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Prenatal stress, the placenta and maternal microbial transmission; implications for health and disease

Thesis presented by

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In the fulfilment of the requirements for the degree of
Doctor of Philosophy

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Declaration:

This is to certify that the work I am submitting is my own and has not been submitted for another degree, either at University College Cork or elsewhere. All external references and sources are clearly acknowledged and identified within the contents. I have read and understood the regulations of University College Cork concerning plagiarism.

Author contributions:

All procedures described in this thesis were carried out accurately, and without bias, to the highest standards. The following methods were performed solely by Katie, with the exception of,

- Section 2.2: Dr Eimear Treacy assisted in the patient recruitment
- Chapter 2.8: Dr Ilaria Carafa preformed the library preparation for metagenomics sequencing
- Section 2.8: Dr Kiera Murphy assisted in the Bioinformatics of microbiome data
- Section 2.9: Ms Loreto Olavarria Ramirez preformed the cortisol ELISAs for the newborn hair samples.

Signed,

_____

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Dissemination of this work:

Published Papers:

- Maternal distress in late pregnancy alters obstetric outcomes and the expression of genes important for placental glucocorticoid signalling.
  Togher KL, Treacy E, Kenny LC, O'Keeffe GW.
  Psychiatry Research (2017), DOI: 10.1016/j.psychres.2017.05.013
- Class-Specific Histone Deacetylase Inhibitors Promote 11-Beta Hydroxysteroid Dehydrogenase Type 2 Expression in JEG-3 Cells.
  Togher KL, Kenny LC, O'Keeffe GW.
  International Journal of Cell Biology (2017), DOI: 10.1155/2017/6169310
- Epigenetic regulation of the placental HSD11B2 barrier and its role as a critical regulator of fetal development.
  Togher KL, O'Keeffe MM, Khashan AS, Gutierrez H, Kenny LC, O'Keeffe GW.
  Epigenetics (2014), DOI:10.4161/epi.28703

In preparation:

- Placental FKBP51 mediates a link between second trimester maternal anxiety and birthweight in female infants.
  Togher KL, O'Keeffe GW, Khashan AS, Clarke G, Kenny LC.
  To be submitted (Scientific Reports, 2017)
- Prenatal distress exposure remodels the maternal microbiome: Implications for offspring gut microbiome assembly.
  Togher KL, Khashan AS, Kenny LC, Stanton C, Carafa I, Murphy K, O'Keeffe GW, Ryan CA, Cryan JF, Dinan TG, Clarke G.
  To be submitted (The journal of Clinical Investigation, 2017)

Abstracts:

- Depression-associated Alterations in the Maternal Microbiome During pregnancy: Implications for infant gut microbiome assembly.

Togher KL, Khashan AS, Kenny LC, Stanton C, Carafa I, Murphy K, O'Keeffe GW, Ryan CA, Cryan JF, Dinan TG, Clarke G.

Society for Neuroscience (SFN) Annual Meeting. Washington DC November 2017.

- Depressive symptoms during pregnancy disrupt gut microbiome dynamics during critical prenatal and postnatal time windows.

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- Depression-associated Alterations in the Maternal Microbiome During pregnancy: Priming for adverse infant outcomes?

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- Psychological wellbeing, HPA activity and sleep patterns in a population of nulliparous pregnant women.

  Togher KL, Khashan AS, O' Keeffe GW, Kenny LC, Clarke G.

  Young Neuroscience Symposium, University College Dublin. November 2016.

- Psychological wellbeing, HPA activity and sleep patterns in a population of nulliparous pregnant women.

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- Examining the relationship between prenatal maternal stress and anxiety with gastrointestinal function in a population of nulliparous pregnant women.

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- Epigenetic regulation of placental HSD11B2 identifies class-specific HDAC and DNMT inhibitors as novel protective pharmacotherapy for the human placenta.

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- Dose-dependent inhibition of 11-beta hydroxysteroid Dehydrogenase type 2 by cortisol in SHSY5Y cells: implications for fetal development.

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Science Communication:

- Happy Mum, Happy Microbes?

  Togher KL

  Science for All Finals. Univerisity College Cork. May 2017

- Stress: I Blame the Parents.

  Togher KL

  FameLab Cork Heats. Triskel Arts Centre, Cork. March 2017

Abstract:

There is an extensive amount of epidemiological evidence showing that prenatal maternal distress (PNMD) is a risk factor for a wide range of poor obstetric and neonatal outcomes, as well as an increased risk for the development of metabolic, immune and nervous system disorders in affected children later in life. Whilst many epidemiology studies have supported these associations, the biological mechanisms linking maternal prenatal distress with adverse outcomes remains understudied, particularly in human cohorts. One potential mechanism, known as the glucocorticoid hypothesis proposes that fetal overexposure to stress-induced maternal cortisol during critical windows of development increases the risk of adverse outcomes in the offspring. At the core of this hypothesis is the placenta, which expresses the enzyme 11beta hydroxysteroid dehydrogenase type 2 (HSD11B2), which ultimately controls the amount of cortisol a fetus is exposed to. Prenatal stress has been shown to reduce the placental expression of this enzyme; however the molecular mechanisms through which this occurs have not been well examined. More recently, the transmission of a suboptimal stressed maternal microbiota is emerging as an alternative mechanism that may mediate the impact of prenatal stress on infant development. However this has not yet been examined in a clinical population.

We first utilized an *in vitro* placenta model, JEG-3 cells, to examine the effects of stress on the placental expression of HSD11B2. JEG-3 cells were cultured with exogenous cortisol and interleukin-1 beta (IL-1β), two potential biological mediators of prenatal stress. This study showed both cortisol and IL-1β can reduce HSD11B2 expression, an effect that could be prevented by co-treatment with a histone deacetylase inhibitor. Having established that cortisol can directly affect the expression of HSD11B2, we moved on to our first clinical study to examine this question in a clinical population by examining the impact of prenatal distress on placental gene expression and infant outcomes. A cohort of 121 pregnant women receiving antenatal care at Cork University Maternity Hospital (CUMH) completed the Perceived stressed scale (PSS), State Trait Anxiety Inventory (STAI) and Edinburgh Postnatal Depression Scale (EPDS) in late pregnancy and donated

placental biopsies at the time of birth. This study identified a significant reduction in HSD11B2 mRNA along with an increase in the glucocorticoid receptor (NR3C1) in placentae from high distressed pregnancies. Additionally prenatal distress was associated with a number of adverse outcomes including delivering via Caesarean section, reduced Apgar scores and reduced birth temperature, supporting a role for placental glucocorticoid signalling in the relationship between prenatal distress and adverse outcomes.

Having reported that stress impacts molecular placental signals and birth outcomes, we moved on to complete the SMArTI (Stressed Microbial Transfer to the Infant) study, a more detailed pregnancy cohort to examine the impact prenatal distress on the maternal and infant microbiome. This study yielded a final cohort off 111 nulliparous pregnant women that were recruited from the IMPROvED consortium at CUMH. Women enrolled in SMArTI completed distress questionnaires and provided saliva and fecal samples in the second and/or third trimester of pregnancy. Vaginal swabs, placenta samples and newborn hair were acquired at birth and infant fecal samples were subsequently collected across the first 5 months of life. We first used this cohort to further examine and validate the relationship between prenatal distress, placental glucocorticoid genes and infant outcomes. We found this relationship to be dependent on the timing of distress, type of distress and infant sex. Most notably we observed second trimester maternal anxiety correlated with reduced birthweight in female infants, a relationship mediated by placental FK506-bind protein 51 (FKBP51) mRNA expression.

We finally used the SMArTI cohort to examine, for the first time, the impact of PNMD on the maternal and infant microbiome, using 16S rRNA gene sequencing. Reduced diversity of the maternal gut microbiome in the second trimester was associated with second trimester distress, most substantially with maternal depressive symptoms, an effect that was no longer apparent by the third trimester. The third trimester gut microbiome appeared relatively resistant to change with only modest alterations observed in women who had high second trimester cortisol. Of interest, third trimester distress had no effect on the third trimester gut microbiome, highlighting the experience of distress specifically in the second

trimester as an important window of vulnerability. Reduced diversity of the vaginal microbiome, just prior to delivery, was associated with second trimester cortisol, with no alterations linked third trimester distress. When examining the infant gut microbiome we found increased diversity across the first 5 months of life to be associated with second trimester stress with corresponding decreases to the important *Bifidobacteriaceae* and *Lactobacillaceae* family.

In conclusion, this thesis indicates the experience of PNMD influences key placental genes involved in glucocorticoid signalling in the placentae. The timing of maternal distress and infant sex are important factors in this relationship. Of particular interest we find placental FKBP51 to mediate a relationship between maternal anxiety and infant birthweight, demonstrating a direct role for placental glucocorticoid signalling underlying the relationship between prenatal distress and infant outcomes. The work presented in this thesis is the first of its kind to prospectively examine the influence of PNMD on the maternal gut, vaginal and infant gut microbiome. Stress-induced alterations in the maternal gut microbiome may contribute to adverse obstetric and birth outcomes albeit via a mechanism other than transmission of a suboptimal maternal microbiota during birth. Taken together, our results identify the second trimester as an especially vulnerable period to stress exposures and implicate the placenta and microbiome in mediating these effects. Counteracting the impact of stress during this critical time window may have important obstetric implications. Additionally understanding the consequence of the altered infant gut microbiome as a result of prenatal distress warrants further investigation.

Chapter 1:

General Introduction

1.0 Introduction:

The concept that *in utero* life experiences, such as the emotional state of the mother during pregnancy, affects child development has been proposed for centuries. Leonardo Di Vinci wrote in his Quaderni; '*the same soul governs the two bodies the things desired by the mother are often found impressed on the members of the child which the mother carries at the time of the desire*'. However it was not until recent decades that the role of the *in utero* environment in the development of disease became a topic of scientific enquiry. The coining of the term 'fetal programming' by Professor David Barker in the 1980s led to a new research effort aiming to understand how *in utero* and early-life experiences alter fetal developmental trajectories that increases the risk for disease later in life (Barker and Osmond, 1986, Barker et al., 1993, Barker et al., 1989). One factor that has received significant attention in this regard is the adverse effects of prenatal maternal distress.

The term 'prenatal maternal distress' (PNMD) is broadly used to refer to the experience of psychological adversities in the prenatal period, including stress, anxiety and depression. There is now an accumulating amount of preclinical and clinical evidence showing PNMD influences fetal and/or infant development that confers disease risk in childhood and adulthood. The effects of PNMD are evident in both the mother and child and manifests as adverse obstetrical outcomes, neonatal outcomes and longer term disease risk. These include (but are not limited to) an increased risk of caesarean delivery, low birthweight (LBW), preterm birth (PTB), intrauterine growth restriction (IUGR) and long term disorders of the immune, metabolic and nervous systems for affected offspring (Ding et al., 2014a, Grote et al., 2010, Dunkel Schetter and Tanner, 2012, Tarabulsy et al., 2014, Entringer et al., 2015).

Whilst the increased risk of adverse obstetric outcomes arising from the experience of PNMD is evident, less is known about the biological mechanisms underpinning this association. Studies in animals have made substantial progress in unravelling these mechanisms, but this has not been well paralleled by clinical investigations. The most studied mechanism is the glucocorticoid hypothesis, which

proposes that fetal overexposure to maternal cortisol adversely alters fetal developmental trajectories to increase disease risk (Reynolds, 2012). At the core of this hypothesis lies the placenta, in particular enzymes/expression of genes within the placenta involved in glucocorticoid signalling namely 11 beta hydroxysteroid dehydrogenase type 2 (HSD11B2). Placental HSD11B2 controls fetal exposure to maternal glucocorticoids acting as a protective barrier for the fetus (Togher et al., 2014). Hence disruptions to this enzyme would be disadvantageous to fetal development. Indeed epigenetic dysregulation of placental HSD11B2 is being increasingly linked to adverse outcomes (Togher et al., 2014).

More recently, focus has been placed on the microbiome to explain the relationship between PNMD and adverse outcomes. The concept that the microbiome is modifiable by stress in not novel (Rea et al., 2016), but little is known about the impact that stress induced microbial dysbiosis may have for pregnancy outcomes and subsequent infant development. Indeed the maternal gut and vaginal microbiome undergoes remodelling throughout the prenatal period to support a healthy pregnancy and changes to the maternal vaginal microbiome has been linked PTB (Stout et al., 2017, Koren et al., 2012). Alterations in the early infant gut microbiome is been increasingly linked to immune disorders in infancy and PNMD is beginning to be recognized as one of the factors that may alter the infant gut microbiome (Jasarevic et al., 2015b). It is hypothesised that PNMD alters the maternal gut and/or vaginal microbiome and that this stressed microbiome is vertically transmitted to the infant during parturition (Jasarevic et al., 2014). This stressed microbial signature then interferes with normal microbial host interactions important for infant development, subsequently conferring an increased risk of disease. However this has yet to be examined in human populations and needs to be considered in the context that the gut microbiome also appears to be a critical regulator of the host stress response and that the gut microbiota acquired early in life may be involved in priming the Hypothalamic Pituitary Adrenal (HPA) axis for appropriate physiological outputs (Clarke et al., 2014a).

The aim of this review chapter is to provide a comprehensive overview of the literature. Firstly I will discuss the current epidemiological evidence linking PNMD to adverse outcomes. Next I will give a detailed account of the biological

mechanisms that may underlie this relationship, briefly mentioning less studied and potentially relevant mechanisms, followed by a detailed account of the glucocorticoid hypothesis and placental HSD11B2. Finally I will discuss the importance of the maternal and infant microbiome. This will precede the aims of the current thesis.

1.1 Evidence from Epidemiological studies:

A significant proportion of women suffer from psychological disorders in the perinatal period and in a systematic review conducted by Bennett and colleagues, the prevalence rate of depression during pregnancy was found to be 7.4%, 12.8% and 12% in the first, second and third trimester respectively (Bennett et al., 2004). Similarly, anxiety is commonly reported during pregnancy with the prevalence depending on the type of anxiety experienced (Ross and McLean, 2006). In a study population of 1522 women in the US, 6% of women reported high, 78% reported low/moderate and 16% reported no psychological stress during the second and third trimester of pregnancy (Woods et al., 2010). Thus, a significant percentage of pregnant women experience some form of PNMD during pregnancy that can potentially affect the developing fetus. There is a growing body of literature available on PNMD, whereby a large number of epidemiological studies have confirmed that PNMD is a risk factor for a number of adverse fetal outcomes and increases the risk in particular for a number of neuropsychiatric disorders in adulthood. Here I discuss the current state-of-the-art of this field with a focus on human studies that have identified PNMD as a risk factor for adverse outcomes.

1.1.0 PNMD alters birth outcomes:

Low Birth Weight, Preterm Birth and Intrauterine Growth Restriction:

The World Health Organization (WHO) defines LBW as infants born less than 2,500 grams, which affects ~15.5% of pregnancies (World Health Organisation, 2004). LBW often arises as a result of PTB (<37 weeks gestation) or IUGR. PTB affects 10% of all births (Beck et al., 2010) and is estimated to account for 35% of all neonatal deaths worldwide (Blencowe et al., 2012). IUGR complicates between 10-15% of all

pregnancies (Saleem et al., 2011). Babies born below the 10th percentile for their gestational age are referred to as Small for Gestational Age (SGA) and IUGR is a common cause of SGA (Saleem et al., 2011). In recent years PNMD has been identified as a prenatal risk factor for LBW, PTB, SGA and IUGR.

The relationship between maternal anxiety and these birth outcomes has been recently reviewed. In this meta-analysis of 15 studies, women who reported anxiety in pregnancy were at a 50% increased risk of PTB and a 76% increased risk of LBW, independent of the timing of anxiety measurement (Ding et al., 2014b). This risk was heighted for women of lower socioeconomic status and lower prenatal care. Additionally Asian women were at an elevated risk as compared to women from European countries (Ding et al., 2014a).

Grote and colleagues undertook a meta-analysis of studies to clarify the risk of adverse birth outcomes following maternal prenatal depression (Grote et al., 2010). This meta-analysis found a relationship between prenatal depression and adverse outcomes to be moderated by the type of psychometric measurement used. When depression was defined categorically to be clinically significant, there was a 39%, 45% and 49% increased risk of PTB, IUGR and LBW respectively. When analysed on a continuous scale every 1 unit increase in depression scores was associated 3%, 2% and 4% increase risk of PTB, IUGR and LBW respectively. The risk of adverse outcomes increased for women who reported depression and were living in developing countries or of low socioeconomic class, highlighting particularity at risk populations (Grote et al., 2010).

To our knowledge the relationship between psychological stress and birth outcomes has not been systematically reviewed. This is likely a reflection of the complex range of psychometric tools used to assess prenatal stress which range from environmental exposures, bereavement, biological assessments to a vast array of different questionnaire based methods. A summary of current studies investigating this relationship between PNMD and birth outcomes can be found summarized in Table 1 of this thesis. It is clear that this relationship is complex and often reports yield inconstant results with variability arising from differences in study design, populations, timing of stress measurement and type of stress measurement. None the less the majority of research highlights an increased risk of

adverse birth outcomes with heightened maternal psychological stress, although a systematic review and meta-analysis of this relationship is warranted.

Other birth outcomes:

Although the incidence of infant mortality has decreased significantly over the past 2 decades, a large portion of infants still die within the neonatal period. In 2009, 3.3 million babies died in the first month of life and 2.6 were stillborn (World Health Organistation, 2014). Whilst there are a wide range of known causes, PNMD has recently been identified as a risk factor. In particular maternal bereavement in both the preconception and prenatal period, have been linked to an increase risk of stillbirth and infant mortality (Class et al., 2013, Laszlo et al., 2013). Additionally in a smaller prospective study in a Danish population, PNMD, assessed using the 12-item general health questionnaire at 16 and 32 weeks' gestation was associated with a 90% increased risk for stillbirth (Wisborg et al., 2008). Miscarriage occurs in 10-15% of observed pregnancies and it is thought that environmental factors, such as PNMD may play a role. Indeed some studies support and increased risk (Boyles et al., 2000, Bashour and Abdul Salam, 2001, Neugebauer et al., 1996), whilst others do not (Nelson et al., 2003). PNMD has also been linked to congenital heart disease (Carmichael and Shaw, 2000) a major global health problem affecting over 1.35 million newborns each year (van der Linde et al., 2011). Finally, while some studies support a link between PNMD and reduced head circumference at birth (Lou et al., 1994, Hansen et al., 2008, Hansen et al., 1996), which is predictor for neurodevelopment disorders later in life (Garcia-Alix et al., 2004), others do not, and indicate that the effect PNMD on child neurodevelopment may not be mediated by an effect on brain size (Obel et al., 2003). However, both studies suffer from a small sample size and to our knowledge, there is currently no *large* cohort study that has examined an associated between PNMD and head circumference at birth. Until such a study is conducted the association between PNMD and head circumference at birth remains unclear.

Table 1:

Author	Design (N)	Prenatal Stressor	Timing	Outcomes	Results
(Khashan et al., 2009)	Prospective (1.3m)	*Maternal bereavement*	Preconception	**PTB**	↑ risk PTB
(Class et al., 2011)	Prospective (2.6m)	*Maternal bereavement*	Month 5&6	**PTB** **LBW** **SGA**	↑ risk PTB & ↓ gestations ↑ risk LBW ↑ risk SGA
(Khashan et al., 2008b)	Prospective (1.4m)	*Maternal bereavement*	Pre-/Post-conception	**SGA**	↑ risk SGA
(Barbosa, 2000)	Prospective (472)	*Maternal bereavement*	During pregnancy	**GL**	↓ gestational length
(Pritchard and Teo, 1994)	Prospective (393)	*Perceived stress*	20 wks.	**PTB** **LBW**	↑ risk PTB ↑ risk LBW
(Hedegaard et al., 1996)	Prospective (8719)	*Psychological*	30 wks.	**PTB**	↑ risk PTB
(Nordentoft et al., 1996)	Prospective (2432)	*Psychological*	20 wks.	**PTB** **IUGR**	↑ risk PTB ↔ IUGR
(Rondo et al., 2003)	Prospective (865)	*Psychological*	3rd trimester	**PTB** **LBW**	↑ risk PTB ↑ risk LBW
(Nkansah-Amankra et al., 2010)	Case control (8064)	*Negative life Events*	During pregnancy	**LBW** **PTB**	↑ risk LBW ↑ risk PTB
(Zhu et al., 2010)	Prospective (1800)	*Negative life Events*	1st trimester 2nd trimester	**LBW** **PTB**	↑ risk LBW ↑ risk PTB
(Copper et al., 1996)	Prospective (2593)	*Psychological*	25-29 wks.	**LBW** **PTB** **IUGR**	↑ risk LBW ↑ risk PTB ↔ IUGR
(Roy-Matton et al., 2011)	Prospective (303)	*Psychological*	10-20 wks.	**PTB**	↑ risk PTB
(Baibazarova et al., 2013)	Prospective (158)	Psychological	15.3 -18.2 wks.	**LBW** **GL**	↑ risk LBW ↓ gestational length
(Khashan et al., 2014)	Prospective (8531)	*Psychological*	20 wks.	**SGA**	↑ risk SGA
(Pagel et al., 1990)	Prospective (100)	*Negative life Events*	31-36 wks.	**LBW** **Apgar Score**	↑ risk LBW ↓ Apgar score
(Sable and Wilkinson, 2000)	Case control (2378)	*Perceived stress*	During Pregnancy	**LBW**	↑ risk LBW
(Wadhwa et al., 1993)	Prospective (90)	*Negative life Events*	28-30 wks.	**LBW**	↑ risk LBW
(Witt et al., 2014b)	Population (9035)	*Negative life Events*	Preconception	**PTB**	↑ risk PTB
(Witt et al., 2014a)	Population (9035)	*Negative life Events*	Preconception	**LBW**	↑ risk VLBW
(Mansour and Rees, 2011)	Prospective (1391)	*Perceived Stress*	28 & 36 wks.	**LBW** **PTB** **SGA**	↔
(Oyarzo et al., 2012)	Case Control (3609)	*Environmental*	1st & 2nd Trimester	**PTB** **SGA**	↑ risk PTB ↑ risk SGA
(Glynn et al., 2001)	Case control (40)	*Environmental*	1st trimester	**GL**	↓ gestational length
(Torche and Kleinhaus, 2012)	Case Control (8064)	*Environmental*	Month 2 & 3	**PTB**	↑ risk PTB

Table 1: Maternal prenatal psychological stress and adverse birth outcomes. *Abbreviations:* Preterm birth (PTB), low birth weight (LBW), small for gestational age (SGA), gestational length (GL) and intrauterine growth restriction (IUGR).

1.1.1 Effects of PNMD on long term outcomes:

As well as poor obstetric outcomes, there is now an extensive body of literature that implicates PNMD in a number of adverse long-term outcomes that affect multiple organ systems later in life. These include immune and cardio-metabolic disturbances, and altered neurodevelopmental trajectories and increased risk of neurodevelopmental and neuropsychiatric disorders in affected infants. Although a large body of pre-clinical evidence supports these findings, which has been extensively reviewed elsewhere (Sanchez et al., 2001), here we briefly summarize studies in human populations where maternal exposure to PNMD in the prenatal period has been linked to disease in adulthood.

Immune related disorder in infants affected by PNMD:

PNMD, particularly when experienced in the third trimester, is associated with an increased risk of wheezing, coughing and breathlessness in infants (Mathilda Chiu et al., 2012, Wright et al., 2013), a major cause of morbidity in preschool children (Bisgaard and Szefler, 2007). Additionally, two large prospective cohort studies have identified maternal bereavement as a risk factor for immune-related disease in adulthood. In a large cohort of 3.2 million Swedish women, maternal bereavement in the first and second trimester was found to be associated with a 48% increase in the incidence of hospitalization for asthma (Khashan et al., 2012). In the second study, involving 1.6 million Danish women, maternal bereavement during pregnancy was associated with a 71% and 39% increased risk for hospitalization with severe and less severe infectious disease respectively (Nielsen et al., 2011).

