer et al., 2015).

Although there were significant differences in methylation between mother and child overall (regardless of EPDS group), when observed across CpG sites, the pattern of methylation were similar. The only difference is that the mothers' methylation levels were slightly higher than that of the children's but this could be explained by the fact that methylation tends to increase with age, especially in CpG contexts (Christensen et al., 2009). Nonetheless, we

validated our findings with respect to raw methylation data and found that our data are in line with that of publicly available data sets (Appendix II and III).

Limitations

There are some important limitations to consider. First, we did not have equal sample sizes in each of the depressive groups and more importantly, the group that made up the persistent PD group was composed of only 17 women. Therefore, caution is required in generalizing our findings to the general population. Secondly, we only collected methylation data at one time point – at 2.9 years postpartum, even though we assessed mothers' depressive symptoms throughout pregnancy and postpartum period. We do not know how the methylation profiles of both mother and child differed over the course of pregnancy or postpartum or whether the methylations patterns we saw were stable and sustained over the perinatal period. And more significantly, since we did not measure OXTR methylation before or after the onset of PD, it is not possible to know whether methylation is a cause or consequence of PD and depression, in general.

Another limitation is that we cannot confirm the downstream biological effects of OXTR hypermethylation – at the gene expression level, nor at the level of circulating OXT. However, previous biological work has been done verifying the relationship between OXTR methylation and gene expression (Kusui et al., 2001), although there is less research confirming how methylation drives protein production. Other studies have examined the relationship between the methylation of other genes and their respective hormone levels, but there remains some inconsistency. For example, one study found that increased methylation of the glucocorticoid receptor (NR3C1) leads to an increase in cortisol levels (Oberlander et al., 2008) while another study found no association between NR3C1 methylation and cortisol response activation (van

der Knaap, Oldehinkel, Verhulst, van Oort, & Riese, 2015). Therefore, there is a need to add to this area of research, possibly by being consistent about the procedural measures used, to be able to confirm the biological endpoints of DNA methylation.

One last limitation is that we did not control for cell type in our analyses. Since one of the principles of epigenetics is cell differentiation[17], methylation patterns could vary depending on the type of cell (Razin & Szyf, 1984). Saliva is composed of epithelial and white blood cells, in varying proportions, depending on the individual. To my knowledge, there is no study that has examined the methylation profiles of the separate cellular components of saliva; however, some studies have found that methylation between saliva and blood are comparable (Cao-Lei et al., 2014; Wu et al., 2014). This may be because saliva contains a higher proportion of white blood cells than epithelial cells and when deriving DNA from blood, the white blood cell layer is typically extracted (Thermo Fisher Scientific, 2016; Waters et al., 2013). In fact, when mapped onto a publicly available consortium of genome-wide epigenetic assays, our methylation data (which has been obtained from saliva) coincides with that of mononuclear/white blood cells from peripheral blood (appendix II and III).

Even though methylation across peripheral tissues is similar, there remains the issue that peripheral markers of methylation may not necessarily coincide with that of the central nervous system. After all, PD and other psychopathologies are manifestations of the brain, so the biomarkers under investigation have to be relevant to various neurobiological processes related to the pathology in question. Since it is not possible to acquire living human brain tissue for the purposes of research and epigenetic analyses, researchers must rely on ethical and non-invasive means to access peripheral tissues, namely blood, saliva and urine. That being said, epithelial and

[17] Cell differentiation is most active in the prenatal period when the embryo is developing; pluripotent stem cells differentiate into specific cell types with the help of epigenetic mechanisms which silence or activate certain processes so that designated cells do not perform the functions of other cell types.

nervous tissue are derived from the same ectoderm layer during development (Lester et al., 2016; Stiles & Jernigan, 2010) and this could explain why the methylation profiles of saliva and brain are more comparable (Nohesara et al., 2011; Smith et al., 2015) than that of blood and brain (Hannon, Lunnon, Schalkwyk, & Mill, 2015; Smith et al., 2015).

Future Directions

The biological relevance of DNA methylation remains theoretical – it is essential to follow up with validation data (e.g., gene expression/mRNA and circulating oxytocin levels) to be able to confirm that OXTR methylation for the particular CpG sites we interrogated indeed have an effect on OXTR gene expression and ultimately, OXT circulation. Validation studies have been done for other genes, whereby hypermethylation has been associated with reduced gene expression (Chen et al., 2015; McGowan et al., 2009). Additionally, it would be of interest to assess other factors that may be influencing the methylation patterns in children, including the maternal behavior (and parental care overall). Finally, we will interrogate the methylation patterns of other candidate genes relevant to stress regulation and maternal behavior, namely: AVPR1a, AVPR1b, AVP and the intergenic region (IGR**;** between OXT and AVP). To date, there are no human studies that have investigated these genes with respect to DNA methylation and depression.