Increased risk of cardio-metabolic disturbances in infants affected by PNMD:

Fetal exposure to maternal stress is also associated with altered cardiovascular function in childhood. In a cohort of 2968 Dutch women, psychological stress experienced at 16 weeks' gestation was associated with increased systolic blood pressure, diastolic blood pressure and mean arterial pressure in children at 5 years of age (van Dijk et al., 2012a). In the same population, maternal stress at 16 weeks' gestation was not associated with an altered cardiac autonomic nervous system

balance (an early indicator of cardiovascular disease) in 5 year old children (van Dijk et al., 2012b). Whether PNMD has a long-term effects on cardiovascular disease in adulthood remains unknown, as no study to date has examined this relationship in a population older than 7 years of age.

The prevalence of obesity has increased dramatically over the past 3 decades, doubling since the 1980s (World Health Organisation, 2014). It has been suggested that exposure to maternal stress during fetal life may be a risk factor for developing obesity. In a large cohort study in a Danish population maternal bereavement during, or up to 6 months before pregnancy was associated with an increased risk of childhood overweight in children aged 7-13. Children whose mothers experienced bereavement up to six months before pregnancy were at the highest risk, being three times more likely to be overweight at 12 years of age (Li et al., 2010b). In a similar cohort study in a Danish population, psychological distress assessed by telephone interview at 16 and 30 weeks gestation was not found to be associated with weight in 7-year-old children. However when stratified based on gender a modest increase of 15% for the risk of overweight was seen in boys, but not girls (Ingstrup et al., 2012). More recently a study has been conducted that supports the risk for overweight in adulthood. In a cohort study among men aged 17-31, maternal bereavement up to 6 months before and during pregnancy was associated with an increased risk for overweight and obesity. The strongest association was seen for men who had a 25% risk of obesity and a 14% risk for being overweight if their mothers were in their first trimester at the time of the bereavement (Hohwu et al., 2014).

PNMD has also been linked to developing other types of metabolic disease. Women whose mothers reported psychosocial stress during pregnancy have an elevated insulin response to a glucose tolerance test, which is an early indicator of insulin resistance (Entringer et al., 2008). Elevated insulin secretion was found in women whose mothers reported high levels of stress as a result of the 1988 Quebec ice storm (Dancause et al., 2013). However, a recent study failed to confirm this association and found no relationship between prenatal maternal stress, depression and anxiety and glucose metabolism in 5-year-old children (van Dijk et al., 2014b). This might be explained by the age difference between the subjects,

suggesting that the effects of early life stress on diabetes risk may only present later in life. Further, the latter study assessed psychological stress at 16 weeks gestation only, a time point that may not represent a window of vulnerability for this effect (van Dijk et al., 2014a). Finally, in a large prospective cohort study in a population of 1.8 million women in Denmark, exposure to maternal bereavement in the second trimester was associated with a 15% increased risk of developing type 2 diabetes in offspring (Li et al., 2013a).The cumulative data in these studies highlights a role for PNMD in the aetiology of cardio-metabolic disorders in affected offspring.

PNMD and disorders of the nervous system:

The programming effect of PNMD is most widely studied in relation to the nervous system. There is a large amount of research linking PNMD to poor neurodevelopmental outcomes in infancy, which can be found summarized in Table 2. A number of studies have found PNMD to confer an increased risk of neuropsychiatric conditions including, but not limited to, autism spectrum disorder (ASD), attention deficit hyperactivity disorder (ADHD), schizophrenia, effective disorders and eating disorders (Table 2). Altered HPA axis activity has been associated with many psychiatric disorders (Belvederi Murri et al., 2014, Baumeister et al., 2014) and prenatal programming of the HPA axis may contribute to the biological mechanism through which PNMD programmes adult psychiatric disease. Indeed altered HPA activity, evident by increased cortisol outputs, has been observed in offspring born to high stress pregnancies (Table 2).

Table 2:

Author	Design (N)	Prenatal Stressor	Timing	Outcomes	Results
(Huizink et al., 2003)	Prospective (170)	(1) Everyday problem list (2) Salivary cortisol (3) PRAQ	(a)15-17 wks. (b)27-28 wks. (c)37-38 wks.	3 & 8 months **BSID**	(1a) ↓ MDI at 8mo (2c) ↓ MDI 3mo; ↓ MDI & PDI at 3mo and 8mo (3b) ↓ MDI & PDI at 8mo, (3c) ↓ MDI at 8mo
(Gutteling et al., 2005b)	Prospective (172)	(1) Daily Hassles (2) PRAQ (3) PSS (4) GHQ (5) Salivary cortisol	During pregnancy	27 months **(a) ICQ** **(b) BSID** **(c) CBCL**	(2a) ↑ restless/disruptive behaviour (3a) ↓ restless/disruptive behaviour (2b) ↑ attention regulation problems (3c) ↑ problem behaviour (3c) ↑ externalizing problems
(DiPietro et al., 2006)	Prospective (94)	(1) POMS-A (2) STAI (3) DSI (4) PSS (5) CES-D (6) POMS-D (7) PES	24-32 wks.	2 years **BSID** (Heartrate)	(3&4) ↑ PDI (1&2) ↑ PDI and MDI (5&6) ↑ PDI and MDI (7) ↓ PDI
(Bergman et al., 2007)	Retrospective (123)	(1) Stressful life events	During Pregnancy	14-19 months **(a) BSID** **(b) Lab-TAB**	(1a) ↓ MDI (1b) ↑ fearfulness
(Bergman et al., 2010b)	Prospective (125)	(1) Amniotic fluid cortisol (2) Stressful life events	15-20wks	14-19 months **BSID**	(1) ↓ MDI (2) ↓ cognitive development (BSID total)
(Davis and Sandman, 2010)	Prospective (125)	(1) PSS (2) Salivary cortisol (3) CES-D (4) STAI (5) PSA (6) PSI	(a) 15 wks. (b) 19 wks. (c) 25 wks. (d) 30 wks. (e) 36 wks.	3, 6 & 12 months **BSID**	(2a) low ↑ MDI (2e) high ↑ MDI (5a) ↓ MDI
(Zhu et al., 2014)	Case Control (38 cases, 114 controls)	PLEC	(1) 1st trimester (2) 2nd trimester (3) 3rd trimester	16-18 months **(a) BSID-CR** **(b) TTS**	(1a) ↓ MDI (1b) ↑ regularity & persistence and attention span
(Bhang and Ha, 2016)	Prospective (648)	(1) PWI (2) CES-D	(a) 1st trimester (b) 3rd trimester	6 months **BSID** ***ASQ**	(1a) ↓ MDI (2a) ↓ MDI
(Karam et al., 2016)	Prospective (71)	PSS	(1) During Pregnancy (2) 2 months postpartum	1 year **BSID-III**	(1) ↑ motor scale core (2) ↓ motor and socio-emotional development
(Henrichs et al., 2011)	Prospective (3139)	Family stress	20 wks.	18 months **(1) MCDI** 24 months **(2) PARCA** 37 months **(3) Snack & gift delay task**	(1)↓ word production (1) ↓ word comprehension (2) ↓nonverbal cognition (3) ↓ snack delay performance (3) ↓ gift delay performance

Table 2 continued:

(Baibazarova et al., 2013)	Prospective (158)	(1) PSS (2) PRAQ (3) STAI (4) Plasma cortisol (5) Amniotic fluid cortisol	15-18 wks.	3 months **IBQ**	(1) ↑ distress to limitation
(Pesonen et al., 2005)	Retrospective (319)	Psychological Stress	During Pregnancy	6 months **IBQ**	↑ Negative temperament
(Davis et al., 2007)	Prospective (247)	(1) CES-D (2) STAI (3) PSS (4) Salivary cortisol	(a) 18-20 wks. (b) 24-26 wks. (c) 30-32 wks.	2 months **IBQ**	(4c) ↑ negative reactivity (1a,b,c) ↑ negative reactivity (2a,b,c) ↑ negative reactivity
(de Weerth et al., 2003)	Prospective (17)	Salivary cortisol	34 wks.	1-20 weeks **(1) Behavioural observations** 7& 18 weeks **(2) ICQ**	(1) ↑ crying, fussing & negative facial expression (2) ↑ scores at 7 weeks only (negative temperament)
(Bergman et al., 2010a)	Prospective (108)	(1) Amniotic fluid cortisol (2) Amniotic fluid testosterone	15-20wks	14-19 months **Lab-TAB**	(1)↔ (2) ↑ fear reactivity ♂
(Austin et al., 2005)	Prospective (970)	(1) Perceived stress to life event (2) STAI (3) EPDS	3rd trimester	4-6 months **SITQ**	(2) ↑ scores (worse temperament) (3) ↑ scores
(van der Wal et al., 2007)	Prospective (8266)	(1) CES-D (2) PRAQ (3) PDH (4) Work stress	16 wks.	3-6 months **Crying**	(1) ↑ crying (2) ↑ crying (3) ↑ crying (4) ↑ crying
(Sondergaard et al., 2003)	Prospective (378)	(1) General distress (2) GHQ	During Pregnancy	Within month 1 **Infantile colic**	(1) ↑ risk
(Wurmser et al., 2006)	Prospective (86)	LES	18 wks.	6 weeks, 3 & 6 months **Crying** **Fussing**	↑ crying and fussing
(Slykerman et al., 2005)	Retrospective (539)	(1) PSS (2) Social support	Late Pregnancy	3.5 years **SBIS**	(1) ↓ intelligence (2) ↑ social support ↑ intelligence
(Laplante et al., 2008)	Retrospective (89)	(1) Storm exposure (objective) (2) IES (subjective) (3) GHQ (4) LES	During Pregnancy	5.5 years **(a) WPPSI** **(b) PPCT**	(1a) ↓ scores (intellectual) (1b) ↓ scores (language)
(Virk et al., 2014)	Prospective (167,900)	Maternal Bereavement	12 month prior to & during pregnancy	18 years **BPP (IQ)**	↓ IQ scores in ♂
(Rodriguez and Bohlin, 2005)	Prospective (290)	PSS	24 – 32 wks. 28 wks. 32 wks.	**ADHD**	↑ risk
(Ronald et al., 2010)	Prospective (2868)	Stressful life events	During pregnancy	**ADHD**	↑ risk in 2 year olds
(Grizenko et al., 2012)	Case Control (71 case 71 controls)	Stressful life events	During pregnancy	**ADHD**	↑ symptom severity in children exposed
(Zhu et al., 2015)	Prospective (2455)	-Stressful life events -Social support -Avoidance coping	2nd trimester	**ADHD**	SLEs, high avoidance coping & low social support ↑ ADHD symptomology in ♂

Table 2 continued:

(Li et al., 2010a)	Prospective (1 million)	Maternal bereavement	(1)Preconception & (2) 3rd trimester	**ADHD**	↑ risk in ♂
(Class et al., 2014)	Prospective (2.4 million)	Maternal bereavement	3rd trimester	**ADHD**	↑ risk
(Grizenko et al., 2008)	Case Control (110 cases 93 controls)	Stressful life events	During pregnancy	**ADHD**	↑ symptom severity in children exposed
(Motlagh et al., 2010)	Case Control (222)	Psychosocial Stress	During Pregnancy	**ADHD**	↑ risk
(Beversdorf et al., 2005)	Case Control (400)	Psychological	21-32 wks.	**Autism**	↑ risk in PNS at 25-28wks
(Kinney et al., 2008)	Case Control (320,686(	Environmental	Months 5, 6, 9, 10	**Autism**	↑ risk related to severity
(Walder et al., 2014)	Prospective (89)	Environmental	During Pregnancy	**Autism**	↑ risk
(Ronald et al., 2010)	Prospective (2868)	Stressful life events	During pregnancy	**Autism**	↑ risk ♂
(Class et al., 2014)	Prospective (2.4 million)	Maternal Bereavement	Third trimester	**Autism**	↑ risk
(Li et al., 2009b)	Prospective (1,492,709)	Maternal Bereavement	12mt prior to & during pregnancy	**Autism**	↔ risk
(Rai et al., 2012)	Prospective (4501 ASD 54,831 control)	Psychological	During Pregnancy	**Autism**	↔ risk
(Spauwen et al., 2004)	Prospective	Maternal stress	During pregnancy	**Childhood Psychotic Experiences**	↑ risk
(Dorrington et al., 2014)	Prospective (5038)	Stressful life Events	During pregnancy	**Childhood Psychotic Experiences**	↔ risk
(Betts et al., 2014)	Prospective (2227)	Negative life Events	During pregnancy	**Psychotic Experiences**	↔ risk
(Abel et al., 2014)	Prospective	Maternal Bereavement	Preconception & postnatal	**Psychosis**	↑ risk when exposed in postnatal or preconception period
(Khashan et al., 2008a)	Prospective (1.38 million)	Maternal bereavement	1st trimester	**Schizophrenia**	↑ risk
(Malaspina et al., 2008)	Prospective (88,829)	Environmental	Month 3	**Schizophrenia**	↑ risk
(Susser and Lin, 1992)	Prospective (40,000)	Environmental	1st trimester	**Schizophrenia**	↑ risk
(Selten et al., 2003)	Retrospective	Environmental	During pregnancy	**Schizophrenia**	↔ risk
(Fineberg et al., 2016)	Case control (95 cases 206 controls)	Stressful life events	During pregnancy	**Schizophrenia spectrum disorders**	↑ risk ♀ only
(Slykerman et al., 2016)	Retrospective (620)	PSS	Late pregnancy	11 years **(CED-S)**	↑ risk moderate depression
(Van den Bergh et al., 2016)	Prospective (86)	STAI	(1) 12-22 wks. (2) 23-32 wks. (3) 32-40 wks.	14-15 year **(a) CDI (b) Salivary cortisol**	(1b) Trait anxiety ↓ daytime cortisol (1a) Trait anxiety ↑ depression in ♀
(Kleinhaus et al., 2013)	Case Control (92,408)	Environmental	First trimester	**Affective disorder**	↑ risk
(Khashan et al., 2011)	Prospective (1.1 million)	Maternal bereavement	2nd trimester	**Affective disorder**	↑ risk
(Class et al., 2014)	Prospective (2.4million)	Maternal Bereavement	12mt before & during pregnancy	**Bipolar Disorder**	↔ risk
(Li et al., 2008)	Prospective (1.5 million)	Maternal bereavement	1st trimester	**Epilepsy**	↔ risk

Table 2 continued:

(Li et al., 2009a)	Prospective (1.4 million)	Maternal bereavement	Preconception	**Febrile Seizures**	↔ risk
(Shang et al., 2010)	Case-control (120 infants)	Stressful life events	Pregnancy	**Infantile spasms**	↑ risk
(Cao et al., 2014)	Prospective (89(	Environmental	During Pregnancy	**Motor Function**	↓ motor functions
(Li et al., 2009c)	Prospective (1.5 million)	Maternal Bereavement	Pre-/post-conception	**Cerebral palsy**	↑ risk in non-IUGR child. ↑ risk in IUGR child.
(Su et al., 2015)	Prospective (5 million)	Bereavement	Preconception	**Eating Disorder**	↑ risk ♂
(Liang et al., 2013)	Prospective (4 million)	Bereavement	Pregnancy	**Substance Abuse**	↔ risk
(Entringer et al., 2009)	Case Control (31 case 30 controls)	Psychosocial	During Pregnancy	**HPA activity**	↑ ACTH levels ↑ cortisol response
(Davis et al., 2011)	Prospective (116)	Psychological	During pregnancy	**HPA Activity**	↔ affect
(Gutteling et al., 2005a)	Prospective (25)	Maternal Cortisol	During pregnancy	**HPA Activity**	↑ cortisol response
(Gutteling et al., 2004)	Prospective (24)	Maternal Cortisol	During pregnancy	**HPA Activity**	↑ cortisol response

Table 2: Prenatal psychological stress and neurological outcomes in offspring. *Abbreviations:* Pregnancy Related Anxiety Questionnaire (PRAQ), Baily Scales of Infant Development (BSID), Mental Developmental Index (MDI), Psychomotor Developmental Index (PDI), Perceived Stress Scale (PSS), General Health Questionnaire (GHQ), Infant Characteristics Questionnaire (ICQ), Child Behavioural Checklist (CBCL), Profile of Mood States-Anxiety(POMS-A), State Trait Anxiety Inventory (STAI), Daily Stress Inventory (DSI), Centre for Epidemiological Studies-Depression (CES-D), Profile of Mood States-Depression Subscale(POMS-D), Pregnancy Experience Scale (PES), Pregnancy Specific Anxiety (PSA), Parenting Stress Index (PSI), Prenatal Life Event Checklist (PLEC), Toddler Temperament Scale (TTS), Psychosocial Well-being Index (PWI), Ages and Stages Questionnaire (ASQ), Minnesota Child Development Inventory (MCDI), Parent Report of Children's Abilities (PARCA), Infant Behavioural Questionnaire (IBQ), Short Infant Temperament Questionnaire (SITQ), Edinburgh Postnatal Depression Scale (EPDS),Pregnancy Daily Hassles (PDH), Depression, Anxiety and Stress Scales (DASS), Stanford Binet Intelligence Scale (SBIS), Impact of Events Scale (IES), Life Event Stress (LES), Wechsler Preschool and Primary Scale of Intelligence (WPPSI), Peabody Picture Vocabulary Test (PPCT), biophysical Profile Score (BPP).

1.1.2 Assessing maternal distress in pregnancy:

Overall the current epidemiological literature outlined above identifies PMND as a risk factor for a number of adverse outcomes for both mother and child. However there is an overwhelming amount of variability between current reports. This is likely a result of the extensive amount of screening tools that researchers use to determine maternal experience of PNMD. These range from environmental exposures to war or storms (Laplante et al., 2008, Kertes et al., 2016), experience of bereavement due to death of a close relative (Class et al., 2014, Khashan et al., 2011) to a vast array of questionnaire based measurements (Khashan et al., 2014). Even within the questionnaire based approach there are a considerable amount of tools available that examine different aspects of distress, including, but not limited to, objective stress, work stress, perceived stress, stress from daily hassles, financial stress, family stress, general anxiety, anxiety specify to pregnancy and depressive symptoms (Lazinski et al., 2008). In a thorough review of psychometric instruments, Nast and colleagues have shown the Perceived Stress Scale (PSS), State Trait Anxiety Inventory (STAI) and Edinburgh Postnatal Depression Scale (EPDS) to be the most reliable indicators of prenatal maternal stress, anxiety and depression respectively (Nast et al., 2013). Indeed a further consideration when assessing questionnaire-based stress readouts arises when deciding on cut-off scores in questionnaires that are clinically relevant to reflect high distress. With regards to the EPDS, an EPDS score ≥ 13 has been reported to have the highest specificity (97.8%), yet low sensitivity (16.8%) for predicting depressive symptoms in pregnancy. Authors have also reported a lower cut off of ≥ 5 for high depression kept both specificity and sensitivity to 70%, suggesting an adequate cut off point for initial screening (Meijer et al., 2014). In other studies EPDS ≥ 9/10 have been used to define clinically relevant depression (Shakeel et al., 2015, Alvarado-Esquivel et al., 2014). For the PSS and STAI, no standardized estimates have been reported and grouping based on cohort quartiles or means have been used (Khashan et al., 2014, Vijayaselvi et al., 2015). In contrast some studies have opted to use the questionnaire scores as continuous, rather that categorical variables which can yield different outcomes. Single time-point measurements represent another factor contributing to variability between studies. It is suggested that when experienced in

early pregnancy women perceive events as more stressful that when experienced in late pregnancy (Glynn et al., 2001). Additionally studies that have prospectively examined stress in pregnancy highlight specific periods of vulnerability (Davis and Sandman, 2010). Therefor examining parameters of distress prospectively across pregnancy would have greater accuracy.

Measuring physiological parameters of maternal distress is also important as stress induced alterations in maternal physiology is most likely responsible for adverse outcomes (Lazinski et al., 2008). Maternal cortisol is most commonly used as a physiological indicator of distress. However many studies report no association between maternal psychological distress and cortisol levels (Baibazarova et al., 2013, Harville et al., 2009, Bergman et al., 2010b, Davis and Sandman, 2010). This could be a result of cortisol measurements, as assessing human cortisol levels is a methodological challenge. Whilst some studies analyse cortisol in maternal serum, others use maternal saliva. Measurements in serum samples can be skewed, as most serum cortisol is bound to proteins and therefore not biologically active. More accurately, cortisol should by analysed in saliva samples as this represents a more precise indicator of free, unbound cortisol levels (Aardal-Eriksson et al., 1998). Limitations still arise as many cortisol samples rely on the compliance of patients to take salivary samples at specific times and slight variations in this compliance can alter accuracy of the cortisol circadian profile (Kudielka et al., 2003). Further, many environmental factors are known to alter cortisol secretion. For example, salivary cortisol concentrations display seasonal variation (Persson et al., 2008). Sleep disturbances decrease the salivary cortisol awakening response (Backhaus et al., 2004). Food intake (Hershberger et al., 2004), caffeine (Lovallo et al., 2005), family history of alcoholism (Sorocco et al., 2006), physical activity (Frey, 1982), smoking (Rohleder and Kirschbaum, 2006) and alcohol consumption (Kokavec et al., 2009) are all known to alter the HPA axis. The maternal HPA is a highly dynamic and adaptive system that displays marked intra- and inter-individual variations and many studies which utilize single time point cortisol assessments fail to account for these variations (Hansen et al., 2008). Of particular importance for pregnant women as there is a natural, yet dramatic increase in circulating cortisol in the third trimester (Carr et al., 1981), measuring fluctuations in this period would be

particularly difficult. Indeed assessing other biological parameters of maternal stress should be considered including analysing maternal catecholamines, cytokine profiles and/or placental signalling (Field et al., 2003, Diego et al., 2006, Coussons-Read et al., 2005).

1.2 Mechanisms of transmission:

The mechanisms underlying the link between prenatal distress and negative infant outcomes are an extensively studied area in preclinical science. Understanding these mechanisms is important as it will aid in developing strategies to counteract the impact of the stress exposures and prevent poor outcomes. The most commonly discussed mechanism is the glucocorticoid hypothesis which suggests stress induced increases in maternal HPA activity and subsequently increased fetal exposure to glucocorticoids alters fetal development. At the core of this hypothesis lies the placenta, which ultimately controls the levels of glucocorticoids the fetus is exposed to. More recently attention is being placed on the role of the microbiota in mediating this link. In this hypothesis and in addition to the possibility that the gut microbiome regulates maternal HPA axis outputs, prenatal distress compromises the maternal microbiome and alters the initial seeding and colonisation of the infants' gut, which interferes with postnatal infant development (including postnatal HPA axis programming). Other mechanisms included stressed induced activation of the sympathoadrenomedullary (SAM) system, activation of the maternal immune system and adverse lifestyle behaviours such as increased smoking and alcohol use, unhealthy eating, reduced physical activity and sleep disturbances (Glover, 2015). Here we review the current literature implicating these mechanisms, with focus on the glucocorticoid hypothesis, placenta and microbiome.

1.2.0 The glucocorticoid hypothesis:

The glucocorticoid hypothesis describes how increased fetal exposure to maternal glucocorticoids such as cortisol as a result of prenatal stress during pregnancy is

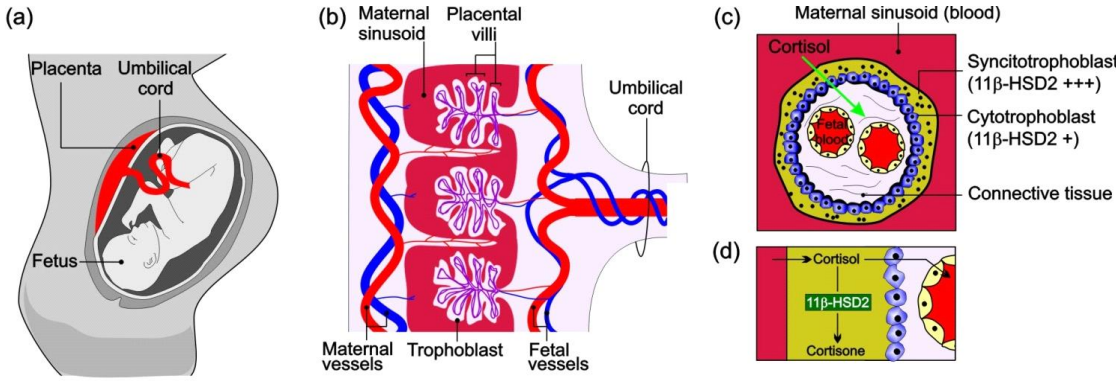
associated with a number of poor birth outcomes and increased risk for neurological and cardio metabolic syndromes in exposed offspring later in life.