Conclusion

Having the technology available to be able to study the interplay of gene-environment interactions offers valuable insight into complex mechanisms that underlie human behavior. The role of epigenetics is crucial in that it offers an explanation for why certain individuals are more vulnerable to disease. In an attempt to contribute to this growing field of research, we conducted one of the first inter-generational human studies to assess the role of epigenetics in relation to

PD. Particularly, we focused on methylation of the OXTR gene using a relatively large sample size of 440 subjects. The results from this study demonstrate that persistent PD is associated increased OXTR methylation in mothers, but that exposure to persistent PD alone did not have an impact on the children's OXTR methylation. There is a need to investigate other factors as well as other genes that could be influencing children's methylation profiles. Once we have a broader understanding of the direct and indirect consequences of depression during pregnancy as well as the biological mechanisms that are implicated, we can focus on relevant treatments and interventions to potentially curb negative outcomes and susceptibility to disease.

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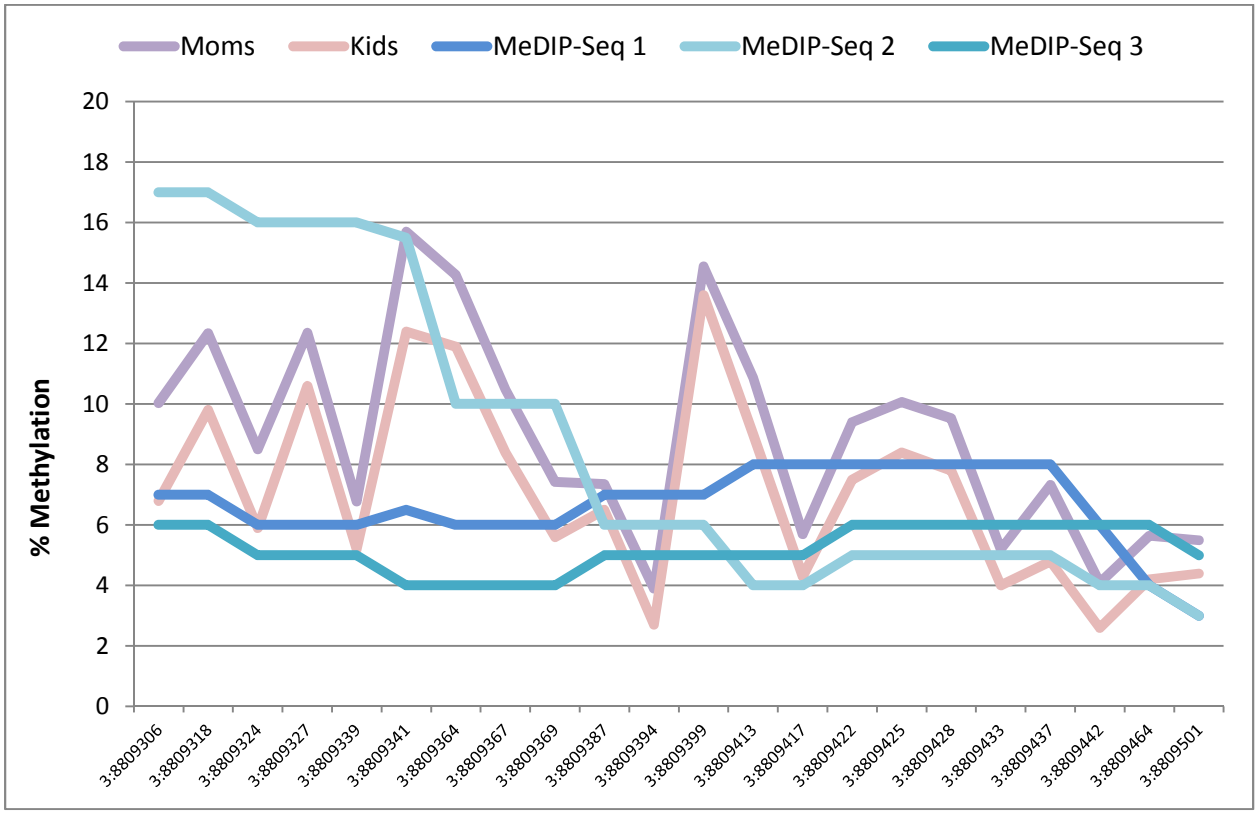
Appendix

Appendix I: OXTR Primer Sequences

OXTR1	F - TT TGT TAA GGT TTT GGG ATT R - CT CCT CCA AAA ACA AAA AAT
OXTR2	F - AT TTT TTG TTT TTG GAG GAG R - CC TAA AAA ACC CCA ACT CTA
OXTR3.1	F - GT TGG GGT TTT TTA GGT AAG R - TC TAA AAC AAC AAA ACA CAA
OXTR3.2	F - GGGGTTTTTTAGGTAAGTGGTTATT R - TCATCTAAAACAACAAAACACAACC
OXTR4	F - GT TTT TGG GAA TGG GAT AAG R - AA AAC ACT ACC ACC ACC AAA
OXTR5	F - TT GGT TAA GTA TTT GTA GGT GGT R - AA TCC CCA AAA CTA AAT AAA
OXTR6.1	F - TA GTT TTG GGG ATT TAA GGT TTA R - AC CAA ACT CCC TCC TCC TAA
OXTR6.2	F- TTTAGTTTTGGGGATTTAAGGTTTATAT R – CCAACCCTAACTACCTTCCTTAAAC

* F = forward; R = reverse
Sequence for CS1 primers = ACA CTG ACG ACA TGG TTC TAC A NNN
Sequence for CS2 primers = TAC GGT AGC AGA GAC TTG GTC T NNN

Appendix II: Comparison of Our Methylation Data with that of WashU Epigenome Browser



Note: *MeDIP-Seq data has been obtained from WashU epigenome browser, a publicly available dataset (MeDIP-Seq data reflects enriched DNA methylation sequences). Peripheral blood (PB) is the tissue for which mononuclear cells were derived; 3 sources were available.*

Appendix III: Raw Methylation Scores

CpG Site	Moms Mean Methylation, %	N	Standard Deviation	Kids Mean Methylation, %	N	Standard Deviation	MeDIP-Seq Meth. PB 1	MeDIP-Seq Meth. PB 2	MeDIP-Seq Meth. PB 3
3:8809306	10.0	218	5.2	6.8	209	4.6	7	17	6
3:8809318	12.3	218	5.1	9.8	210	5.4	7	17	6
3:8809324	8.5	218	4.7	5.9	210	4.1	6	16	5
3:8809327	12.3	218	5.3	10.6	210	6.2	6	16	5
3:8809339	6.8	218	4.0	5.2	210	4.2	6	16	5
3:8809341	15.7	218	5.7	12.4	210	6.7	6.5	15.5	4
3:8809364	14.3	218	5.5	11.9	210	6.0	6	10	4
3:8809367	10.5	218	4.8	8.4	210	5.0	6	10	4
3:8809369	7.4	218	4.4	5.6	210	4.2	6	10	4
3:8809387	7.3	219	4.2	6.5	210	4.6	7	6	5
3:8809394	3.9	219	3.1	2.7	210	2.5	7	6	5
3:8809399	14.6	219	5.7	13.6	210	6.3	7	6	5
3:8809413	10.9	218	5.2	9.0	210	5.0	8	4	5
3:8809417	5.7	218	3.9	4.3	210	3.7	8	4	5
3:8809422	9.4	218	4.6	7.5	210	4.9	8	5	6
3:8809425	10.1	218	4.5	8.4	210	4.8	8	5	6
3:8809428	9.5	218	4.7	7.8	210	4.3	8	5	6
3:8809433	5.2	218	3.4	4.0	210	3.8	8	5	6
3:8809437	7.3	218	4.7	4.8	210	3.4	8	5	6
3:8809442	4.1	218	2.9	2.6	210	2.3	6	4	6
3:8809464	5.6	218	3.5	4.2	210	3.6	4	4	6
3:8809501	5.5	218	3.2	4.4	209	3.1	3	3	5
AVG Exon 3	**9.0**	**219**	**3.8**	**7.1**	**210**	**2.5**	**6.7**	**8.6**	**5.2**

Note: *MeDIP-Seq data has been obtained from WashU epigenome browser, a publicly available dataset (MeDIP-Seq data reflects enriched DNA methylation sequences).Peripheral blood (PB) is the tissue for which mononuclear cells were derived; 3 sources were available.*