As early as the 11-12th week of pregnancy, there are profound changes in the activity of the maternal HPA, which leads to increased production of maternal cortisol. The levels of cortisol continue to rise during each trimester to reach almost three times those of non-pregnant women by the third trimester (D'Anna-Hernandez et al., 2011, Demey-Ponsart et al., 1982, Jung et al., 2011). The HPA axis is regulated by the release of Corticotrophin-releasing hormone (CRH) from the hypothalamus, which stimulates the release of adrenocorticotrophin hormone (ACTH) from the anterior pituitary gland which in turn stimulates the production of cortisol from the adrenal glands. Normally, cortisol regulates its own secretion through a negative feedback loop that inhibits the production of CRH and ACTH (Myers et al., 2012). However, during pregnancy, the placenta begins to release CRH into the maternal bloodstream and levels reach 1,000 -10,000 times that of a non-pregnant woman. This further increases the production of cortisol by the adrenals (Duthie and Reynolds, 2013, Petraglia et al., 1987). Although usually cortisol negatively regulates the secretion of hypothalamic CRH, in contrast, a rise in maternal cortisol actively stimulates the release of placental CRH, which further raises the levels of maternal cortisol leading to the progressive increase in maternal serum cortisol levels seen during pregnancy (D'Anna-Hernandez et al., 2011, Demey-Ponsart et al., 1982, Jung et al., 2011). This increase in maternal glucocorticoid levels has been proposed to be required for normal fetal organogenesis (Smith and Shearman, 1974) and in keeping with this suggestion, antenatal synthetic glucocorticoid administration to pre-term infants has been shown to significantly decrease mortality and morbidity in infants born before 34 weeks of gestation by accelerating lung maturation (Saccone and Berghella, 2016). However, excessive fetal exposure to maternal glucocorticoids have been proposed as the causative mechanism linking prenatal stress to adverse impacts on birth outcomes and result in long-term health consequences in affected infants (Barbazanges et al., 1996). Elevated maternal cortisol levels during pregnancy is associated with a larger amygdala volume and more affective disorders in girls aged 7, directly linking maternal cortisol to long term programming effects (Buss et al.,

2012a). However, no study to date has examined if maternal stress is directly associated with elevated fetal cortisol levels during pregnancy or at term.

Normally the fetus is protected from the high levels of maternal cortisol by molecular mechanisms in the placenta that convert active cortisol to its inactive metabolite, cortisone and as a consequence, fetal cortisol levels have been shown to be ten to thirteen times lower than those found in the maternal circulation (Edwards et al., 1993, Gitau et al., 1998). This barrier function of the placenta is achieved by the two epithelial layers that cover the chorionic villi of the human placenta which serve as the primary barrier between the maternal and fetal circulation (Huppertz, 2008). The first of these epithelial layers is the *syncytiotrophoblast*, a large multi-nucleated terminally-differentiated syncytium (many nuclei in the same cytoplasmic mass), that is formed by the differentiation and fusion of cells derived from second epithelial layer, the underlying *cytotrophoblast (Huppertz and Borges, 2008).* Within these trophoblast layers, there is strong expression of the enzyme HSD11B2 which catalyses the conversion of active cortisol into inactive cortisone thus protecting the fetus from excessive cortisol exposure (Brown et al., 1996, Krozowski et al., 1995, Sun et al., 1997) (Figure 1).

Figure 1: The placental HSD11B2 shield



(a): During pregnancy the placenta acts as a critical regulator that limits fetal exposure to maternal glucocorticoids. (b): Cross sectional image of the human placenta. Blood travels from the fetus through the umbilical arteries (blue) to the chorionic villi where exchange of nutrients and waste products with the mother occurs. The chorionic villi are made up of two epithelial layers; the syncytiotrophoblast and the cytotrophoblast. (c): Longitudinal section of the chorionic villi. HSD11B2 is highly expressed in the synctiotrophoblast (+++) and to a lesser extent in cytotrophoblast (+).Here HSD11B2 converts cortisol to its inactive product cortisone thereby protecting the fetus from glucocorticoid overexposure.

Evidence for glucocorticoid overexposure in fetal programming in humans:

The glucocorticoid hypothesis has been supported by many studies evaluating the relationship between maternal cortisol levels during pregnancy and fetal outcome. In a longitudinal study conducted by Bolton et al., high maternal cortisol levels in the second and third trimester was associated with reduced birthweight and body length, with cortisol accounting for a 19.69% variance in bodyweight and a 9% variance in body length (Bolten et al., 2011). Similarly, high maternal cortisol levels late in pregnancy was associated with shorter gestations as compared to mothers with low cortisol levels (de Weerth et al., 2003) In contrast, maternal cortisol levels in the second trimester were not found to be associated with infant birthweight and reduced gestations (Baibazarova et al., 2013), indicating the third trimester as window of vulnerability for this effect, a time frame coinciding with a critical period for fetal growth and fetal weight gain (Sparks et al., 1980, Archie et al., 2006). There is also evidence to suggest that glucocorticoids can program cardiovascular disease. Rondo et al. found a positive association between maternal cortisol in late pregnancy with low arterial elasticity (Rondó et al., 2010) and systemic vascular resistance (Rondo et al., 2010) in children 5-8 years old.

In relation to brain function, maternal cortisol levels at 15 weeks gestation was positively associated with larger right amygdala volumes and more affective symptoms in girls at 7 years of age (Buss et al., 2012a). Large amygdala volumes is a characteristic of ASD children (Schumann et al., 2004), suggesting a potential role for GC programming of the amygdala be involved in the aetiology ASD. Further, a recent study involving a Danish cohort identified elevations in cortisol and Δ4 sex steroids in the amniotic fluid of infants who were later diagnosed with ASD (Baron-Cohen et al., 2014). High maternal cortisol levels in late second and early third trimester are associated with an elevated cortisol response to the heel prick test in infants and a slower rate of behavioural recovery following the test, indicating HPA axis alterations (Davis et al., 2011). Infants whose mothers have high cortisol levels late in pregnancy display more crying, fussing, negative facial expressions and no vocalization as compared to infants from mothers with low cortisol. These infants, from the high cortisol group had a more negative temperament as compared to the

low cortisol control (de Weerth et al., 2003). High cortisol concentrations in late but not early or mid-gestation were associated with lower Mental Developmental Index (MDI) at 3 months and lower psychomotor developmental Index (PDI) at 3 and 8 months (Huizink et al., 2003). Discordantly, low cortisol levels at 13 weeks' gestation and high cortisol levels at 38 weeks' gestation was associated with a better MDI scores in infants at 12 months. However the slope of maternal cortisol levels across pregnancy was the strongest predictor of infant MDI. This implies that the profile of maternal cortisol across gestation, so that adequate amounts are available at critical times during development, may be the best predictor of infant development (Davis and Sandman, 2010).

Whilst many studies support a role for elevated maternal cortisol, others do not (Bergman et al., 2010a, Baibazarova et al., 2013). However there are methodological issues that need to be considered in the assessment of HPA axis functions as discussed at the beginning of this chapter. Given these large number of potential confounders, perhaps a better measure of fetal cortisol overexposure would be to assess fetal cortisol levels directly. In keeping with this, fetal amniotic cortisol levels in mid gestation have been associated with decreased birth weight (Baibazarova et al., 2013). However performing an invasive procedure during a vulnerable time does not represent an optimal collection method for assessing fetal glucocorticoid overexposure. Hair cortisol analysis is becoming increasingly used as a method to assess HPA activity. Hair growth occurs at an approximate rate of 1cm/month in adults and is thought to be useful as a retrospective calendar of cortisol secretion (Thomson et al., 2010). This highlights the possibility of using neonatal hair as a novel methodological approach to evaluate *in utero* cortisol overexposure (Kapoor et al., 2014b).

An alternative method would be to screen the placenta, given its role as a protective barrier for the developing fetus. Within the syncytiotrophoblast of the placenta an enzyme HSD11B2 coverts active cortisol to inactive cortisone, protecting the fetus from maternal cortisol. Therefore, anything that reduces the expression of this enzyme has the potential to allow excess fetal glucocorticoid overexposure. In addition to increased cortisol levels, stress is often accompanied by elevations in pro-inflammatory cytokines (Coussons-Read et al., 2007), and IL-1β,

IL-6, or TNF-α have been shown to reduce HSD11B2 expression and activity in term human trophoblast cells (Kossintseva et al., 2006, Chisaka et al., 2005). Both noradrenalin and adrenaline, which are secreted as part of a normal stress response, have been shown to reduce HSD11B2 activity and expression *in vitro* (Sarkar et al., 2001). Taken together, this indicates that there are multiple mechanisms through which PNMD may affect placental HSD11B2, which may not be directly related to stress induced elevations in maternal cortisol. Therefore, failure to find an association between maternal cortisol levels and fetal outcomes would not necessarily eliminate a role for maternal glucocorticoids in the programming affect.

1.2.1 Placenta:

HSD11B2:

There is increasing preclinical and clinical evidence to suggest that maternal stress during pregnancy can lead to alterations in placental HSD11B2 expression. For example, direct cortisol infusion into pregnant mice between embryonic day (E) 12 and E15 resulted in an upregulation of HSD11B2 mRNA and protein expression in the placenta at E14.5. However, by E17 placental HSD11B2 mRNA was significantly reduced, indicating an adaptive mechanism whereby acute cortisol exposure initially upregulates this placental barrier to protect the fetus but chronic cortisol exposure reduces its expression resulting in over exposure of the fetus to maternal glucocorticoids (Cuffe et al., 2012). In agreement with these findings, it has also been shown that pregnant rats subjected to acute restraint stress for one hour on gestational day 20, have increased placental HSD11B2 expression (Welberg et al., 2005). In contrast, chronic restraint stress in pregnant rat dams from gestational day 14 to 20 was found to decrease HSD11B2 protein and mRNA expression in the placenta (Welberg et al., 2005, Mairesse et al., 2007). This was accompanied by reduced fetal birth weight and reductions in circulating ACTH levels and decreased adrenal weight (Mairesse et al., 2007). Similarly, pregnant rat dams that were food restricted from gestational day 10 to 20, which causes an increase in maternal corticosterone levels, have reduced placental HSD11B2 mRNA expression and reduced fetal birthweight (Belkacemi et al., 2011b). These offspring display reduced

circulating ACTH, adrenal atrophy and decreased mineralocorticoid and glucocorticoid mRNA expression in the fetal hippocampus indicating alterations in the fetal HPA axis (Lesage et al., 2001). Findings in human pregnancies have been less consistent. Amniocentesis in pregnant women, which is associated with increased anxiety levels and is a form of acute stress in humans, is associated with increased HSD11B2 activity in the placenta (Ghaemmaghami et al., 2014), whereas chronic prenatal anxiety and depression in late pregnancy were found to be associated with reduced activity and expression of placental HSD11B2 (O'Donnell et al., 2012, Seth et al., 2015).

The precise mechanism(s) linking prenatal stress to reduced placental expression of HSD11B2 is unclear, but it has been shown that increased circulating glucocorticoid concentrations as a result of stress stimulates the sympathoadrenal system causing elevated adrenaline and noradrenaline release from the adrenal medulla (Kvetnansky et al., 1995). These stress-induced catecholamines have shown to decrease HSD11B2 mRNA in the BeWo human choriocarcinoma cell line and in primary trophoblastic cells (Sarkar et al., 2001). Women experiencing high levels of stress during pregnancy also have increased circulating levels of pro-inflammatory cytokines (Coussons-Read et al., 2005). Pro-inflammatory cytokines, TNF-α and IL-1β have been shown to decrease the activity and mRNA expression of HSD11B2 *in vitro (Chisaka et al., 2005, Kossintseva et al., 2006, Suzuki et al., 2005).* These studies indicate that there are multiple mechanisms through which prenatal stress may affect placental HSD11B2 expression and/or activity. Collectively, these data highlight an important point; acute stress during pregnancy may not be harmful to the developing fetus due to the ability to upregulate the expression of HSD11B2, however, chronic stress can result in a significant decrease in placental HSD11B2 expression, meaning that the fetus is exposed to excessive levels of maternal cortisol. Although the precise mechanisms mediating these changes in placental HSD11B2 expression remain to be fully elucidated, it has recently emerged that epigenetic regulation may play a crucial role.

The cell biology of epigenetic regulation of gene expression:

The past two decades have been a revolutionary era for the study of epigenetics and epigenetic mechanisms. Broadly, epigenetics refers to the study of heritable changes in gene function that are not caused by changes to DNA sequence (Alikhani-Koopaei et al., 2004). The most intensively studied modes of epigenetic regulation are DNA methylation and histone modification. In the nucleus, stretches of 140-150 base pairs of DNA are wrapped around proteins call histones into structures called nucleosomes (Marsit et al., 2012). The entire collection of DNA and associated histone proteins are called chromatin (Marsit et al., 2012). Normally DNA is tightly coiled around histone proteins (Alikhani-Koopaei et al., 2004). This is a relatively closed structure that restricts transcription factor binding and as a result is associated with a reduced level of gene transcription. Histone modifications involve the phosphorylation, acetylation and methylation of these histone proteins (Lister et al., 2013). Such modifications to histone proteins either increase or decrease how tightly the DNA interacts with the histones and therefore regulates transcription factor binding(Jensen Pena et al., 2012).For example, the addition of an acetyl group by histone acetyl transferase (HAT) enzymes to the histone, loosens the electrostatic bonds between it and the DNA, resulting in a more open configuration and thus renders DNA more accessible to transcription factor binding (Conradt et al., 2013, Nylen et al., 2013, Goffin and Eisenhauer, 2002), while histone deacetylases (HDAC) remove them (Santini et al., 2001a, Santini et al., 2001b). DNA methylation is carried out by enzymes called DNA methyltransferase (DMNT) (Wagner et al., 2010), which attach methyl groups to cysteine residues that are located in cysteine/guanine rich stretches of DNA located in CpG Islands (Cisneros et al., 2003). Methylation of these CpG islands is largely associated with gene silencing by inhibition of transcription factor binding and/or recruitment of transcriptional repressor complexes (Wagner et al., 2010). It is now largely accepted that epigenetics can be modified by environmental exposures – ‘environmental epigenetics’, particularly during development where the epigenome undergoes profound changes (Ding et al., 2012) (Figure 2).

Figure 2: Epigenetic regulation of gene expression.

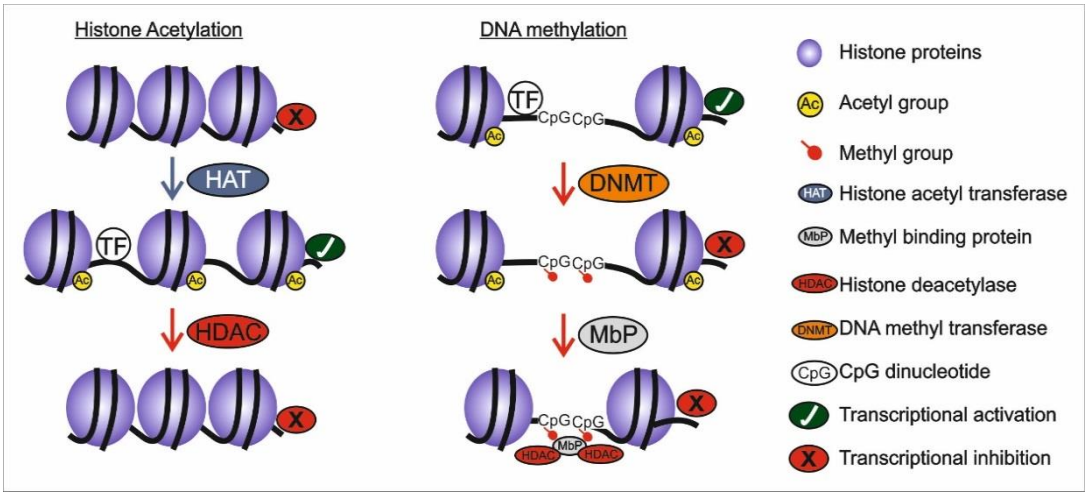


Figure 2: Epigenetic regulation of gene expression. Epigenetic changes are physical modifications to DNA structure that change gene expression. Histone acetylation and DNA methylation are the most common epigenetic changes. Histone acetylation occurs when enzymes called histone acetyltransferases add lysine groups to the N terminal tail of histone proteins. This neutralizes the positive charge on the histone tail, decreasing their interaction with negatively charged DNA. This results in loosening of the DNA around the histones, rendering the DNA more accessible to transcription factor binding and enhanced gene transcription. Histone deacetylation by histone deacetylases removes these lysine residues resulting in reduced gene transcription. DNA methylation is another mode of epigenetic modifications. This is carried out by DNA methyltransferases at CpG islands. This densely methylated island can directly prevent transcription factor binding and therefore reduce gene expression. They can also recruit transcriptional repressor complexes with prevent gene transcription by deacetylation.

Epigenetic regulation of HSD11B2 expression:

The human *HSD11B2* gene contains four CpG islands, two located in the promoter and exon 1 and two in exon 5 and the downstream region (Marsit et al., 2012, Alikhani-Koopaei et al., 2004). Hypermethylation of normally unmethylated CpG islands is indicative of transcriptional repression of the relevant gene (Lister et al., 2013). Interestingly, hypermethylation of *HSD11B2* CpG islands correlates with lower levels of HSD11B2 expression (Alikhani-Koopaei et al., 2004). Low methylation levels of *HSD11B2* were observed in the placenta (were HSD11B2 expression is high), whereas in skeletal muscle (low HSD11B2 expression) the *HSD11B2* promoter was hypermethylated (Alikhani-Koopaei et al., 2004). It has been shown that the activity of the *HSD11B2* promoter is directly regulated by methylation (Alikhani-Koopaei et al., 2004). These data show that exposures that change *HSD11B2* methylation status in the placenta can result in transcriptional repression of the gene, potentially causing the fetus to be exposed to higher levels of cortisol that may adversely affect fetal development. In agreement with this idea, Marsit and colleagues determined the methylation status of *HSD11B2* in 186 placentae and examined any correlation with fetal birth and neurodevelopmental milestones. The methylation status of 4 CpG islands in placental *HSD11B2* was examined, showing a statistically significant, albeit moderate, negative correlation with placental HSD11B2 mRNA expression, indicating that changes in the methylation status of placental HSD11B2 can regulate its expression (Marsit et al., 2012). Intriguingly, a negative correlation was found between infant birth weight and ponderal index (ratio of weight for length) and *HSD11B2* methylation, demonstrating that smaller and leaner/thinner infants showed increased levels of *HSD11B2* methylation (Marsit et al., 2012). When the extent of *HSD11B2* methylation was examined in IUGR infants compared with non-IUGR infants, IUGR infants showed a significantly greater extent of *HSD11B2* methylation than non-IUGR infants (Marsit et al., 2012). They also examined infant neurobehavioral outcomes and found that the extent of *HSD11B2* methylation was greatest in infants with reduced quality of movement scores as assessed using the NICU Network Neurobehavioral Scales (NNNS) examination (Marsit et al., 2012). This

study is important as it was the first one to link growth, epigenetic alterations of placental genes, and early-life neurobehavioral outcomes in humans. Indeed since these work others have confirmed the link between HSD11B2 methylation and newborn neurobehavior (Appleton et al., 2015, Paquette et al., 2015). Clearly, longer follow-up of infants, beyond the neonatal period is required to determine if methylation status of placental *HSD11B2* can help identify those children who may develop neurobehavioral or learning difficulties later in life (Marsit et al., 2012). This suggests that the epigenetic status of placental *HSD11B2* could also be a useful predictor of other adverse outcomes.

Recent data using a rat model of prenatal stress has now demonstrated that prenatal maternal anxiety caused a reduction of HSD11B2 placental expression and, importantly, that this was associated with increased CpG methylation of placental *HSD11B2 (Jensen Pena et al., 2012)*. This study also attempted to assess whether methylation at certain CpG sites within placental *HSD11B2* positively predicted the methylation status of *HSD11B2* in the fetal cortex and hippocampus. This raises the intriguing possibility of using the epigenetic status of placenta to predict corresponding changes in the fetal brain (Jensen Pena et al., 2012). Prenatal maternal anxiety was reported to result in a small but statistically significant increase in *HSD11B2* methylation at CpG sites 4, 7 and 8, and reduced expression of placental HSD11B2. When methylation at these same sites was examined in the fetal hypothalamus, significant changes in methylation were observed, but these were discordant and in the opposite direction as observed in the placenta, apparently causing no effect on HSD11B2 mRNA levels (Jensen Pena et al., 2012). However, it should be noted that significant differences in methylation at other CpG sites in the placenta were also observed that were not found in the fetal brain, suggesting that there may not be a complete direct, inverse relationship between both tissues. No differences in DNA methylation were observed in the fetal cortex of stressed animals compared with controls (Jensen Pena et al., 2012), suggesting that regional differences in the effects of prenatal maternal anxiety on the methylation status of *HSD11B2* in the fetal brain may exist. This raises the intriguing possibility that the epigenetic status of *HSD11B2* in the placenta could predict changes in the fetal brain. However, it is clear that much work remains to be done

to investigate causality, and that the effects of prenatal maternal anxiety on the entire epigenome, rather than individual genes, need to be examined. Such studies are vital, given the recent demonstration that global epigenomic reconfiguration is observed during human brain development, which suggests a key role of DNA methylation in brain development and function (Lister et al., 2013).

Recently, the impact of maternal prenatal anxiety on the placental epigenetic status of *HSD11B2* has been reported (Conradt et al., 2013). Conradt and colleagues used a structured chart review to collect information about whether a mother reported depression and/or anxiety during pregnancy. Of the 482 participants recruited for the study, 13.7% reported depression or anxiety during pregnancy, which is consistent with the estimated prevalence rates (Nylen et al., 2013). In agreement with findings from animal models of prenatal stress (Jensen Pena et al., 2012), maternal anxiety during pregnancy resulted in a statistically significant increase in methylation of CpG site 4 of the *HSD11B2* promoter (Conradt et al., 2013). Infants of mothers who were anxious during pregnancy were more hypotonic, and placentae from these pregnancies had greater methylation of CpG site 4 in *HSD11B2 (Conradt et al., 2013)*. While correlations suggest that the epigenetic status of placental *HSD11B2* may help identify infants at risk for poor neurodevelopmental outcomes, a key challenge that remains is to prove cause and effect. Specifically, whether the increase in *HSD11B2* methylation in the placenta following prenatal anxiety in fact leads to the behavioural changes observed in affected infants needs to be further examined. One possible approach could be to examine fetal neurobehavioral outcomes following prenatal stress in mice that express a drug-inducible, conditional placental-specific knockdown of the methyltransferase *DNMT3a*, whose expression has been shown to be increased in the placenta following prenatal stress; (Jensen Pena et al., 2012) or, more specifically, the generation of mice carrying methylation resistant CpG sites in *HSD11B2*. It is worth noting here that whilst the methylation status of placental HSD11B2 is being increasingly investigated, the potential role of acetylation of HSD11B2 is being overlooked. Just like DNA methylation, histone acetylation plays a crucial role during fetal development (Volmar and Wahlestedt, 2015) and histone

acetylation has been shown to associated with HSD11B2 expression in the placenta (Li et al., 2013b).

The clinical perspective: extrinsic factors that can modify placental HSD11B2 expression:

If epigenetic down regulation of placental HSD11B2 expression is the mechanistic link between the experience of maternal stress and the resultant long-term health consequences for affected offspring, then strategies that increase placental HSD11B2 expression have the potential to improve fetal outcome. Here we highlight two ways that extrinsic factors could modify the expression of placental HSD11B2; 1) the use of pharmacological epigenetic modifiers, and 2) dietary considerations that may affect placental HSD11B2.

Effects of pharmacological epigenetic modifiers on HSD11B2 expression:

A defining feature of epigenetic modifications is that they are reversible. Thus, drugs that interfere with the enzymes that produce these changes may help maintain or restore normal gene expression levels. One class of drugs that alter DNA methylation is that of DNMT inhibitors. Many types of DNMT inhibitors have been produced and some of these have been used clinically for the treatment of leukemias, myelodysplastic syndromes and hemglobinopathies (Goffin and Eisenhauer, 2002). These compounds exert their effects by covalently trapping methyltransferases, thereby preventing full methylation of a DNA strand (Santini et al., 2001a, Santini et al., 2001b). A second class of molecules is that of HDAC inhibitors. These compounds inhibit the activity of HDACs, thus preventing histone deacetylation of lysine residues on histone tails and, as a consequence, enhancing gene transcription. HDAC inhibitors have also been used in epigenetic therapy and two of these compounds have recently gained FDA approval as anti-cancer therapies (Wagner et al., 2010).

DNMT inhibitors 5-aza-2'-deoxycytidine (5-AZA) and procainamide have been shown to demethylate the *HSD11B2* promoter, enhancing both HSD11B2 expression and activity in the SW620 human colon carcinoma cell line, MCF-7

breast adenocarcinoma cell line, and JEG-3 placental carcinoma cell line (Alikhani-Koopaei et al., 2004). When given orally to rats for seven days, DNMT inhibitors were shown to reduce *HSD11B2* methylation and to increase HSD11B2 expression (Alikhani-Koopaei et al., 2004), which highlights the potential impact of such classes of epigenetic pharmacological modifiers on HSD11B2 expression. Similar studies have also examined the effects of HDAC inhibition on HSD11B2 expression and found that the HDAC inhibitor trichostatin A had no effect by itself on these cell lines, but increased HSD211B2 expression in SW620 and MCF-7 cells when added only 48 h after 5-AZA treatment, indicating that DNA methylation is the dominant form of epigenetic modification on this gene (Alikhani-Koopaei et al., 2004).

These classes of pharmacological inhibitors have been proposed to be potentially useful in reversing placental epigenetic change. However, a major challenge with these approaches will be to specifically target the placenta without affecting the developing fetus. This is particularly pertinent as recent data shows that a global reconfiguration of the fetal neural epigenome occurs during development (Lister et al., 2013), suggesting that drugs that interfere with these processes may have profound consequences for normal fetal development. These drugs have significant detrimental effects on both the mother and fetus. For example, intraperitoneal (i.p) injection of pregnant mice with 5-AZA on gestational day 10 has been shown to reduce birth weight in affected offspring, an effect that persists in males at 5 months of age (Cisneros et al., 2003). This reduced weight is also associated with reduced IGF-1 levels in the serum of these offspring (Cisneros et al., 2003). Furthermore, males and females who were exposed to 5-AZA in utero display a 70% reduction and a 30% reduction in mating capacity and fertility, respectively, as measured by the presence of a vaginal plug and viable pregnancy (Cisneros et al., 2003). In a recent study by Ding and colleagues, Kunming mice were subjected to i.p. injection of 5-AZA or saline on gestational days 1, 2 and 4, followed by embryo implantation on day 4. Mice that received 5-AZA showed a significant reduction in embryo implantation, as compared with the saline group. This was associated with a cell specific reduction in mRNA levels and an increase in the methylation levels of many genes involved in endometrial change, as well as a decrease in stromal cell proliferation and differentiation (Ding et al.,

2012).Intravenous infusion of 5-AZA in patients with metastatic lung carcinomas induced haematopoiesis toxicity (Momparler et al., 1997), thus limiting their use in pregnant women. Given these significant problems and challenges associated with their use, the administration of pharmacological epigenetic modifiers to modify the placental epigenome is not feasible at the present time.

Effect of dietary composition on placental HSD11B2 expression:

The influence of dietary composition as a modifier of placental epigenetic machinery is becoming increasingly apparent (Gabory et al., 2012, Gallou-Kabani et al., 2010). Mice fed a high fat diet showed reduced placental expression of a number of methyltransferases involved in epigenetic modification (Gabory et al., 2012, Gallou-Kabani et al., 2010). This is a very important finding when one considers that, in western societies, 50% of all pregnant women are either overweight (BMI of 25–29.9 kg/m^2) or obese (BMI of ≥30 kg/m^2) (Ogden et al., 2006), have a dietary caloric excess predominantly derived from fat (Allegri et al., 2011), or show a higher rate of adverse pregnancy outcomes (Smith et al., 2007, Triunfo and Lanzone, 2014).

DNA methylation is dependent on the availability of methyl group donors and cofactors provided by the methionine and folate metabolisms, as well as dietary-derived vitamin B6 and B12, suggesting that dietary modifications can affect the fetal and placental epigenome (Anderson et al., 2012). This is highlighted by a 12-wk dose-response choline feeding study conducted in third-trimester pregnant women that investigated the effect of a maternal choline intake of 930 mg or 480 mg per day. Higher maternal choline intake significantly increased the average methylation levels in the glucocorticoid receptor gene promoter in placenta and fetal leukocytes, with infants showing lower levels of circulating cortisol in the higher choline group (Jiang et al., 2012). Although limited data exist regarding the impact of dietary composition on the epigenetic modification of placental *HSD11B2*, Takaya et al. examined the methylation of individual CpG dinucleotides in the promoter region of hepatic *HSD11B2* in offspring of pregnant rats fed a magnesium-deficient diet from 2wk prior to mating and throughout the gestational period

(Takaya et al., 2011). The average methylation of the *HSD11B2* promoter in the offspring of dams fed a magnesium deficient diet was significantly higher than that from controls (Takaya et al., 2011). However, it is important to note that contrary to what was expected given previous findings in the placenta (Marsit et al., 2012), *HSD11B2* methylation was positively correlated with HSD11B2 mRNA expression (Takaya et al., 2011). However, responsiveness to environmental exposures in utero cannot be generalized across genes and tissues (Loke et al., 2013), suggesting that epigenetic modification of the *HSD11B2* promoter in different tissues may result in different effects on gene expression. Together, these studies show that maternal diet can exert gene-specific epigenetic changes that can effect placental mRNA expression. The effect of diet on placental epigenetic regulation of *HSD11B2* is a key question for future research. However, a number of studies have examined the effect of both dietary intake and composition on placental HSD11B2.

A recent study investigating the impact of maternal caloric restriction on placental HSD11B2 expression reported that when dietary intake was reduced by 50% in Sprague Dawley rats from gestational day 10 to 20, these rats showed higher maternal corticosterone levels than controls and a reduction in HSD11B2 expression in the labyrinth zone of the placenta (Belkacemi et al., 2011b, Belkacemi et al., 2011a). Furthermore, specific reduction in protein content of the maternal diet during early and mid, but not late, gestation in Wistar rats caused a reduction of placental HSD11B2 expression (Bertram et al., 2001). These findings have been supported by some (Langley-Evans et al., 1996, Whorwood et al., 2001a, Whorwood et al., 2001b), though not all (Garbrecht and Schmidt, 2013), studies and collectively suggest that adequate protein intake is required to maintain optimal expression of placental HSD11B2. However, an excess of calories, predominantly derived from high fat and high sugar foods largely define Western dietary habits (Allegri et al., 2011). Therefore, an area that demands further investigation is the effect of such high calories, high fat/sugar diets on placental HSD11B2 expression. A recent study by Sferruzzi-Perri et al. reported altered placental phenotypes in mice fed a high fat, high sugar diet that affected placental nutrient handling, which has implications for fetal development and pregnancy outcomes (Sferruzzi-Perri et al., 2013). In addition, the high fat, high sugar diet influenced fetal growth trajectory

(initial growth restriction followed by catch-up growth), which may have implications for later-life outcomes. However, one potential caveat of this well-designed study (acknowledged by its authors) was the difference in protein content between the two diets (the standard diet consisted of 26% protein compared with the high fat, high sugar diet, which consisted of 17% protein) (Sferruzzi-Perri et al., 2013). It has previously been shown that even mild protein restriction can contribute to changes in placental expression of HSD211B2 and development of HPA dysfunction and hypertension (Allegri et al., 2011). Therefore, the data from Sferruzzi-Perri and colleagues need to be interpreted within the context of unmatched maternal protein intakes. However, given that mice and rats fed a high fat diet during pregnancy display alterations in the placental transcriptome and have dysregulated placental epigenetic machinery (Gabory et al., 2012, Gallou-Kabani et al., 2010), a much greater understanding of the effects of diet on placental HSD11B2 is required. When we analysed the supplementary data available in the study by Mao et al. looking at the placental transcriptome at day 12.5 of gestation in mice fed a high fat diet, robust changes were found in the placental transcriptome however *HSD11B2* was not in their list of differentially expressed genes (Mao et al., 2010). This does not mean that this gene does not change in response to a high fat diet, it only means that changes in *HSD11B2* gene expression were not detected at the particular time at which they did their microarray profiling. In addition, transient changes with long-lasting effects cannot be ruled out, especially given the data from Sferruzzi-Perri and colleagues (Sferruzzi-Perri et al., 2013).

Future research should focus on addressing the effects of such diets, as well as on understanding the correct balance of nutrients that may mitigate the effects of increased maternal stress. For instance, could optimal protein levels throughout pregnancy protect against the placental morphological and phenotypical changes associated with a high fat, high sugar diet. In addition, we know that a reduction in total maternal calories downregulates HSD11B2 in the labyrinth zone and preliminary evidence suggests that a high fat, high sugar diet influences labyrinth zone development; therefore, devising maternal dietary interventions that may influence both placental development and ensure optimal fetal outcomes may be

an exciting area of research. Could maternal dietary manipulation represent a mechanism that could protect or counteract the effect of maternal stress (environmental or emotional) on HSD11B2 expression and fetal outcomes? These questions warrant further investigation.

Whether nutritional interventions can specifically prevent or reverse prenatal stress-induced epigenetic change in placental *HSD11B2* remains to be elucidated, but it represents an exciting and, more importantly, feasible approach to potentially reverse or prevent the programming effect of prenatal stress on the placental and fetal epigenome.

Other placental genes:

Whilst the majority of the current literature focuses on HSD11B2 as the core regulator of fetal glucocorticoid exposure, two other placental genes have recently emerged as important contributors; the glucocorticoid receptor (NR3C1) and FK506-bind protein 51 (FKBP51). NR3C1 codes for the glucocorticoid receptor (GR) which when bound by cortisol, translocates into the nucleus where is regulates gene transcription (Clifton et al., 2017). FKPB51, is a chaperone protein which regulates nuclear transport of NR3C1, thus reducing the amount of receptor available for cortisol binding (Zhang et al., 2008a). Similar to HSD11B2 the placental expression of these genes has been linked to prenatal distress and infant neuro-behaviours (discussed below), with the majority of the literature again focusing on the epigenetic regulation.

A number of studies have now shown that placental NR3C1 can be altered by prenatal distress. In cohort of 67 healthy women, low levels of education, a potential indicator on elevated prenatal distress was associated with increased placental NR3C1 and HSD11B1 mRNA expression, a combination that may increase cortisol productivity in the placenta and also increased glucocorticoid sensitivity (Raikkonen et al., 2014). Maternal depressive symptoms in the third, but not first or second trimester were associated with increased placental NR3C1 mRNA expression (Raikkonen et al., 2015). Most interestedly NR3C1 partly mediated the relationship

between maternal prenatal depressive symptoms and infants behaviours (Raikkonen et al., 2015).

More often, the methylation status of placental NR3C1 has been linked with neurological deficits in newborns. In a cohort of 186 infants increased methylation of placental NR3C1, which correlated with reduced mRNA expression, was associated with reduced attention in neonates (Bromer et al., 2012). Methylation of NR3C1 in the placenta has also been linked with variations in newborn cry, which can be an early indicator of poorer neurobehavioral outcome in infancy (Sheinkopf et al., 2016). Altered methylation patterns of both placental NR3C1 and HSD11B2 have been shown to jointly contribute to infant neurobehavior in another study cohort (Appleton et al., 2015).

It has also been shown that placental NR3C1 can mediate a link between prenatal mental health and infant neuro-behaviours. Maternal depression, but not anxiety (both measured retrospectively after birth) was shown to be related to increase CpG2 methylation of placental NR3C1, and this increased methylation was associated with hypotonic behaviours, and poor self-regulation in infants assessed using the NNNS (Conradt et al., 2013). Condradt et al. observed a significant interaction between maternal depression, NR3C1 methylation and poor self-regulation, hypertonia and lethargy, in that the relationship between NR3C1 methylation and these behaviours was only evident for infants of depressed mothers, and not for infants of non-depressed mothers (Conradt et al., 2013). Indeed other studies have shown that the link between increased NR3C1 methylation and neurobehavioral deficits is still evident in infancy. In a cohort of 128 infants, Conradt and colleges demonstrated that increased placental NR3C1 methylation was predictive of increased cortisol reactivity and self-regulation in response to stress at 5 months of age (Conradt et al., 2015).

There is also some evidence to suggest a sex divergent effect of placental NR3C1 in response to stress. In mice 60h exposure of corticosterone starting at E14.5 is associated with increased expression of NR3C1 in male offspring but not females (Cuffe et al., 2012). In humans', increased maternal cortisol was found to be associated with increased NR3C1 mRNA expression in males, but not females

(Mina et al., 2015). This suggests cortisol may programme placental NR3C1 expression is a sex dependant manner with males being more vulnerable to the effect.

FK506 binding proteins are a class of immunophilins that are well known for their ability to bind immunosuppressive drugs (Kang et al., 2008). Of particular interest is FKBP51 which has been shown to interact with the androgen, progesterone and glucocorticoid receptor. Whilst FKBP51 is a positive regulator of the androgen receptor, it inhibits the activation of the progesterone and glucocorticoid receptors (O'Leary et al., 2013). FKBP51 is emerging as an important contributor regulating to the stress response, and mutations in FKBP51 has been linked to a number of psychiatric illnesses (O'Leary et al., 2013, Binder et al., 2008, Sinclair et al., 2013). FKBP51 has long been known to be abundantly expressed in the human placenta (Baughman et al., 1997), however until very recently its role in the placenta and in fetal development has not been widely studied.

One of the first studies to examine the role of FKBP51 in the placenta hypothesised that its expression was crucial role for neurodevelopment (Paquette et al., 2014). FKBP51 methylation was quantified in 509 placenta samples and neurobehavioral outcomes were assessed in newborns using the NNNS. This study reported high placenta FKBP51 methylation, and thus reduced mRNA expression was associated with poorer neurobehavioral outcomes (Paquette et al., 2014). It should be noted however that a follow-up study using a different analytically approach reported no association between FKBP51 methylation and newborn neurobehavior (Paquette et al., 2015).

To date only two clinical studies have examined the impact of prenatal distress on placental FKBP5, and both of these have focused on methylation levels and have not examined FKBP5 placental mRNA expression. The first of these was conducted in a cohort of 24 month-infant dyads in the conflicted region of Congo, and used war trauma as an indicator of prenatal exposure to stress. This study report a positive correlation between placental FKBP5 methylation and exposure to war stress (Kertes et al., 2016). More recently perceived stress, anxiety and depressive symptoms (but not maternal cortisol) measured in the third trimester

were shown to be associated with greater methylation of placental FKBP5, HSD11B2 and NR3C1. Indeed increased methylation of HSD11B2 and FKBP5 were associated with reduced fetal coupling, which is predictive of neurodevelopmental outcomes (DiPietro et al., 2010), and HSD11B2 methylation was found to mediate the relationship between prenatal stress and fetal coupling (Monk et al., 2016). This further supports the hypothesis that alterations in the expression of genes in the placenta that are involved in glucocorticoid signalling, may underlying the adverse effects of prenatal distress on adverse obstetric outcomes.

1.2.2 Focus on the Gut Microbiome:

The glucocorticoid hypothesis remains the most extensively studied area of research linking PNMD with adverse outcomes. However, recently another theory is emerging that proposes the experience of PNMD alters the maternal prenatal microbiota and that this stressed maternal microbiota is transmitted to the infant during parturition which then interferes with postnatal infant development. The human body is host to trillions of microorganisms that reside on the skin, oral cavity, nasal cavity, reproductive tract and gastrointestinal tract. The number of bacterial cells in the human body is estimated to be $3.8x10^{13}$, existing at an equal ratio to that of human cells (Sender et al., 2016). Over the past few decades our knowledge of the human microbiome has expanded with the establishment of sequencing technologies (Hooper et al., 2001). There are two primary ways to analyse the microbiome; shotgun metagenomics and 16S ribosomal RNA (rRNA) gene amplicons, both affording their own weaknesses and strengths (Jovel et al., 2016). Both technologies require complex bioinformatics that allow the classification of bacterial taxa, as well as the diversity of bacteria within (alpha (α) diversity) and between samples (beta (β) diversity) (Jovel et al., 2016). During taxonomic classification bacteria are first classified into *phylotypes* (phyla) and then clustered into operational taxonomic units (OTUs; family and genus) (Jovel et al., 2016). The optimization of these sequencing technologies over the past two decades has expanded our knowledge of the human microbiome (Hooper et al., 2001), and it is now clear that the microbiome plays a crucial role in human health, with perturbations in the microbiome, or microbial dysbiosis being identified in a

vast array of human disease (Sidhu and van der Poorten, 2017, Dinan and Cryan, 2017). Of particular interest, the gut microbiome has been shown to influence brain function, a relationship that is now known to be bidirectional. This has led to the coining of the term, microbiota-gut-brain-axis (Dinan and Cryan, 2017, Rogers et al., 2016). Communication between the gut and the brain occurs through hormonal, immunological and neural pathways and disruption to this communication is suggested to contribute to the pathophysiology of neurological disorders, particularly stress related psychiatric illness, although much of our current knowledge comes from preclinical studies (Cryan and Dinan, 2012, Rogers et al., 2016, Deans, 2017).

Stress and the gut brain axis:

The idea that the microbiota may play a role in the biology of PNMD comes from the extensive literature showing the gut microbiota is important regulators of the HPA axis and stress response. Preclinical studies have shown the composition of the gut microbiota can be altered by stress (Bailey, 2014, Bailey et al., 2011, De Palma et al., 2015, Rea et al., 2016). Additionally in humans, microbial dysbiosis is evident in stress related disorders including Irritable Bowel syndrome (Jeffery et al., 2012) and depression (Kelly et al., 2016a, Jiang et al., 2015). The relationship between the gut bacteria composition and HPA functioning has been demonstrated by studies using germ free (GF) animals. GF animal are born and reared in a sterile environment and so have no microbiota, therefore are commonly utilized to examine the impact of an absent microbiota on host physiology and behaviour. GF animals display increased anxiety type behaviour, as well as alterations in brain neurochemistry in the amygdala and hippocampus (Neufeld et al., 2011). GF animals have an increased ACTH and cortisol response to stressors, an effect that can be reversed by reconstituting with *Bifidobacteria infantis* or by receiving a fecal transplant from control mice (Sudo et al., 2004). The most notably effect reported in this study was that fecal transplantation was effective at reversing HPA deficits, when transplanted in the postnatal period, but not if transplanted later in adolescents. This highlights that the early developmental period as a critical

window whereby microbial changes may impact physiology (Sudo et al., 2004). Despite the extensive literature implicating the gut microbiota as a critical regulator of the stress response, the influence of stress on maternal microbial communities in pregnancy has hardly been examined. Indeed the increasing evidence highlighting the importance of the microbiome in health has triggered interest in understanding how the microbiome is acquired, and how it develops and changes throughout the lifespan. Establishment of the microbiome in infancy has subsequently emerged as a critical period of microbe host interactions, and an increasing body of work is attempting to understand how microbial disruptions in early life, may influence host physiology and confer disease risk (Mackie et al., 1999, Borre et al., 2014). For these reasons there is a need to describe and to understand the functional importance of the microbiome during the prenatal and postnatal period in determining obstetric and health outcomes.

Prenatal maternal gut microbiome:

Pregnancy is characterised by a period of substantial physiological changes which include weight gain, insulin resistance, increased glucose, hormonal changes and immune system modulation which all support the development of a healthy fetus (Nuriel-Ohayon et al., 2016). Recently the importance of gut microbial changes throughout gestation is being acknowledged and is now accepted to be integral part in maintaining a healthy pregnancy. One of the pioneering studies demonstrating this, examined the gut microbiota in 91 pregnant women in the first and third trimester of pregnancy (Koren et al., 2012). Here β-diversity was found to expand with advancing gestation whilst α-diversity indices were reduced. This was explained by increasing abundance of Proteobacteria and Actinobacteria and changes in the gradient of Bacteroidetes and Firmicutes. When compared to non-pregnant controls from the Human Microbiome Project Database, the microbiome from the first trimester samples was closely related to the controls, whereas the β-diversity of the third trimester samples was much greater (Koren et al., 2012). When transplanted into GF animals, recipients of the third trimester microbiota had increase markers of inflammation, gained more weight, had increased blood

glucose and insulin suggesting the microbiome plays a direct role in contributing to the metabolic state that is typical of pregnancy to support the nutritional demands of the growing fetus (Koren et al., 2012). Unfortunately the authors did not report any correlations between specific bacterial species and physiological symptoms of pregnancy. Such information will be important in understanding precisely what bacteria are necessary to maintain and support a normal healthy pregnancy. Of note one study that thoroughly sampled the gut, vaginal and oral microbiome weekly throughout pregnancy reported the gut microbiome to remain stable across pregnancy (DiGiulio et al., 2015a). This highlights the need for further studies examining the composition and functional role of the microbiome across pregnancy, and at different body sites.

Just like the non-pregnant population, the gut microbiota in pregnancy can be influenced by environmental factors. Preclinical studies have shown that prenatal stress disrupts bacterial communities in the maternal gut (Jasarevic et al., 2017), however this has yet to be examined in human populations (Rea et al., 2016, Foster et al., 2017). The most well studied factor that has been shown to impact on bacteria communities in the maternal prenatal gut is maternal diet and weight. When compared to normal weight women, women who are overweight, according to their pre-pregnancy body mass index (BMI), have high levels of *Bacteroides* and *Staphylococcus*. Interestingly the levels of *Bacteroides* showed a positive correlation with weight, BMI and weight gain over pregnancy. Additionally, there was a negative correlation observed between weight gain and *Bifidobacterium* such that women who gained less weight had increased *Bifidobacterium* counts (Collado et al., 2008). Women who were classified as obese in pregnancy based on their pre-pregnancy BMI, were found to have a reduced species richness and evenness along with higher abundance of Firmicutes and Actinobacteria and reduced Tenericutes in their gut microbiota in early pregnancy (Gomez-Arango et al., 2016). *Lachnospiraceae* and *Rikenellaceae* were found to positively correlate with maternal BMI in these women. Additionally this study found a number of correlations between metabolic hormones involved in glucose metabolism and energy metabolism to correlate with the maternal gut microbiota. Notably insulin and C-peptide levels positively correlated with *Coriobacteriaceae* of the

Actinobacteria phyla and higher abundance of *Lachnospiraceae* and *Ruminococcaceae* correlated with increased plasma leptin (Gomez-Arango et al., 2016). It is worth noting that reduced *Lachnospiraceae* and *Ruminococcaceae* is a microbial signature observed in patients with depression (Jiang et al., 2015), suggesting the potential for PNMD to alter important bacteria that interact with maternal physiology in pregnancy.

The impact of prenatal antibiotic use in pregnancy on the maternal gut microbiome has not being well studied in humans despite the fact that one in four women are prescribed antibiotics in their pregnancy, with antibiotics accounting for 80% of prescribed medications in pregnancy (Bookstaver et al., 2015). In rodents prenatal exposures to commonly administered antibiotics Azithromycin, Amoxicillin and Cefaclor altered the maternal gut microbiome, notably reducing species diversity, increasing the abundance of Proteobacteria and reducing Firmicutes with corresponding alterations at family and genus level including increased growth of the pathogenic *E.Coli*. Antibiotic use was also associated with weight gain over the course of the pregnancy (Khan et al., 2016). Use of antibiotics in pregnancy is associated with an increased risk of childhood obesity (Mueller et al., 2015b) and asthma (Lapin et al., 2015) and the infant gut microbiome is altered by perinatal use of antibiotics (Persaud et al., 2014), which could be linked to alterations in maternal microbes. This highlights the need for more research to examine the impact of antibiotics use on the maternal gut microbiome.

A growing body of research linking maternal microbial perturbations to adverse pregnancy outcomes highlights the importance of the maternal gut microbiome in pregnancy. Infection with *Helicobacter pylori* in pregnant women is associated with symptoms of dyspepsia and hyperemesis, two of the most common conditions that affect pregnant women (Poveda et al., 2014, Shaban et al., 2014). Reduced α-diversity, mainly decreased microbial richness, has been reported in the gut of women with gestational diabetes mellitus (GDM) in the first trimester (Koren et al., 2012). Although no direct link has been shown between the maternal gut bacteria and preterm birth, bacteria abundant in the gastrointestinal tract have been isolated from the amniotic fluid of pregnancies complicated by preterm pre-labour rupture of membranes (DiGiulio et al., 2010). The route by which maternal

gut microbes might enter the fetal compartment to influence development is not well known. Possible mechanisms include contamination of the maternal vaginal canal, which ascends through the birth canal or translocation from the gut into the blood stream (Dunlop et al., 2015). This may be particularly important in women with heightened stress, as stress can impair intestinal barrier function and integrity which could result in greater permeability and escape of bacteria into the circulation (Li et al., 2017, Cameron and Perdue, 2005).

Vaginal microbiome:

The vaginal microbiome plays an important role in protecting against infections, a function achieved through the bactericidal activities of the *Lactobacilli* species (Boris et al., 1998, Martin et al., 1999, Nardis et al., 2013, O'Hanlon et al., 2013). These bactericidal properties are produced by utilization of glycogen deposited in the vaginal epithelium by *Lactobacilli* to produce lactic acid and maintain a low vaginal PH (Vasquez et al., 2002, Mirmonsef et al., 2012). The importance of vaginal *Lactobacilli* is further highlighted by a study showing that greater than 50% of lactic acid found in vaginal secretions are of D isoform. As human vaginal epithelial cells can only produce the L isoform of lactic acid, and *Lactobacilli* produce both isoforms, this study emphasises the importance of bacteria, particularly *Lactobacilli* in maintaining vaginal health (Boskey et al., 2001). The majority of vaginal microbiomes are dominated by a species of *Lactobacilli*. This has led to the grouping of vaginal communities into five 'community state types' (CTSs). Four of these communities are characterised by their dominance from particular *Lactobacillus* species, *L.crispatus, L.gasseri*, *L.iners* or *L.jensenii*-dominant. The fifth CST (CST VI) represents a more even and taxonomically diverse state with low levels of *Lactobacillus* (Romero et al., 2014, DiGiulio et al., 2015b, Charbonneau et al., 2016) that is more commonly associated with a high vaginal PH and the development of bacterial vaginosis (Romero et al., 2014).

In pregnancy the vaginal microbiome is important, not only protecting the developing fetus from infections, but also in the initial seeing of the infant microbiome upon rupture of the amniotic membranes. Some studies have reported

a substantial change in the vaginal microbiome throughout pregnancy reporting reduced species richness and diversity with advancing gestation and predominance of *Lactobacillales, Clostridiales, Bacteroidales* and *Actinomycetales* (Aagaard et al., 2012), whilst others do not studies do not identify these changes (DiGiulio et al., 2015b, Romero et al., 2014). Indeed the latter two studies do report a more stable vaginal microbiome in the pregnant population switching between *Lactobacillus* dominant CSTs during pregnancy but rarely to the more diverse CSTs (DiGiulio et al., 2015b, Romero et al., 2014, Walther-Antonio et al., 2014). The reason why the vaginal microbiome undergoes this switching between CSTs in pregnancy is unknown, however enhanced vaginal glycogen stores as a results of increased estrogen levels in pregnancy, is thought to favour *Lactobacilli* growth which may explain why the vaginal microbiome in pregnant women favours a *Lactobacilli* dominant CST (Prince et al., 2015). Of note, psychological stress in women has been linked to reduced estrogen levels (Roney and Simmons, 2015), which may consequently, reduce vaginal *Lactobacilli* growth.

The vaginal microbiome remains altered in the postpartum. In the study by Giulio and colleagues, a noticeable alteration in the vaginal microbiome was found in the postpartum period. This involved depletion of *Lactobacillus,* and the introduction of various anaerobic bacteria more common to the gut than the vagina, that persist up to one year after birth (DiGiulio et al., 2015b). This change in the postpartum vaginal microbiome has also been reported in other studies (MacIntyre et al., 2015, Huang et al., 2015). Whether this change in vaginal flora in the postpartum period is important for maternal health or is just a mechanical disruption from childbirth is unknown. Further studies examining this by assessing the vaginal flora in women after Caesarean delivery would help to elucidate this.

The functional importance of the vaginal microbiome in pregnancy is supported by a number of studies linking vaginal microbiome dysbiosis to adverse obstetric outcomes. Decreased vaginal richness and diversity of the vaginal microbiome in the first and second trimester has been linked to an increased risk of PTB (Stout et al., 2017, Nelson et al., 2016). Further women with a *Lactobacillus*-poor CST in the first trimester of pregnancy were reported to be more likely to delivery PTB (DiGiulio et al., 2015b). Interestingly the duration and proportion of

time a women had this vaginal profile, was predictive of preterm birth (DiGiulio et al., 2015a). At taxonomic level, a *Lactobacillus* depleted microbiota, increased abundance of *Gardnerella* or increased abundance of *Ureaplasma* increased the risk of PTB (DiGiulio et al., 2015b). The influence that PNMD has on vaginal microbial communities has yet to be examined clinically, although in rodents prenatal stress was found to decreases vaginal *Lactobacilli*, highlighting the need for research examining if stress related changes in the vaginal microbiome occur during pregnancy and if so, the consequence that these changes may have for pregnancy outcomes (Jasarevic et al., 2015a).

Infant microbiota development:

The early microbial communities that colonise the gut of an infant are important for infant health as this period of first microbial succession overlaps with critical periods of immune and nervous system development. Alterations in the development of an infant's microbiome may interfere with developmental processes that lay the groundwork for poor health and increased disease risk in later in life. Whilst the adult microbiome is stable and ultimately resilient to change, the infant microbiome can be altered. Therefore microbiome-based interventions in early infancy could be a valuable therapeutic approach to restore aberrant microbiome development in order to minimise later disease risk.

Until recently, the *in utero* environment was believed to be completely sterile, a paradigm referred to has the 'sterile womb hypothesis', whereby neonates receive their first microbes from the mother by vertical transmission during the process of childbirth and then horizontally from the environment after birth (Funkhouser and Bordenstein, 2013). Many studies have now reported the presence of bacteria in the meconium (Jimenez et al., 2008), placenta (Aagaard et al., 2014), amniotic fluid (Collado et al., 2016) and umbilical cord blood (Jimenez et al., 2005). These studies have challenged this view, and a new theory has emerged proposing colonization of the infant gut begins *in utero*, which has become known as the '*in utero* colonisation hypothesis' (Perez-Munoz et al., 2017). Despite these recent studies, it is still unclear whether bacteria are present *in utero*, or if the

detection of microbes is due to contamination upon specimen collection (Perez-Munoz et al., 2017, Hornef and Penders, 2017). Additionally, how commensal microbes might overcome the structural and immunological barriers of the placenta to enter the fetus is unknown. Therefore when and how an infant acquires their first microbes remains a topic of scientific debate.

In the first few years of life, the infant microbiota displays large inter-individual variation. As time progresses and by the age of one, infants tend to converge towards an adult-like level of complexity. Interestingly at this age the microbiota of infants is no more similar to their parents than to that of other parents, inferring a greater role for environmental over genetic influences on infant microbial development (Palmer et al., 2007). A large portion of microbial colonization occurs at birth, as babies are exposed to the bacteria present in the maternal vagina, fecal matter, skin and associated surroundings. Infant meconium samples are often dominated by the facultative anaerobes such as *Enterococci* and *Staphylococci* (Jimenez et al., 2008). Within the first few days of life these bacteria utilize up most oxygen stores, enabling them to be outcompeted by strict anaerobes such as *Bifidobacteria*. The establishment of breastfeeding allows such bacteria to thrive as maternal breast milk is a rich source of *Bifidobacteria* and *Lactobacillus* (Solis et al., 2010), which enables a direct transfer of these bacteria. Breast milk also contains human milk oligosaccharides (HMOs) which promote the growth of *Bifidobacteria* (Wickramasinghe et al., 2015). *Bifidobacteria* are important colonisers of the infant gut as they downregulate genes associated with inflammation in epithelial cells (Wickramasinghe et al., 2015), inhibit pathogens (Simone et al., 2014), modulate intestinal barrier integrity (Chichlowski et al., 2012) and ultimately aid in immune system development. *Bifidobacteria* remain the dominant species in the first year of life but decline with increasing age. Other dominant species during this time include *Lactobacillus, Streptococcus* and *Lactococcus* (Yatsunenko et al., 2012). The cessation of breastfeeding and introduction of solid foods marks the next developmental milestone of the infant gut bacteria as microbes involved in food degradation start to increase (Backhed et al., 2015). By the end of the first year of life the microbial ecosystem starts to resemble that of an adult (Palmer et al., 2007), and by age three the microbiome is

believed to be stabilized into its adult-like phenotype (Voreades et al., 2014). There are now a number of prenatal and postnatal factors that are known to influence this early life microbial assembly.

Factors influencing infant microbiome development:

Mode of Delivery:

Delivery mode is a key factor influencing the initial microbial assemblage of the infant gut. Infants born vaginally (VD) are exposed to the bacteria along the birth canal, and so are initially dominated by species resembling that of the maternal vaginal and faecal microflora. Conversely, infants born via caesarean section (CS) are first exposed to the bacteria on maternal skin and in the environment of the theatre, and thus have a different initial colonization. A recent systematic review compiled 7 studies that assessed infant microbiota at multiple points over the first year of life to show colonization patterns differ based on delivery mode (Rutayisire et al., 2016). In the first 7 days of life infants born by CS have lower total diversity, with marked reductions of *Bifidobacteria*, *Bacteroides* and *Enterobacteriaceae* (Gronlund et al., 1999, Hesla et al., 2014, Mitsou et al., 2008, Dogra et al., 2015, Kabeerdoss et al., 2013, Jakobsson et al., 2014). *Lactobacilli* are observed more frequently in VD neonates (Mitsou et al., 2008, Kabeerdoss et al., 2013) whilst CD neonates are more often colonized with members of the pathogenic *Kliebsiella* and *Veillonella* species (Hesla et al., 2014, Dogra et al., 2015). By the end of the first month of life, *Bifidobacteria* are the most dominant microbe regardless of delivery mode and at this age both *Bifidobacteria and Enterobacteriaceae* are no longer different between infants (Gronlund et al., 1999, Kabeerdoss et al., 2013, Jakobsson et al., 2014). However it should be noted that most studies still report lower *Bacteroides* in CS delivered infants at this time (Gronlund et al., 1999, Hesla et al., 2014, Kabeerdoss et al., 2013). By the age of 3 months, VD infants still display increased levels of *Bacteroides* (Hesla et al., 2014, Jakobsson et al., 2014, Gronlund et al., 1999, Kabeerdoss et al., 2013). By 6 and 12 months of age, delivery mode no longer appears to impact microbial colonization (Rutayisire et al., 2016), with only one report still identifying increased *B.fragilis* in VD infants at 6 months (Gronlund

et al., 1999). Further by adulthood, mode of delivery no longer appears to influence microbiome composition (Falony et al., 2016). Microbiome alterations specifically in the early gut colonization is suggested to be one potential mechanism underlying the increased risk of metabolic and immune related disorders in infants born via CD (Thavagnanam et al., 2008, Bager et al., 2008, Li et al., 2014, Kuhle et al., 2015).

Mode of feeding:

The infant diet is another important factor shaping gut colonization patterns. Infants who are exclusively breast-fed (BF) at 4 months have increased *Lactobacilli* and *Bifidobacteria* compared to those that are exclusively formulae-fed (FF). FF infants display elevated levels of other bacteria such as *Citrobacter spp.*, *C.difficile* and *Enterobacteria cloacae* (Backhed et al., 2015). Infants who are still being breastfed at 12 months are still dominated by *Bifidobacteria* and *Lactobacillus*, whereas infants whose breastfeeding has ceased have a shift towards a more adult phenotype enriched with *Bacteroides* and *Bilophila*, suggesting that cessation of breastfeeding rather than introduction of solids is a critical determinant of the shift towards a more adult like composition (Backhed et al., 2015). Of interest, BF may be a means to compensate for the reduction in *Bifidobacteria* and *Lactobacilli* associated with CS, as infants who are exclusively breastfed following CS have a restoration of *B. bifidum* and *L. gasseri* at 3 months that is comparable to VD infants (Martin et al., 2016a). Additionally changes in microbiome as a result of the mode of feeding in infants, is associated with changes to the host transcriptome. Increased microbiome virulent characteristics in BF infants are associated with decreases in inflammatory related genes in infant epithelial cells (Schwartz et al., 2012), inferring a direct consequence of altering the microbiome by mode of feeding on the host. Further some bacteria may be directly transferred from breast-milk to the infant gut as microbes such as *Staphylococci, Lactobacilli* and *Bifidobacteria* are present in breastmilk (Fernandez et al., 2013). Additionally breastmilk contains HMOs which promote *Bifidobacteria* growth (Asakuma et al., 2011).

Gestational Age and birthweight:

In a study of 50 LBW infant, α-diversity was initially reduced in LBW infants and found to increase over time from one week to one month to two months (Drell et al., 2014). The major phyla were Proteobacteria, whose abundance increased from one week to one month, Firmicutes which decreased from one week to one month, and Bacteroidetes (Drell et al., 2014). *Bifidobacterium* was undetectable and *Bacteroides* and *Lactobacillus* were barely detectable in these LBW infants. Additionally α-diversity was found to positively correlate with gestational age and negatively with the start of enteral feeding (Drell et al., 2014). Further in this study infants born to mothers' with chorioamnionitis had a more diverse gut microbiome, which is possibly a reflection of exposure to bacteria in the amniotic fluid (Drell et al., 2014). These findings suggest an additive role of maternal infection during pregnancy on early infant microbial colonization.

Antibiotics:

A large portion of fetuses are exposed to antibiotics during pregnancy, usually for the treatment of Group B Streptococcus, premature rupture of membranes or to reduce infections prior to CS delivery. An estimated 37% of women receive antibiotics during pregnancy with 33% receiving antibiotics in the intrapartum period (Stokholm et al., 2013). Intrapartum antibiotic prophylaxis (IAP) with ampicillin, is associated with increased *Enterobacteriaceae* and reduced *Bifidobacteria* in both exclusively or partially breastfed infants and lower bacterial diversity in bottle fed infants at day 7 of life. By day 30 *Bifidobacteria* had normalized but *Enterobacteriaceae* remained elevated in antibiotic treated infants (Mazzola et al., 2016). Prenatal antibiotic exposure may also interact with mode of delivery and feeding to influences the infant gut microbiome composition. Neonates delivered vaginally whose mothers received IAP, had reduced richness at 3 months, whilst those delivered via emergency CS (with IAP) had increased diversity at 1 year. Also at 3 months infants exposed to IAP had reduced Bacteroidetes, which was 46% in unexposed infants, 24% in exposed delivered vaginally and <1% exposed and delivered by CS. Exposed infants delivered via

emergency CS had increased Firmicutes and Proteobacteria which persisted at 1 year, at this time there were no differences in the microbiota of infants exposed born vaginally or by elective CS. These relationships were further influences by feeding, in that infants who were exclusively breastfed at 3 months were less likely to display microbiome alterations at 1 year (Azad et al., 2016). These data suggest the potential and usefulness of breastfeeding to counteract the impact of maternal adversity on infant gut microbiome composition.

Pregnancy stress:

Colonization of microbial communities within the infant gut can also be influenced by prenatal maternal stress. One of the first studies to demonstrate this, exposed pregnant rhesus macaques to an acoustic startle in early and late pregnancy and examined the intestinal bacteria in the offspring up to 24 weeks of life. Exposed offspring displayed reductions in *Lactobacilli* when exposed in early pregnancy, and reductions in *Lactobacilli* and *Bifidobacteria* when exposed in late pregnancy (Bailey et al., 2004). More recently the influence of prenatal stress on offspring microbiota has been replicated using an early prenatal stress model in rodents (Jasarevic et al., 2015a). Here *Lactobacilli* were again reduced in prenatally stressed offspring, with the reduction corresponding to a similar reduction of vaginal *Lactobacilli* in the stressed mothers, implying direct vertical transmission from mother to offspring. The authors also identified sex dependent microbial effects with increased *Bacteroides* and *Clostridium* in exposed males at postnatal day (PD) 2, who overall more closely resemble control females than males (Jasarevic et al., 2015a). Offspring of prenatally stressed mothers also displayed distinct alterations in colonic and plasma metabolites as well as changes in amino acid profiles with the hypothalamus, providing a route by which prenatal stress may impact developmental outcomes (Jasarevic et al., 2015a). Using the same stress paradigm Jasarevic and colleagues further showed sex dependant microbial alterations by prenatal distress that persisted up to PD28. They also examined maternal gut microbial colonisation and found distinct alterations in bacterial communities, along with disruptions to bacterial interactions among prenatally stressed dams (Jasarevic

et al., 2017). Additionally further evidence from preclinical models suggests that prenatal stress induced changes in intestinal microbiota can persist into adulthood, and that these microbial changes correlate with alterations in HPA axis function (Golubeva et al., 2015), providing a direct link between stress-induced microbial alterations and offspring physiological functioning.

Only one study to date has examined the relationship between prenatal stress and offspring gut colonization in humans, which largely supports the preclinical findings. In this study pregnant women completed psychological distress questionnaires and provided salivary samples for cortisol analysis in the third trimester of pregnancy, and infant microbial structure was assessed at 7, 14, 28, 80 and 110 days of life. Infants born from higher stressed pregnancies had high levels of pathogenic bacteria including *Escherichia* and *Enterobacter* and reduced lactic acid bacteria, of which *Lactobacilli* was included. *Bifidobacteria* was also reduced in these infants. Additionally α-diversity over this time period was elevated in infants from high stress pregnancies (Zijlmans et al., 2015a). Further infants born from the high stressed pregnancies displayed a high prevalence of adverse gastrointestinal symptoms (38%) compared to just 22% of those in the low stressed. Notably infants who developed gastrointestinal problems had lower abundances of lactic acid bacteria, a microbial phenotype also observed among infants with allergic reactions; again directly linking prenatally stressed induced microbial alterations to altered infant development (Zijlmans et al., 2015a). To date the impact of prenatal stress on the maternal gut and vaginal microbiome has yet to be examined in humans. As such if vertical transmission of a 'stressed microbial signature' during parturition accounts for the altered microbial structure in infants, or if other mechanisms are involved in this process remains to be determined.

Altered microbiome development and disease risk:

Early establishment of the infant microbiome coincides with critical periods of immune, metabolic and nervous system development (Borre et al., 2014), thus forming the rationale behind the extensive body of literature examining factors that might interfere with this process.

Infant Microbiome and disorders of the Metabolic System:

In support of this concept, one longitudinal study has examined the association between infant microbial development and the development of type 1 diabetes (T1D) in a cohort of 33 children. In this study infants who went on to develop T1D displayed a flattening of α-diversity before 2 years of age, a time when the diversity of non-convertors and semi-convertors continued to rise. At the compositional level, these infants were overrepresented by potentially pathogenic bacteria *Ruminococcus* and *Streptococcus* and had reductions in the dominant *Lachnospiraceae* (Kostic et al., 2015).

Infant microbiome and Immune System development:

The link between infant microbial colonization and immune system disorders has been prospectively studied in clinical populations, and is mechanistically paralleled by preclinical work strongly supporting a role for bacteria is regulating immune system development (Kelly et al., 2007). In two longitudinally designed cohorts, infants with greater amounts of *Lactobacillaceae* and *Bifidobacterium* species in their gut during the first 2 months of life were less likely to develop allergic symptoms by the age of 5 (Sjogren et al., 2009, Johansson et al., 2011). Early colonization with *Bifidobacteria* can influence systemic immune responses. Infants with higher numbers of *Bifidobacteria* species in the first two months of life have higher levels of secretory IgA (SlgA) at 6 months of age. Additionally *Bacteroides fragilis has been shown to* negatively correlated with toll like receptor 4 in peripheral blood mononuclear cells (PBMCs) at 12 months of age, which are important in pathogen recognition and immunity (Sjogren et al., 2009). *Bifidobacteria* have been shown to play a significant role in immune system development in young rats including promoting maturation of dendritic cells, influencing T-cell development and increasing IL-10 production from PBMCs (Dong et al., 2010). Of note, manipulating the gut microbiota by supplementation with probiotics during pregnancy and in infants 6 months postnatally, increases *Lactobacilli* and *Bifidobacteria* in the infant gut at 2 years of age and significantly reduces the risk of eczema (Kukkonen et al., 2007). Importantly, prenatal stress-

induced alterations in the infant microbiome have been linked to allergic symptoms in infants (Zijlmans et al., 2015a). Further the current evidence demonstrates a predominant role for *Lactobacilli* and *Bifidobacterium* species in immune system development, and these bacterial species are the ones that are most commonly reduced in offspring exposed to prenatal stress (Jasarevic et al., 2015a, Bailey et al., 2004, Zijlmans et al., 2015a). This provides further evidence that microbial dysbiosis may underlie the link between prenatal stress and immune system disorders.

Infant Microbiome and the Nervous System:

The relationship between early microbial colonization and neurodevelopmental outcomes has not been extensively studied in humans, and much of our knowledge comes from preclinical studies using GF animal models (sterile animals that have never been exposed to microbes). One of the earliest studies examining this demonstrated an exaggerated HPA response in GF animals when compared to specific pathogen free (SPF) mice, a phenotype that was reversed when the mothers of the GF animals were fed a single *Bifidobacterium infantis* strain. Importantly reconstitution of the GF mice with faeces from the SPF mice could also rescue the exaggerated HPA response, but only when reconstituted in early life (Sudo et al. 2004). This highlights an important window during which gut microbes can impact on nervous system functioning (Sudo et al., 2004). This exaggerated HPA phenotype has also been demonstrated by another study, where the GF animals also displayed reduced anxiety like behaviours along with altered neurochemistry in the brain that was specific to male offspring including reduced BDNF and increased tryptophan and serotonin. Again *early* colonization, immediately post weaning, could restore the behavioural deficits in GF animals but not the altered neurochemistry (Cryan et al., 2012). Alterations in the gut microbiome in children with ASD adds clinical support to the idea that the gut microbiome plays a role in neurodevelopment (Mulle et al., 2013). Indeed a recent exploratory trial has shown promise for fecal microbiota transplant in improving ASD behaviours and associated gastrointestinal function in a cohort of 18 ASD children (Kang et al., 2017). Delivery via CS, which is known to produce profound alterations early microbial colonization,

increases the risk of ASD (Curran et al., 2015). Recently the first report directly linking the infant microbiome to neurological outcomes was published. In this study the microbiota was characterised from fecal samples from 89 infants at 1 years old and cognitive testing was performed at 1 and 2 years old as well as structural MRI (Carlson et al., 2017). Infants were clustered into three groups based on their microbiome; high abundance of *Faecalibacterium (C1),* high abundance of *Bacteroides (C2)* and high abundance of *Ruminococcaceae* (C3). At 2 years old infants from C2 has the highest cognitive performance with infants from C1 showing the lowest. Alterations in grey matter volume were also observed based on microbiome clustering. Further higher α-diversity was associated with poorer cognition at 2 years of age (Carlson et al., 2017). This work highlights the need for more longitudinal studies directly examining early infant microbial colonization and its association with neurobehavioral outcomes in childhood and adulthood. This will elucidate the importance of *early* gut colonization on neurodevelopmental disorders.

These reports raise the issue of how infant gut microbiota affect the developing brain. Microbial communication via the vagus nerve represents one mechanism by which the gut bacteria can communicate with the brain. Indeed when the vagus nerve is severed, the antidepressant and anxiolytic effects of *Lactobacillus rhamnosus* in rodents are prevented (Bravo et al., 2011). Further the same bacterial strain was found to increase firing and activity of vagal afferents ex vivo (Perez-Burgos et al., 2013). Conversely behavioural alterations induced in mice by antibiotic depletion of the gut microbiota occurred regardless of vagotomy (Bercik et al., 2011), highlighting other methods of communication also exist between the gut bacteria and the brain.

Microorganisms produce and secrete a variety of metabolites and neurotransmitters that can enter the blood stream and in some case, cross the blood brain barrier to gain access to the brain (Sharon et al., 2014). Of particular interest short chain fatty acids (SCFAs), produced by the microbiome have widespread effects on the brain (Oleskin and Shenderov, 2016). Of note, SCFAs, butyrate and propionate have potent HDAC inhibitory activity (Waldecker et al., 2008, Stilling et al., 2016), which could have significant impacts on brain

development, as HDACs are import regulators of brain development (Volmar and Wahlestedt, 2015).

1.3 Aims and Objectives of Thesis

This thesis aims to investigate the biological mechanisms by which prenatal maternal distress can influence fetal development. In particular this work focuses on two key mechanisms: 1. placenta mechanisms, and 2. the maternal-infant microbiome. Currently, literature regarding the effect of prenatal distress on placental glucocorticoid signalling has produced conflicting findings and the molecular mechanisms that are involved in this effect have not been well studied. The concept that the microbiome may play a role in the programming effects of prenatal distress is novel and has only been investigated a handful of times in studies that have primarily focused on the effect on the infant microbiome in rodents. The impact that prenatal mental health has on the maternal microbiome has not been investigated in humans. Therefore the overall objective of this thesis was to establish a longitudinal study cohort that would allow a thorough investigation into the biological underpinnings linking prenatal distress to adverse outcomes.

1.3.0 Investigate the biological and molecular mechanisms by which prenatal stress alters placental HSD11B2 expression

Over the past decade placental HSD11B2 has emerged as a critical regulator of fetal development. There has been an explosion of descriptive clinical studies suggesting prenatal distress may influence the regulation of HSD11B2, however, the precise molecular mechanisms that regulate HSD11B2 under conditions of stress have not been studied. This chapter used an *in vitro* model of placental cells to examine the biological mechanism by which prenatal distress might influence placental HSD11B2 expression, with particular focus on cortisol, and IL-1β. Both compounds have been found to be elevated in the serum of pregnant women experiencing stress and may have the potential directly influence HSD11B2 expression. Additionally, whilst many studies are focusing primarily in the role of DNA methylation in HSD11B2 regulation, this chapter explored the part that histone acetylation plays in controlling HSD11B2 placental expression.

1.3.1 Examine the effects of prenatal distress on birth outcomes and placental gene expression in a preliminary human cohort

To date most studies have examined the effect of prenatal distress on the methylation status of HSD11B2, with limited studies investigating the effect at level of mRNA expression. This goal of this work was to examine the prevalence and impact of prenatal distress on birth outcomes and placental gene expression in women receiving antenatal care at Cork University Maternity Hospital (CUMH).

1.3.2 Establish a comprehensive study cohort to examine the multiple influences of prenatal distress

In order to examine the effect of prenatal distress on the microbiome we aimed to establish a longitudinal study cohort of nulliparous healthy pregnant women at CUMH. We aimed to measure multiple variables of psychological distress and collect and biobank maternal stool and saliva samples at multiple times throughout pregnancy. Additionally we intended to acquire vaginal swabs, placental samples and newborn hair at birth and collect and biobank infant stool samples across the first 5 months of life. Data and samples collected from this cohort would provide a comprehensive database to examine the biological underpinnings of PNMD.

1.3.3 Use this cohort to further understand how prenatal distress influences placental gene expression

The current thesis will first use this second cohort to further examine the influence of PNMD on genes involved in glucocorticoid signalling in the placenta. This cohort will address previous limitations by exploring both psychological and physiological measures of stress at multiple time-points throughout pregnancy.

1.3.4 Investigate the influence of prenatal distress on maternal gut, vaginal and infant gut microbiome development

As the microbiome is emerging as important determinant of maternal and infant health we next sought to examine the influence of psychological and physiological readouts of stress on the maternal and infant gut microbiome. The current hypothesis from preclinical work suggests PNMD impairs the maternal gut and

vaginal microbiota and this stressed microbial signature is then transferred to the infant at birth. With this in mind the current thesis aims to explore this hypothesis in humans by examining the impact of PNMD on the maternal gut microbiome across pregnancy, the vaginal microbiome just prior to parturition and the infant microbiome as it is seeded and develops over the first 5 months of life.

Chapter 2:

Materials and Methods

2.1.0 Cell culture

All experiments involving cell culture were performed in a sterile laminar-flow Class II Microflow Biological Safety Cabinet under aseptic conditions. Materials used were purchased sterile or sterilized by autoclaving. Liquids were sterilized by passing them through a 0.20 µM polyethersulfone filter (Sigma). For all cell culture experiments JEG-3 cells (ATCC) were used as an *in vitro* model of placental trophoblast cells (Orendi et al., 2011). JEG-3 cells were stored in Dulbecco's modified Eagle Medium Nutrient Mixture F-12 (DMEM F-12; Sigma), with 20% fetal calf serum (FCS; Sigma) and 10% dimethyl sulfoxide (DMSO; Sigma) in liquid nitrogen. Prior to use, cells were thawed and centrifuged at 500 rpm for 5 min. The supernatant was removed and the cells were resuspended in 'growth media' consisting of DMEM F-12 containing 100nM L-Glutamine (Sigma), 100U/ml penicillin (Sigma), 10µg/ml streptomycin (Sigma) and 10% FCS. The cells were then added to a T75 flask (VWR) with 20 ml of pre-warmed growth media and incubated at 37°C in a humidified atmosphere of 5% CO_2 (ThermoForma Series II, Thermo Electron Corporation). Growth media was removed and replaced with fresh growth media every 48 h until 80-90% confluency was reached.

Once cells reached confluency, growth media was removed from the flask and the cells were washed in Hank's Balanced Salt Solution (HBSS; Sigma) to remove any residual media. The cells were enzymatically dissociated by adding 2 ml of 0.2 % Trypsin (Sigma) to the cells and incubating for 5 min at 37°C. 2 ml of growth media was then added to the flask to neutralize the trypsin. The cell suspension was removed from the flask and placed in a 15ml conical flask (Sigma) and centrifuged at 500 rpm for 5 min. The supernatant was removed and the remaining cell pellet was resuspended in 1 ml of growth media. Mechanical dissociation was performed by triturating the cell suspension with a plugged flame polished Pasteur pipette (Sarstedt). A 1:10 dilution was made using growth media for cell counting. 10 µl of the diluted cell suspension was added to a haemocytometer (Marenfield Superior) and 5 grids were counted using the following formula:

Cells per ml =

$$\frac{\text{Number of cells counted x dilution factor } \mathbf{(10)} \text{ x haemocytometer constant } \mathbf{(10000)}}{\text{Number of squares counted } \mathbf{(5)}}$$

Cells were then added to a T75 flask at a density of 1 x 10^6 cells with 20ml of growth media. For the thiazolyl blue tetrazolium bromide (MTT) assay, immunocytochemistry and RNA extraction, cells were seeded into 24-well tissue culture plates (VWR) at a density of 1 x 10^5, 3 x 10^4 or 1 x 10^5 cells/well respectively along with 500 µl of growth media. Plated cells were incubated at 37°C and 5% CO_2 for 24 h prior to treatment. On the day of treatment the growth media was removed and replaced with 500 µl of fresh pre-warmed growth media. Cells were treated with 1, 5 or 10 µM of MC1568, MS275 or SAHA (Selleckchem) for 24 h. Where indicated, 10ng/ml IL-1β (Promokine) or 2µM Cortisol (Santa Cruz) were added to the cells for 24 h prior to the HDAC inhibitor.

2.1.1 MTT Assay

To assess cell viability, MTT solution was made by dissolving MTT (Sigma) in HBSS to a final concentration of 0.5 mg/ml. The media was removed from the cells and 300 µl of MTT solution was added per well. Following a 2 h incubation at 37°C and 5% CO2, the MTT solution was removed and 100 µl of DMSO was added to each well to lyse the cells. 75 µl of the DMSO solution was pipetted into a 96 well plate (VWR) and the absorbance of each sample was determined using a plate reader (Tecan sunrise) at a wavelength of 595nm with a reference wavelength of 630nm.

2.1.2 Fixation, blocking and Immunocytochemistry

At the experimental end point, the 24 well plates were removed from the incubator and the media was aspirated. 500 µl of ice-cold 4% paraformaldehyde (PFA) dissolved in 10mM phosphate buffered solution (PBS) was added to the cells and the plates were incubated at -20°C for 10 min. Following fixation, the PFA was removed and the cells were washed three times in 10mM PBS containing 0.02%

Triton X-100 (PBS-T) for 5 min. Cultures were incubated in a blocking solution of 5% bovine serum albumin (BSA; Sigma) in PBS-T for 1 h at room temperature. After blocking, the BSA solution was removed and the cells were incubated with 200 µl of the desired primary antibodies, which were diluted in 1% BSA in PBS-T at 4°C overnight. The following primary antibodies were used: rabbit HSD11B2 (1:250; Santa Cruz), rabbit AcH3 (1:250; Santa Cruz), rabbit GR (1:250; Santa Cruz) or rabbit IL1R1 (1:250; Invitrogen). Following primary antibody application the primary antibodies were removed and the cells were washed three times in PBS-T for 5 min. The cells were then incubated in 250 µl of Alexa Fluor 488 or 594, conjugated secondary antibodies (1:1000, Invitrogen) reactive to the species of the primary antibodies and diluted in 1% BSA in PBS, at room temperature for 2 h in the dark. The secondary antibodies were removed, and three 5 min PBS-T washes were carried out before cultures were counterstained with DAPI (1:3000; Sigma) diluted in PBS for 5 min at room temperature. The cells received a final three 5 min washes in PBS, and were stored in PBS at 4°C in the dark until imaged on an inverted fluorescent microscope (FV1000, Olympus) with AnalysisD™ software. Negative controls in which the primary antibody was omitted were also prepared. The fluorescence intensity of individual cells was measured using Image J analysis software (Rasband, WJ, http://rsb.info.nih.gov/ij/). The relative fluorescence intensity was calculated as the intensity of each individual cell after subtraction of the background noise.

2.1.3 RNA Extraction

RNA was extracted from JEG-3 cells or term human placental tissue (for placental tissue see collection and detailed extraction protocol below) using Trizol Reagent (Life Technologies). For RNA extraction from JEG-3 cells, media was removed and cells were washed in PBS. 50 µl of Trizol was added to each well and cells were incubated on ice for 10 min. Cells were removed from the plate by scraping and transferred into Eppendorf tubes (Sarstedt). 10 µl of chloroform (Sigma) was added to each sample at left at room temperature for 5 min, followed by centrifugation at 12,000 rpm for 15 min at 4°C. The upper aqueous phase was transferred into fresh

Eppendorf tubes and 25 µl of propanol (Sigma) was added to each tube and were left at room temperature for 10 min. Samples were then centrifuged at 12,000 rpm for 10 min at 4°C. The supernatant was removed and the RNA pellet was washed in 50 µl of ice-cold 70% ethanol. The sample was vortexed and centrifuged for a final time at 7,500 rpm for 5 min at 4°C. The supernatant was removed and the RNA pellet was left to air-dry for 15 min. The pellet was resuspended in 15 µl of RNAse free water (Sigma). RNA concentration in ng/µl was determined by using a Spectrophotometer (ND1000; NanoDrop Technologies, Inc).

2.1.4 cDNA synthesis and real time Polymerase Chain Reaction (PCR)

500 ng of RNA was reverse transcribed using a high capacity cDNA Reverse Transcription Kit (Applied Biosystems) in a 20µl reaction mixture consisting of 2.0µl 10× RT Buffer, 0.8µl 25× dNTP mix (100mM), 2.0µl 10× RT Random Primers, 1.0µl Reverse Transcriptase, and 4.2µl Nuclease-free H_2O, using the following parameters: 25 °C for 10 min; 37 °C for 120 min; 85 °C for 5 min; 4 °C for at least 10 min. The cDNA was stored at −80 °C prior to use. For real-time PCR, samples were run in triplicate using the following parameters 50 °C for 2 min; 95 °C for 10 min; 50 repetitions of 95 °C for 15 s and annealing/elongating at 60 °C for 1 min.

2.1.5 Immunohistochemistry

Histological placental sections (6µM) were incubated in blocking solution (5% bovine serum albumin (BSA)) for 1 h at room temperature. Sections were treated with 10% H_2O_2 for 5 min, washed in 10 mM Phosphate Buffered Saline (PBS) and blocked for 1 h in 10% normal goat serum in 10 mM in PBS-T. Sections were incubated in primary antibody to HSD11B2 (1:250; Santa Cruz) in 1% normal goat serum in 10 mM PBS with 0.4% Triton-X overnight at 4°C. Following a 3 × 10 min wash in 10 mM PBS, sections were incubated with a biotinylated secondary antibody (1:200; Vector Labs) for 2 h at room temperature. Following another 3 × 10 min wash in 10 mM PBS, sections were incubated in ABC solution (1:200; Vector Labs) for 45 min at room temperature followed by immersion in diaminobenzidine substrate/chromogen reagent for 2–3 min at room temperature.

Sections were dehydrated, cleared, mounted and imaged using an Olympus AX70 Provis upright microscope.

2.2.0 Participant recruitment (chapter 4)

This study was carried out with full ethical approval from the Research Ethics Committee of Cork Teaching Hospital. Participants attending antenatal care at Cork University Maternity Hospital (CUMH), Cork, Ireland between July 2015 and September 2016 were invited to participate in this study. The inclusion criteria were; *1)* 18 years of age or older, *2)* English speaking, *3)* having a current singleton pregnancy and *4)* plans to give birth in in the maternity hospital. The participants were recruited when they were greater than 28 weeks' gestation. Written informed consent was obtained from all women who agreed to take part and participants were asked to complete a combination of questionnaires used to assess maternal distress (Khashan et al., 2014) and donate a small biopsy of their placenta following delivery. For placental collection a kit was inserted into the patients' medical chart with written instructions for the midwives on the delivery ward. Detailed clinical and demographic data were collected from the medical notes of patients once the entire cohort had given birth. This data included information on maternal age, BMI, previous obstetric complications, previous psychiatric history, medical conditions, current obstetric complications, birthweight, gender, gestational age, Apgar score, birth temperature, neonatal resuscitation (if any), admissions to the Neonatal Intensive Care Unit (NICU) and mode of feeding on discharge from the hospital.

2.2.1 Questionnaires

This study used the 10-item Perceived Stress Scale (PSS). The PSS is a popular tool used to measure psychological stress and how individuals appraise stressful life events. Higher Scores on the PSS are indicative of a higher level of perceived stress. In this study, a score of greater than or equal to 20 was used as the cut-off for the 'high stress' group. Maternal anxiety was measured using the 6-item version of State Trait Anxiety Inventory (STAI). The 6 item STAI is a frequently used brief psychological measure of anxiety and the 6 item version which has been validated

for use during pregnancy (Marteau and Bekker, 1992). As there are currently no recommended cut off scores for the STAI during pregnancy, women were deemed anxious if they scored in the top 25% of the cohort. Depressive symptoms were assessed using the Edinburgh Postnatal Depression Scale (EPDS). Consistent with previous studies, we used a score of 13 or greater to indicate a high probability of depression (Rubertsson et al., 2011, Cohen et al., 1983). These self-reported questionnaires are marked by a 4-point Likert Scale. In this study, the Cronbach's alpha of the PSS, STAI and EPDS were 0.867, 0.838 and 0.894 respectively.

Figure 1: Timeline of recruitment for cohort 1

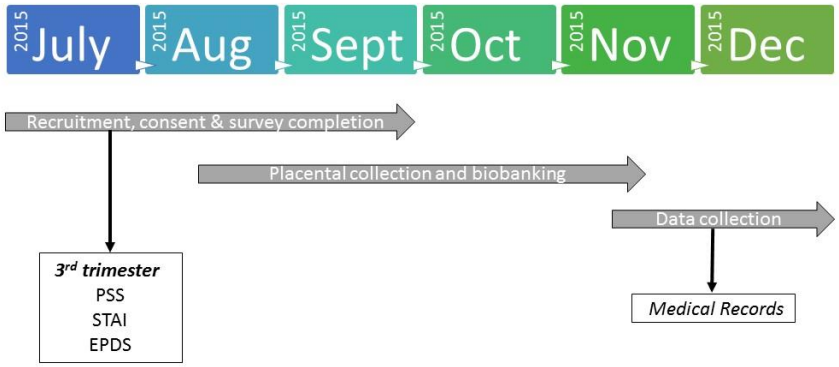


Figure 1: Recruitment of third trimester pregnant women from Cork University Maternity Hospital proceeded through July 2015 to September 2015. The inclusion criteria were; *1)* 18 years of age or older, *2)* English speaking, *3)* having a current singleton pregnancy and *4)* plans to give birth in in the maternity hospital. Placental samples were collected and bio-banked until November 2015 when the final women in the cohort gave birth. Data collection from storage medical records and then commenced and finished in December 2015 closing the study collection phase.

2.2.2 Placental Collection

Participants agreeing to donate their placenta had a 'placental collection kit' (Appendix A) inserted into their medical records. Midwives on the delivery ward were asked to place the placenta into a yellow biohazard bag (supplied) immediately after delivery and label this bag with a patient identifier. The bag containing the placenta was placed into a collection bucket located on the delivery ward at room temperature. Midwives were then asked to contact Katie as soon as possible. All placental samples were collected from the delivery ward within 2 hours of delivery. The placenta was brought to the lab and placed on a sterile aluminium bench. The placenta was cleaned with sterile water and the membranes and umbilical cord were removed. Placental weight was measured in kg. 5 cross-sectional samples were randomly excised from each placenta to incorporate both the maternal and fetal sides. The samples were washed in dH_2O and immediately stored at -80 °C for further analysis.

2.2.3 RNA extraction, cDNA synthesis and PCR

RNA was extracted from placental samples using the Trizol method as per the manufacturer's instructions. Briefly, placental sample was homogenised in 3 ml of Trizol reagent and left on ice for 10 minutes. Samples were centrifuges at 4°C from 5 minutes and 1 ml of the supernatant was transferred into a new Eppendorf. 200ul of chloroform was added, mixed and left at room temperature for 3 minutes. The sample was centrifuged at 4°C 12000g for 15 min. The upper clear aqueous phase was removed and placed in a new 1.5ml Eppendorf, 500ul of isopropanol was added and left at room temperature for 10 min. The sample was centrifuged at 4°C 12000g for 10 min. The supernatant was removed and the pellet was washed in 1 ml of 70% ethanol. The sample was centrifuged, supernatant removed and the pellet was left to air dry and re-suspended in 70ul of RNase free H_20. RNA concentration and quality was assessed using the Nanodrop 8000. 500 ng of placental RNA was reversed transcribed using the high capacity cDNA reverse transcription kit (Applied Biosystems) using the following parameters 25 °C for 10 min; 37 °C for 120 min; 85 °C for 5 min; and held at 4 °C until storage at 80 °C. Real time PCR was performed for HSD11B2 under the following cycling parameters;

50 °C for 2 min; 95 °C for 10 min; 50 repetitions of 95 °C for 15 s and annealing/elongating at 60 °C for 1 min. All samples were run in duplicate, cycle threshold values were recorded and analysis was performed using the 2−ΔΔcycle threshold method (Livak and Schmittgen, 2002).

2.3.0 Participant recruitment for the SMArTI Study

The SMArTI (stress microbial transfer to the Infant) study was carried out with full ethical approval from the Research Ethics Committee of Cork Teaching Hospital (Appendix A). Patients participating in the IMPROvED study at CUMH between October 2014 and August 2015 were invited to take part in SMArTI. Exclusion criteria for the IMPROvED study can be found at (Navaratnam et al., 2013). Women were referred to SMArTI from midwives working on the IMPROvED study or through posters and advertisements (Appendix A). Women who were approached to participate but declined were noted to produce recruitment estimates. All patients participating must agree to provide *1)* questionnaires for at least one visit *2)* saliva samples for at least one visit, *3)* vaginal swab at birth (if vaginal delivery) and *4)* fecal samples from their infant for as many time points as possible (see Figure 2 for breakdown of visits). Maternal fecal specimens, placenta and newborn hair samples were optional. For full study protocol see Appendix A. Detailed clinical and demographic data were collected from the IMPROvED database once the entire cohort had given birth. Additionally participants were asked about the mode of feeding of their infant when providing infant fecal samples. Details on postnatal maternal and infant antibiotic use and diagnosis of colic was also collected.

Figure 2: Flowchart of SMArTI visits

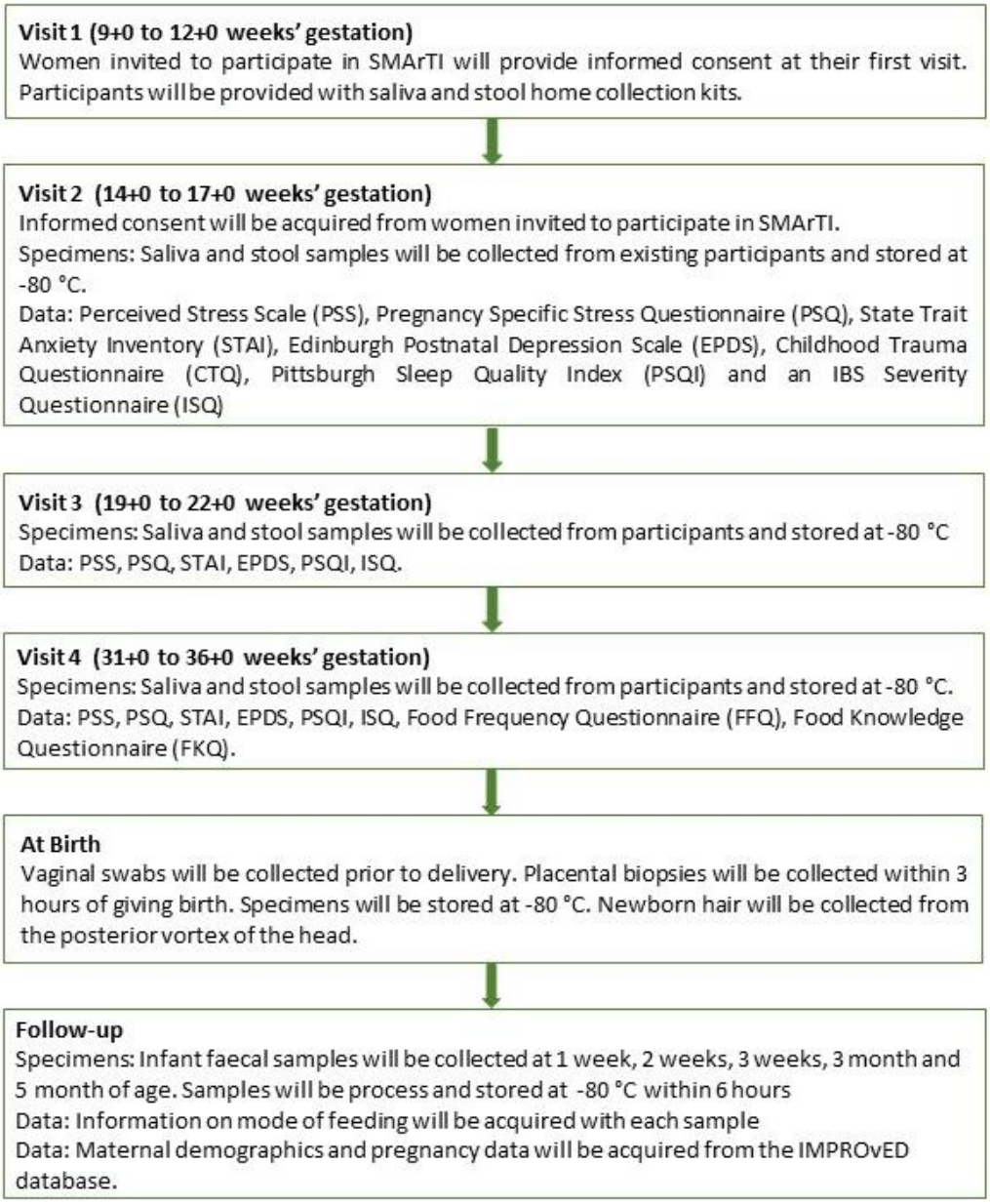


Figure 2: Flowchart of participant visits for the SMArTI study

Figure 3: Timeline of SMArTI study

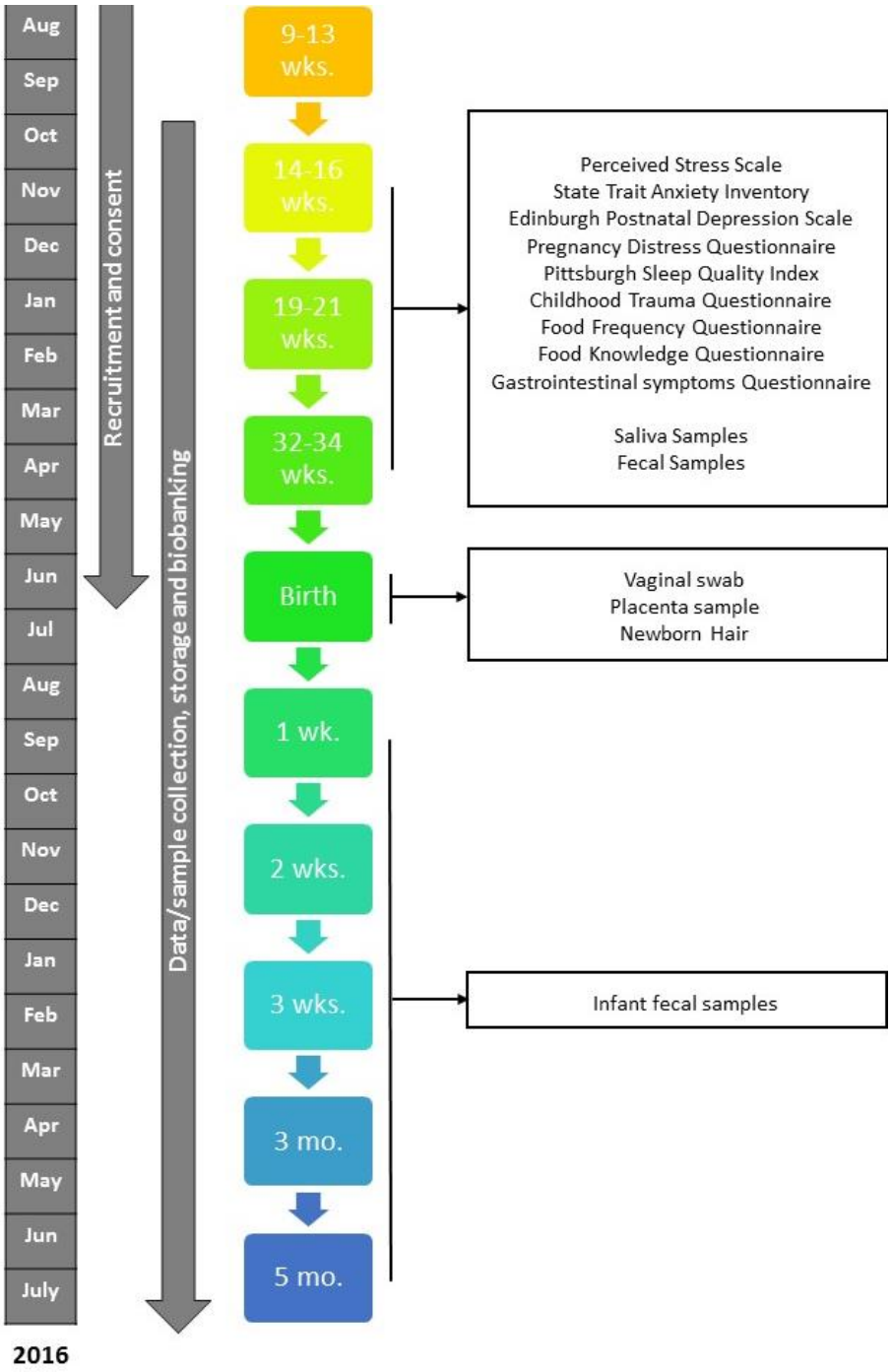


Figure 3: Timeline and overview data collected during the SMArTI study. This study began in August 2014 with the final sample collected in July 2016. Analysis of data and samples began thereafter.

2.3.1 Questionnaires

Questionnaires were completed by participants online using Survey Monkey (https://www.surveymonkey.com/). As well as the PSS, STAI and EPDS as used in the pilot study participants completed the Pregnancy Distress Questionnaire (PDQ), Pittsburgh Sleep Quality Index (PSQI), Childhood Trauma Questionnaire (CTQ), an IBS Severity Questionnaire, Food Frequency Questionnaire (FFQ) and a Food Knowledge Questionnaire (FKQ) (Appendix A). The mean gestational age at which the surveys were completed were 15.62 ± 0.136, 20.30 ± 0.087 and 32.67 ± 0.125.

Perceived Stress Scale

This study used the 10-item PSS. The PSS is a popular tool used to measure psychological stress and how individuals appraise stressful life events. Higher Scores on the PSS are indicative of a higher level of perceived stress (Cohen et al., 1983). In the SMArTI cohort a PSS score below or above the cohort mean was used to indicate low and high stress respectively. For both the second and third trimester PSS < 13 indicated low stress and PSS > 14 indicated high stress (Appendix A).

State Trait Anxiety Inventory

Maternal anxiety was measured using the 6-item version of STAI. The 6 item STAI is a frequently used brief psychological measure of anxiety and the 6 item version which has been validated for use during pregnancy (Marteau and Bekker, 1992). In the SMArTI cohort a STAI score below or above the cohort mean was used to indicate low and high anxiety respectively. . For both the second and third trimester STAI < 4 indicated low anxiety and STAI > 5 indicated high anxiety (Appendix A).

Edinburgh Postnatal Depression Scale

Depressive symptoms were assessed using the EPDS. For both the second and third trimester EPDS < 8 was used to indicated low depressive symptomology and EPDS > 9 was used to indicate high depressive symptomology (Appendix A).

Pregnancy Distress Questionnaire (PDQ)

The PDQ is a short questionnaire that measures any stress and anxiety specific to pregnancy (Alderdice et al., 2013) (Appendix A).

Pittsburgh Sleep Quality Index (PSQI)

The present study used the PSQI to assess sleep quality across pregnancy. The PSQI consists of 19 items which are then grouped into seven components; subjective sleep quality, sleep latency, sleep duration, habitual sleep efficiency, sleep disturbances, sleep medications and daytime dysfunction (Buysse et al., 1989). Each score is weighted on a 0-3 scale and summed to form a global PSQI score. A PSQI global score < 5 is indicative of good sleep quality, whereas a global PSQI score of ≥ 5 indicates poor sleep quality (Tsai et al., 2016) (Appendix A).

Childhood Trauma Questionnaire (CTQ)

114 participants completed the 28-item version of CTQ during their pregnancy. The CTQ assesses 5 scales of childhood maltreatment; physical abuse, emotional abuse, sexual abuse, physical neglect and emotional neglect (Bernstein et al., 2003). The measure includes a three-item Minimization/Denial scale indicating the potential underreporting of maltreatment. Participants respond to each item in the context of "when you were growing up" and answer according to a five-point Likert scale ranging from "never" = 1 to "very often" = 5, producing scores of 5 to 25 for each trauma subscale. The three items comprising the Minimization/Denial scale are dichotomized ("never" = 0, all other responses = 1) and summed; a total of one or greater suggests the possible underreporting of maltreatment (Appendix A).

IBS Severity Questionnaire

To measure gastrointestinal function we used the Rome III criteria for assessing gastrointestinal function (Ford et al., 2014) (Appendix A).

Food Frequency Questionnaire (FFQ) and Food Knowledge Questionnaire (FKQ)

Food intake over the course of pregnancy was measured using the FFQ. Additionally participants' knowledge on their food intake was assess by the FKQ (Appendix A).

2.3.2 Sample Collection and Storage

Maternal fecal samples

Participants were provided with 'at home' sample collection kits (Appendix A) and asked to collect the fecal samples at home, as close as possible to the time of their next SMArTI study appointment. The collection kits contained disposable gloves, a plastic container with an AnaeroGen sachet (Thermo Fisher Scientific) attached to the inside lid, disposable gloves, a zip lock bag and an envelope (Appendix A). Participants were instructed to put on the gloves, place the opened plastic container inside the toilet bowl and preform a bowel movement into the container. Participants were asked to tear the top of the AnaeroGen sachet open and firmly secure the lid of the container. The container was then placed into the zip lock bag and sealed in the envelope. The date and time of sample collection was noted on the envelope and participants were ask to keep the samples as cool as possible until bringing it with them to their SMArTI appointment at CUMH. Once acquired, samples were immediately aliquoted into five Eppendorf tubes in a Microflow Biological Safety Cabinet and stored at -80°C.

Maternal saliva samples

Participants were provided with 'at home' saliva collection kits (Appendix A) and asked to collect the saliva samples at home, the morning of, or before their SMArTI study appointment. To avoid contamination, participants were asked to abstain from brushing their teeth until all samples had been collected, and to abstain from eating for at least 30 minutes prior to collecting the samples. Participants were instructed to keep the saliva samples as cool as possible until bringing them to their appointment. Saliva collection kits consisted of 4 Salivettes (Sarstedt) in a zip-lock bag. Saliva samples were collected *1)* immediately upon waking, *2)* 30 min after waking, *3)* 1 h after waking and *4)* 3 h after waking. For each sample participants were asked to remove the lid of the Salivette tube and tip the 'bud' into their

mouth without using their hands. Participants were asked to chew the bud for 1-2 min and transfer the bud directly back into the Salivette tube without touching. The Salivette tube was labelled with the time and date, placed in the zip lock bag and kept cool until their appointment at CUMH. Once acquire, Salivette tubes were centrifuged for at 5000 rpm for 5 min at 4°C and each sample was aliquoted into 2x Cryovials and stored at -80°C.

Vaginal Swab and placental samples

Participants agreeing to these samples had a collection kit containing sterile swabs (Epicentre) and a biohazard bag inserted into their medical notes (Appendix A). Midwives in the delivery ward were asked to collect these samples. Vaginal swabs were collected after the patients consent when admitted to the delivery ward. A sterile swab was inserted into the vagina and turned a couple of times before being removed and sealed into its plastic tube. The tube containing the swab was then sealed in a zip lock bag and labelled with a patient identifier before being stored at 4°C. Vaginal swabs were collected from the delivery ward every day and stored at -80°C. Placental collection proceeded as per section 2.2.2.

Newborn hair

All participants were visited in CUMH within 24 hours after birth. Mothers were asked to donate a small sample of newborn hair from their infant. For infants whose mothers consented, a small section of hair was cut from the newborns head, as close to the scalp as possible and immediately placed in aluminium foil. Hair was stored at room temperature until processing.

Infant fecal samples

All women in the study were provided with collection kits containing sterile collection tubes with scoops (Sarstedt), zip lock bags and envelopes (Appendix A). Participants were asked to collect infant fecal samples at 1 week (w), 2 w, 2w, 3 months (m) and 5 m. Participants received a SMS reminder the evening before and morning of sample collection. When a dirty nappy was produced mothers were

asked to collected fecal matter with the scoop and seal in the sterile tube. The tubes were labelled with time and date, placed into the zip lock bag and kept as cool as possible. Participants were asked to contact Katie as soon as possible once the sample was collected and collection from the participants home was arranged immediately. Samples were transported on ice to CUMH. Samples were aliquoted into Eppendorf tubes in a Microflow Biological Safety Cabinet and stored at -80°C. Sample collection and freezing time were noted.

2.4 Cortisol Concentration

On the day of processing, samples were thawed on ice and cortisol concentration was determined by Enzyme linked immunosorbent Assay (ELISA) according to the manufactures instructions (Enzo Life Sciences). Prior to use reagents were brought to room temperature. Assay buffer solution was prepared by pipetting 5 ml of assay buffer 45 ml of dH_2O (1:10). Wash buffer solution was made by pipetting 10ml of wash buffer into 190 ml of dH_2O (1:20). The cortisol standard (100, 00 pg/ml) was diluted in assay buffer to produce a standard curves of concentrations 0, 156, 313, 625, 1250, 2500, 5000, 10 000 pg/ml. Once fully thawed saliva samples were vortexed and diluted in Assay buffer solution (1:3) to a final volume of 240 µl. 100 µl of assay buffer was pipetted into the NSB and B0 wells. Samples were vortexed and 100 µl of each sample was pipetted into the appropriate wells of the plate. 100 µl of standards was pipetted into the appropriate wells. All samples and standards were run in duplicate. 50 µl of blue conjugate was added to each well except the TA and Blank wells. 50 µl of yellow conjugate was pipetted into each well except the blank, TA and NSB well. The plate was sealed and incubated at room temperature for 2 h on a shaker at 500 rpm. After the incubation, the contents of the wells was emptied and the wells were washed with 400 µl of wash solution three times. The wells were emptied and dried. 5 ml of blue conjugate was added to the TA wells. 200 µl of pNpp substrate was pipetted into each well. The plate was covered with aluminium foil and left at room temperature. After 1 h, 50 µl of stop solution was added to each well and the plate was immediately read at 405 nm using the Varioskan Flash Instrument 4.00.51. Blank measurements were

subtracted and a four parameter logistic calibrator curve was constructed of the standards to determine cortisol concentration in each sample using the SkanIt Software (2.4.3 RE for Varioskan Flash). CV values were also recorder (%).

2.5 DNA Extraction from Maternal Fecal Samples

DNA was extracted from the maternal fecal samples using the Qiagen DNA stool Mini Kit in a sterile laminar-flow Class II Microflow Biological Safety Cabinet. Prior to use AW1 and AW2 buffer was prepared by adding 25 ml and 30ml of 100% molecular grade EtOH (Sigma) to each buffer respectively. Fecal samples were thawed on ice and 0.2 g of faeces was added to 1 ml Qiagen Lysis Buffer in a 2 ml screw cap tube containing a mix of 0.1 mm, 2.0 mm, 2.3 mm and 3.5 mm sterile zirconia beads (Thistle Scientific). The sample was homogenised for 90 seconds using the MagnaLyser Instrument (Roche) and incubated at 90 °C for 5 min. Samples were centrifuged at 16,000 g for 2 min and the supernatant was removed and transferred to a fresh 2 ml Eppendorf tube and placed on ice. 500 µl of Qiagen lysis buffer was added to the 2 ml screw cap tubes containing the pellet and the sample was homogenised again for a further 90 seconds using the MagnaLyser, followed by a 5 minute incubation at 90°C and centrifugation . The supernatants were then pooled and an *InhibitEX tablet* was added and vortexed until completely dissolved. Samples were centrifuged at 16,000 g for 2 min and the supernatant was removed. 200 µl of the supernatant was added to a new 1.5 ml Eppendorf containing 15 µl of Proteinase K and 200 µl of Buffer AL. The samples were vortexed briefly and incubated at 70 °C for 10 minutes. Following incubation the complete lysate was removed and placed in a QIAamp spin column. The column was centrifuged at 16 000 f for 1 min. The supernatant was discarded and the column was placed in a new 2 ml collection tube. 500 µl of AW1 buffer was added and the column was centrifuged at 16 000 g for 2 min. The supernatant was discarded and the column was again placed in a new 2 ml collection tube and 500 µl of AW2 buffer was added before centrifugation for 3 min at 16 000 g. The column was placed in a new collection tube and centrifuged for a further 1 min at 16 000g to remove any buffer carryover. The collection tube was discarded and the column was placed into

a new 1.5 ml Eppendorf tube. 200 µl of AE buffer was added and the sample was left the sit at room temperature for 2 min. The column underwent a final centrifugation at 16 000 g for 1 min to elute the DNA. Extracted DNA quantity (ng/ml) and quality (280/280) was determined using the Nanodrop 1000 and elutes were stored at -80 °C.

2.6 DNA Extraction from Infant Samples

Microbial DNA was extracted from 0.2 g stool samples using the Repeat Bead Beating method described by Yu and Morrison (Yu and Morrison, 2004), with some modifications. Lysis buffer was prepared to a desired concentration of 1M Sodium Chloride (NaCl), 500mM Tris hydrochloride (Tris-HCL), 500mM Ethylenediaminetetraacetic acid and 13% SDS in dH_2O. Infant fecal samples were thawed on ice and 0.2 g of faeces was added to 1 ml Qiagen Lysis Buffer in a 2 ml screw cap tube containing a mix of sterile zirconia beads (described section 2.6). The tubes were sealed and homogenised for 90 seconds (MagnaLyser). Samples were removed and cooled on ice for 60 seconds before another 90 seconds of homogenisation. Samples were incubated at 70 °C for 15 minutes. Samples were centrifuged at 16,000 g for 2 min, the supernatant was removed and placed in an Eppendorf on ice. 200 µl of lysis buffer was added to the remaining pellet. The tubes were homogenised again for 90 s in the MagnaLyser, followed by a 60 s incubation on ice and another 90 s in the MagnaLyser. Samples were heated at 70 °C for 15 min and centrifuged for 2 min at 16,000 g. The supernatant was removed and pooled with the supernatant form the first homogenisation. 260 µl of 10M ammonium acetate (Sigma) was added to the pooled supernatant and placed on ice for 5 min. The samples were centrifuged at 16,000 g for 10 min at 4°C. The supernatant was pipetted into 2 x 1.5 ml Eppendorf tubes and 250 µl of isopropanol (Sigma; molecular grade) was added to precipitate the nucleic acids and left on ice for a further 30 min. The sample was centrifuged for 10 min at 16,000g at 4°C. The supernatant was removed and the nucleic acid pellet was washed in 350 µl of 70% EtOH. Sample were vortexed and centrifuged for 5 min at 16,000 g at 4 °C. The supernatant was removed and discarded at the pellet was allowed to air-dry for 10 min followed by resuspension in 100 µl of TE buffer (Sigma). For purification, 1.5 µl

of DNase free RNase was added to each sample and left at 37 °C for 15 min. The duplicate samples were pooled and 15 µl of proteinase K (Qiagen) and 200 µl of AL buffer (Qiagen) were added. The mix was vortexed and incubated at 70 °C for 10 min. The samples were incubated wit 200 µl of 100 % EtOH, vortexed and transferred to a QIAamp spin column. Samples were cleaned with AW1 and AW2 (Qiagen) buffer as described in section 2.6. DNA was dissolved in 150 µl of AW buffer. Extracted DNA quantity (ng/ml) and quality (280/280) was determined using the Nanodrop 1000. DNA was separated into two 75 µl aliquots and stored at -80 °C.

2.7 DNA extraction from vaginal Samples

Collected swabs were removed from -80°C and immediately placed in a 2ml screw cap tube containing zirconia beads (as described above) with 1 ml of lysis buffer (described in Section 2.7). Samples were left at room temperature for 20min. A spatula was used to drain residual liquid from the swab and the swab was discarded. The tubes were sealed and homogenised for 90 seconds (MagnaLyser). Samples were removed and cooled on ice for 60 seconds before another 90 seconds of homogenisation. Samples were incubated at 70 °C for 15 minutes. Samples were centrifuged at 16,000 g for 2 min, the supernatant was removed and placed in an Eppendorf on ice. 200 µl of lysis buffer was added to the remaining pellet. The tubes were homogenised again for 90 s in the MagnaLyser, followed by a 60 s incubation on ice and another 90 s in the MagnaLyser. Samples were heated at 70 °C for 15 min and centrifuged for 2 min at 16 000 g. The supernatant was removed and pooled with the supernatant form the first homogenisation. DNA extraction proceeded as outlined above in Section 2.6.

2.8 16S rRNA Sequencing Library Preparation

Extracted DNA samples were quantified using the Nanodrop 1000^{TM} and stored at -80 °C until amplification. Preparation of samples for 16S Metagenomics Sequencing was done according to the manufactures instructions (Illumina). DNA was amplified to the V4 region of the bacterial 16S rRNA under the following parameters; 95°C for

3 min, 25 cycles of 95°C for 30 sec, 55°C for 30 sec and 72°C for 30 sec and finally 72°C for 5 min before being held at 4°C. The PCR product was cleaned using AMPure XP beads and 5µl of supernatant was transferred to a new PCR plate where PCR was performed with Nextera XT index primers under the same conditions as mentioned above. The product was cleaned again using AMPure XP beads and the final product was analysed on a Bioanalyser DNA 1000 chip for quality assurance (size ~630bp). Samples were diluted and sequenced by the Miseq Illumina System (See Figure 4).

2.9 Newborn hair sample Preparation

1mg of hair was suspended in 1 ml of methanol in a 2ml Eppendorf tube. The hair was incubated at 50°C for 24 h. Following incubation samples were placed on the sonicator for 30 min at 37 °C followed by another 24 h at 50°C. After incubation the supernatant was removed and placed in a clean 2 ml Eppendorf. The supernatant was evaporated under nitrogen until completely dry and the pellet was resuspended in 220ul of PBS. Cortisol concentration was determined using the ENZO ELISA kit as described in section 2.5, without diluting the samples.

Figure 4: Process from fecal sample collection to microbiome bioinformatics.

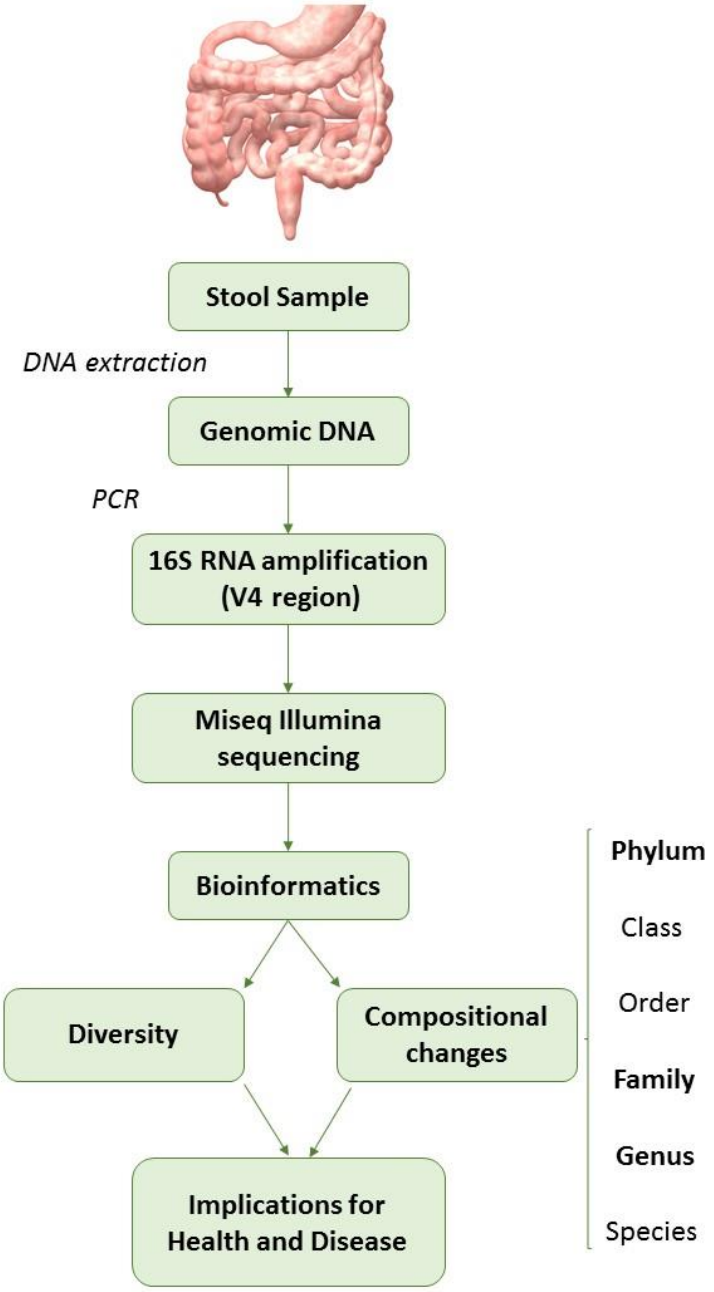


Figure 4: Microbiome work-package form stool sample to data analysis. DNA is extracted from stool samples, amplified against the V4 region of the 16S gene followed by Miseq Illumina sequencing. Bioinformatics preformed to determine the impact of the composition and diversity of microbiome on health.

2.10 Statistical analysis

For chapter 3, where appropriate, unpaired students *t*-test and one-way ANOVAs with Tukey post-hoc were used for statistical analysis. Data were deemed significant when $p < 0.05$. Data analysis was performed using Graphpad prism 5. For chapter 4, data analysis was performed using Graphpad prism 5 and SPSS version 22. Participants with greater than 3 missing values in their questionnaire were removed. Participants were also removed when medical records were unavailable. Kolmogorov-Smirnov tests were used to test for normality. Birthweight, birthweight centiles and placental weight ratios were normally distributed and analysed using an unpaired student *t*-tests or one-way ANOVA with Tukey's post hoc testing. Placental weight, Apgar scores and birth temperature displayed a non-normal distribution, therefore non parametric tests were used. Binary logistics regression with 95% confidence intervals were used to analysis nominal data. Multivariate data was analysis by ordinal logistic regression. Analysis was adjusted for maternal age, social class and BMI where indicated. Data were deemed significant if $p < 0.05$. For chapter 5 data analysis was performed using SPSS v22 and RStudio 3.4.1. Normality was tested using Kolmogorov-Smirnov tests. Data were deemed not normal when $p < 0.05$. Outliers were identified using a Grubbs test. Relationships were determined using linear regression analysis and were adjusted for maternal BMI where indicated. For chapter 6, data analysis was performed using RStudio 3.4.1 and SPSS v22. Kolmogorov-Smirnov tests were used to test for normality. α-diversity was normally distributed and was analysed using unpaired Student's *t*-tests. Phyla, Family and Genus measures were not normal and were subsequently analysed by Mann Whitney U-tests. As scoring in maternal distress questionnaires did not correlated with maternal salivary cortisol we treated these as independent variables. This resulted in a total of 8 predictor variables, stress (PSS), anxiety (STAI), depressive symptoms (EPDS), and maternal cortisol measurements from the second and third trimester. Only vaginally delivered infants were included in analysis involving infant samples. Newborn cortisol levels were not normally distributed and were therefore log transformed prior to analysis. The relationship between newborn cortisol and α-diversity of the infant gut was subsequently determined by linear regression analysis. Data were significant if $p < 0.05$.

Chapter 3:

Class-Specific Histone Deacetylase Inhibitors Promote 11-Beta Hydroxysteroid Dehydrogenase Type 2 Expression in JEG-3 Cells.

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Published in *Epigenetics 2017*

3.1 Abstract:

Exposure to maternal cortisol plays a crucial role in fetal organogenesis. However, fetal overexposure to cortisol has been linked to a range of short- and long-term adverse outcomes. Normally, this is prevented by the expression of an enzyme in the placenta called 11-beta hydroxysteroid dehydrogenase type 2 (HSD11B2) which converts active cortisol to its inactive metabolite cortisone. Placental HSD11B2 is known to be reduced in a number of adverse pregnancy complications, possibly through an epigenetic mechanism. As a result, a number of pan-HDAC inhibitors have been examined for their ability to promote HSD11B2 expression. However, it is not known if the effects of pan-HDAC inhibition are a general phenomenon or if the effects are dependent upon a specific class of HDACs. Here, we examined the ability of pan- and class-specific HDAC inhibitors to regulate HSD11B2 expression in JEG3 cells. We find that pan-, class I, or class IIa HDAC inhibition promoted HSD11B2 expression and prevented cortisol or interleukin-1*β*-induced decrease in its expression. These results demonstrate that targeting a specific class of HDACs can promote HSD11B2 expression in JEG3 cells. This adds to the growing body of evidence suggesting that HDACs may be crucial in maintaining normal fetal development.

3.2 Introduction:

The glucocorticoid hypothesis proposes that overexposure of the fetus to glucocorticoids may produce long lasting effects on fetal development that subsequently increase disease risk later in life (Reynolds, 2013). The glucocorticoid hypothesis is affirmed by studies that have shown that elevated maternal cortisol is associated with heightened HPA activity (Davis et al., 2011) and alterations in brain structure (Buss et al., 2012a) in affected offspring. At the core of this process is the placental enzyme 11β-hydroxysteroid dehydrogenase type 2 (HSD11B2), an enzyme that is expressed primarily within the syncytiotrophoblast of the placenta where it catalyses the conversion of active cortisol into its inactive product cortisone thereby controlling the levels of cortisol that reach the fetus (Chapman et al., 2013). A number of preclinical and clinical studies have demonstrated a reduction in the placental expression of HSD11B2 following exposure to prenatal stress (Jensen Pena et al., 2012), anxiety (Conradt et al., 2013) and following maternal infection (Straley et al., 2014). In addition to this, placental *HSD11B2* mRNA levels are reduced in pregnancy complications such as preeclampsia, (Hu et al., 2014) intrauterine growth restriction (IUGR), (Dy et al., 2008) preterm birth (PTB) (Kajantie et al., 2003) and low birth weight (LBW) (Mericq et al., 2009).

A complex repertoire of molecular pathways has been shown to be involved in regulating placental *HSD11B2* expression. Inhibition of the mitogen-activated protein kinases (MAPK), ERK1/2 increases *HSD11B2* expression (Guan et al., 2013), whilst supressing p38 reduces HSD11B2 activity (Sharma et al., 2009). *HSD11B2* is increased by activation of peroxisome proliferator-activated receptor delta (PPARδ) (Julan et al., 2005) through recruitment of the SP1 transcription factor (TF) (He et al., 2014). Similarly, activation of the hedgehog signalling (Zhu et al., 2016) and forskolin-induced activation of the cyclic AMP (cAMP) pathway increases *HSD11B2* expression (Ni et al., 2009). More recently epigenetic mechanism have been linked to HSD11B2 regulation. The most widely studied epigenetic mechanisms are DNA methylation and histone acetylation. Histone acetylation is regulated by histone acetyl transferase (HATs) and histone deacetylase (HDACs) enzymes. HATs add acetyl groups onto the N-terminal tail of histone proteins which increases gene expression (Yang, 2004). HDACs remove them thereby repressing transcription

(Murakami, 2013). In humans 18 HDACs have been discovered and they are classed into four main families; class I (HDAC 1, 2, 3 and 8), class II (HDAC 4, 5, 6, 7, 9 and 10), class III (SIRT1, 2, 3, 4, 5, 6 and 7) and class IV (HDAC 11) (Brandl et al., 2009).

Recently a significant emphasis has been placed on *in vitro* studies to tease apart the precise epigenetic mechanisms involved in regulating placental HSD11B2 expression. Global knock down of DNA methylation using the demethylating agent 5-aza-2'-deoxycytidine (5-aza) in JEG-3 cells has been shown to increase the expression of a number steroidogenic genes including *HSD11B2*, indicating a direct link for regulation of *HSD11B2* expression by methylation (Hogg et al., 2014). Despite advancements being made in understanding the role of methylation in HSD11B2 expression, little focus has been placed on examining the role that HDACs play in regulating HSD11B2. The present study aimed to investigate the role of histone acetylation in regulating basal and stressor-induced changes in HSD11B2 protein expression in an *in vitro* placenta model using small molecule pharmacological inhibitors.

3.3 Methods:

3.3.0 Cell Culture and Treatment

JEG-3 cells were grown in Dulbecco's modified Eagle's medium (DMEM):F12 (Sigma), with 10% foetal calf serum (FCS), 100nM L-Glutamine, 100U/ml penicillin and 10μg/ml streptomycin (Sigma). Cells were maintained at 37°C in a humidified atmosphere of 5% CO_2. 50,000 cells per well were plated on a 24 well plate and were treated with 1, 5 or 10 μM of MC1568, MS275 or SAHA (Selleckchem). Where indicated, 10ng/ml interleukin-1β (IL-1β; Promokine) or 2μM Cortisol (Cort; Santa Cruz) were added for 24 h before HDAC inhibitor (HDI).

3.3.1 MTT Assay

To assess cell viability, a thiazolyl blue tetrazolium bromide (MTT) solution was added to the cells at a concentration of 1mg/ml in HBSS (Sigma). Following a 2 hour (h) incubation at 37°C, the cells were lysed in DMSO (Sigma). Absorbance was measured at a wavelength of 540nm with a reference wavelength of 630nm.

3.3.2 Immunocytochemistry

At the experimental end point, cultures were fixed in 4% paraformaldehyde (PFA) in PBS for 10 min. Following 3 × 5min washes in 10mM PBS containing 0.02% Triton X-100 (PBS-T), cultures were incubated in blocking solution (5% BSA in PBS-T) for 1 h at room temperature. Where indicated cultures were incubated in the following primary antibodies; HSD11B2 (1:250; Santa Cruz), AcH3 (1:250; Santa Cruz), GR (1:250; Santa Cruz) or IL1R1 (1:250; Invitrogen) diluted in 1% BSA in 10mM PBS at 4 °C for 16h. Following 3 × 5min washes in PBS-T, cells were incubated in the appropriate Alexa Fluor 488- or 594-conjugated secondary antibodies (1:1000; Invitrogen) diluted in 1% BSA in 10mM PBS at room temperature for 2 h. Cultures were counterstained with DAPI (1:3000; Sigma). Cells were imaged under an Olympus IX70 inverted microscope with Olympus DP70 camera and AnalysisD™ software.

3.3.3 RNA Extraction and Real-Time PCR

RNA was extracted from JEG-3 cells 24 hours after seeding and term human placental tissue using Trizol Reagent (Life Technologies). Placental tissue was homogenised with a pestle and mortar and JEG-3 cells were removed from flasks by scraping, and incubated in Trizol for 10 min and RNA extraction proceeded according to the manufacturers' instructions. 500 ng of RNA was reverse transcribed using a high capacity cDNA Reverse Transcription Kit (Applied Biosystems) in a 20µl reaction mixture consisting of 2.0µl 10× RT Buffer, 0.8µl 25× dNTP mix (100mM), 2.0µl 10× RT Random Primers, 1.0µl Reverse Transcriptase, and 4.2µl Nuclease-free H_2O, using the following parameters: 25 °C for 10 min; 37 °C for 120 min; 85 °C for 5 min; 4 °C for at least 10 min. The cDNA was stored at −80 °C prior to use. For real-time PCR, samples were run in duplicate using TaqMan® Gene Expression Assay (Applied Biosystems) for *HSD11B2* using *18S* as a reference gene under the following parameters; 50 °C for 2 min; 95 °C for 10 min; 40 repetitions of 95 °C for 15 s and annealing/elongating at 60 °C for 1 min.

3.3.4 Immunohistochemistry

Histological placental sections (6µM) were incubated in blocking solution (5% bovine serum albumin (BSA)) for 1 h at room temperature. Sections were treated with 10% H_2O_2 for 5 min, washed in 10mM Phosphate Buffered Saline (PBS) and blocked for 1 h in 10% normal goat serum in 10mM PBS with 0.4% Triton-X. Sections were incubated in primary antibody to HSD11B2 (1:250; Santa Cruz) in 1% normal goat serum in 10mM PBS with 0.4% Triton-X overnight at 4°C. Following a 3 × 10 min wash in 10mM PBS, sections were incubated with a biotinylated secondary antibody (1:200; Vector Labs) for 2 h at room temperature. Following another 3 × 10 min wash in 10mM PBS, sections were incubated in ABC solution (1:200; Vector Labs) for 45 min at room temperature followed by immersion in diaminobenzidine substrate/chromogen reagent for 2–3 min at room temperature. Sections were dehydrated, cleared, mounted and imaged using an Olympus AX70 Provis upright microscope.

3.3.5 Statistical Analysis

For real time PCR, expression levels were calculated using the 2 delta Ct threshold method (Schmittgen and Livak, 2008). For immunocytochemistry the fluorescence intensity of individual cells that were immunopositive for HSD11B2 or AcH3 were measured by densitometry using Image J analysis software (Rasband, WJ, http://rsb.info.nih.gov/ij/). The relative fluorescence intensity of HSD11B2 or AcH3 was calculated as the average fluorescence intensity after subtraction of the background noise. Data was analysed using GraphPad Prism v 5 (GraphPad Software Inc, San Diego, California). Where indicated data was analysed (as per section 2.3) with unpaired Student's *t*-test or one-way ANOVA with Tukey's post-hoc testing. Values of $p < 0.05$ were considered statistically significant.

3.4 Results:

3.4.0 Distribution of HSD11B2 in the Human Placenta and JEG-3 Cells

We utilized the BioGPS database, an online platform that enables the examination of relative levels of gene expression across multiple human tissues (Wu et al., 2009). Using this directory we confirmed the highest levels of *HSD11B2* in the placenta, followed by the kidneys, with very little expression seen in other tissues (Fig. 1a), which was confirmed by immunohistochemistry on human term placental samples (Fig. 1c). We next aimed to validate the use of the human choriocarcinoma cell line, JEG-3 cells. JEG-3 cells are a widely used *in vitro* model of placental trophoblast cells and have previously been demonstrated to be an abundant source of endogenous HSD11B2 (Alikhani-Koopaei et al., 2004, Tremblay et al., 1999). In agreement with this, real-time PCR confirmed the expression of *HSD11B2* mRNA in JEG-3 cells, with placental RNA used as positive control (Fig. 1b). Immunohistochemical staining preformed 24 hours after seeding also confirmed abundant expression of expression of HSD11B2 protein in JEG-3 cells (Fig. 1d).

3.4.1 Pan-HDAC Inhibition Increases HSD11B2 Expression in JEG-3 Cells

HDACs can be divided into four distinct families, of particular interest are class I (HDAC1, 2, 5, 8) and class II (HDAC5, 6, 7, 9 and 10) HDACs (Haberland et al., 2009). We used the BioGPS database to examine the relative expression levels of these different HDACs in the human placenta. Class I and class II HDACs were widely expressed in the placenta (Appendix B, Supplementary Fig. 1), however HDAC1 (Class I) and HDAC5 (Class IIa) had the highest relative levels of expression in the placenta compared to other tissues (Fig. 3a, e). Given the widespread expression of HDACs, we next sought to determine the effect of global HDAC inhibition on placenta HSD11B2 protein expression. We treated JEG-3 cells with SAHA, a competitive inhibitor of both class I and class II HDACs (Xu et al., 2007). An initial dose response experiment was carried out 24 hours after seeding where JEG-3 cells were treated with concentrations of SAHA ranging from 1-10μM for 24h, followed by immunocytochemical staining for HSD11B2. The relative expression of HSD11B2 protein was quantified using densitometry. A one-way ANOVA revealed a significant

overall effect of SAHA treatment on HSD11B2 expression ($F_{(3,8)}$=5.5, p=0.02). Tukey's post-hoc test revealed a significance difference between the vehicle- and 10µM SAHA group (p<0.05) (Fig. 2a). As the effects of SAHA were significant at 10µM, we also immunocytochemically stained for p-Ac-histone H3 (S11/K15) (pAcH3) in this group and found a significant increase in the levels of pAcH3 in cells treated with 10µM SAHA for 24h (p<0.001) (Fig. 2b). Overall these data indicate that pan-HDAC inhibition increases the levels of pAcH3 (which has been shown to correlate with gene expression) and HSD11B2 expression in JEG-3 cells.

3.4.2 Class-Specific HDAC Inhibitors (HDI) Promote HSD11B2 Expression in JEG-3 Cells

We next investigated if the effects of pan-HDAC inhibition on HSD11B2 expression was class specific using a class I-specific HDI (MS275) (Bracker et al., 2009), and a class IIa-specific HDI (MC1568) (Collins et al., 2014). JEG-3 cells were treated with increasing concentrations (0-10µM) of MS275 or MC1568 for 24 h before being immunocytochemically stained for HSD11B2 and quantified using densitometry. A one-way ANOVA revealed a significant overall effect of both MS275 ($F_{(3,8)}$=95.89, p<0.0001) and MC1568 ($F_{(3,8)}$=53.69, p<0.0001) treatment. Tukey's post-hoc test showed that MS275 or MC1568 promoted a significant increase in HSD11B2 protein expression with a significant difference observed between the control and HDI-treated groups at concentrations of 1µM (p<0.05), 5µM (p<0.0001) and 10µM (p<0.0001) (Fig. 3b, f). We also examined pAcH3 levels using densitometry and found a significant increase in the levels of pAcH3 in cells treated with 10µM MC1568 or MS275 for 24h (p<0.001) (Fig 3c, g). These data show that class-I and class-IIa inhibition can promote HSD11B2 protein expression in JEG-3 cells.

3.4.3 Cortisol and IL-1β Decrease HSD11B2 Expression Which Is Prevented by MC1568

Given that alterations in placental *HSD11B2* expression are seen in pregnancies complicated with stress or infection (Straley et al., 2014, Jensen Pena et al., 2012), we next sought to determine if the biological mediators of stress (Cort) and

infection (IL-1β) altered HSD11B2 protein expression at the cellular level. Having confirmed using immunocytochemistry that the glucocorticoid receptor (GR) and interleukin 1 receptor, type I (IL1R1) were expressed in JEG-3 cells (Fig. 4a), we carried out an MTT assay to establish a concentration of Cort and IL-1β that did not affect cell viability. JEG-3 cells were treated with Cort (0-10μM) or IL-1β (0-100ng/ml) for 24h and MTT assays were performed. An ANOVA showed an overall effect of Cort and IL-1β treatment on cell viability, with a difference observed with 10μM Cort (Fig. 4b) and 100ng/ml IL-1β (Fig. 4c) groups ($P<0.05$). JEG-3 cells were then treated with 2μM of Cort or 10ng/ml IL-1β (concentrations that did not affect cell viability) for 24h before being fixed and immunocytochemically stained for HSD11B2. Using densitometry, we observed a reduction in HSD11B2 protein expression following exposure to Cort and IL-1β (Fig. 4d).

3.4.4 HDIs Can Restore HSD11B2 Expression in an Environment of Stress and Inflammation

After identifying Cort and IL-1β as potential biological mediators causing a decrease in HSD11B2 protein expression we next aimed to determine if HDIs could counteract these effects of cortisol and IL-1β on HSD11B2 protein expression. After plating for 24 hours, JEG-3 cells were treated with 10μM of SAHA, MC1568 or MS275 followed by cortisol or IL-1β before being fixed and immunocytochemically stained for HSD11B2 protein. Densitometry revealed that pre-treatment of JEG-3 cells with non-specific inhibitor SAHA attenuated the effect of IL-1β and Cort (SAHA: 2.8±0.15; SAHA+ IL-1β: 3.0±0.2; SAHA + Cort: 3.350±0.19) (Fig. 5a, e) on HSD11B2 expression. Similarly treatment of JEG-3 cells with either class I- specific HDI, MS275 (MS275: 2.2±0.06; MS275+ IL-1β: 3.18±0.06; MS275 + Cort: 2.2±0.09) (Fig. 5b, f) or class II a-specific HDI MC1568 (MC1568: 1.8±0.08; MC1568+ IL-1β: 2.3±0.1; MC1568 + Cort: 1.4±0.07) (Fig. 5c, g) was sufficient to attenuate the effect of both Cort and IL-1β on HSD11B2 expression. These data show that exposure to heightened levels of Cort and IL-1β can reduce the levels of HSD11B2 protein in JEG-3 cells, and that this effect that can be prevented by HDAC inhibition.

3.5 Figures and Figure Legends:

Figure 1:

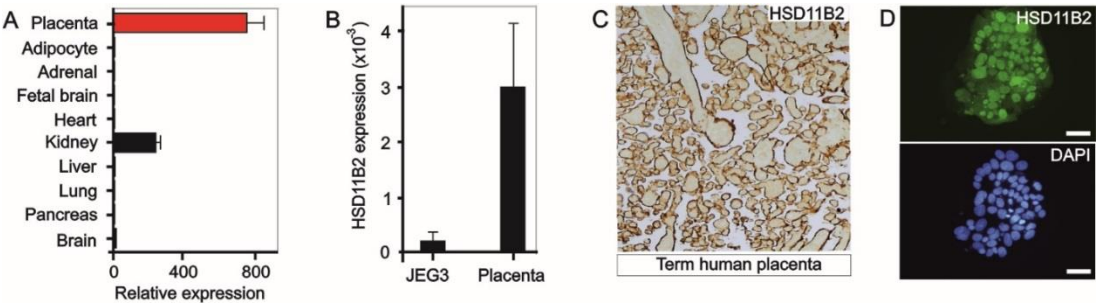

Figure 1: (A) Expression of data derived from the BioGPS database showing relative *HSD11B2* expression across multiple human tissues. (B) Real-time PCR showing *HSD11B2* expression in the term human placenta and in JEG-3 cells using the 2 delta-Ct method (N=3, $p > 0.05$, Unpaired Student's *t*-test; Housekeeping gene 18S). Representative photomicrographs of (C) a term human placenta and (D) JEG-3 cells immunocytochemically stained for HSD11B2. Scale bar = 50µm.

Figure 2:

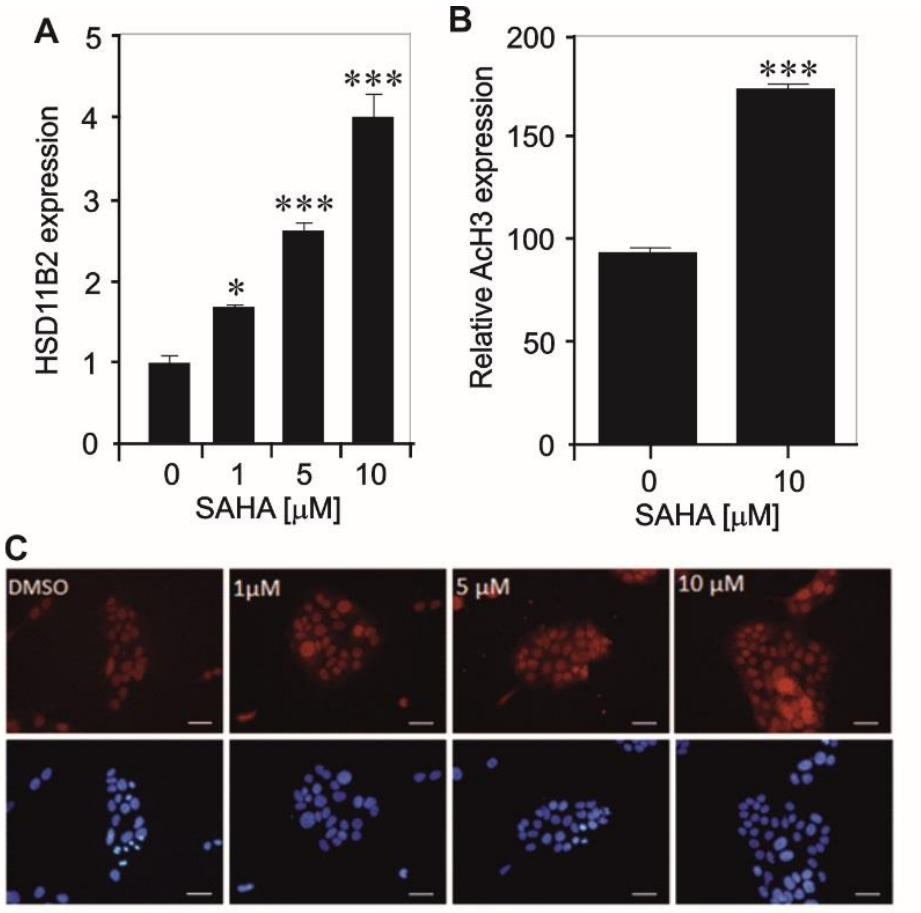

Figure 2: Epigenetic regulation of HSD11B2 expression. Graphical representation of (A) HSD11B2 and (B) AcH3 expression in JEG-3 cells treated with 0-10μM of SAHA for 24h. Data are expressed as mean ± SEM. (C) Representative photomicrographs of JEG-3 cells immunocytochemically stained for HSD11B2 *(* $p < 0.05$, *** $p < 0.001$* compared to 0μM. (A) One-way ANOVA with post hoc Tukey's and (B) Unpaired Student's *t*-test; 25 cells per group per experiment; N=3). Scale bar = 50μm.

Figure 3:

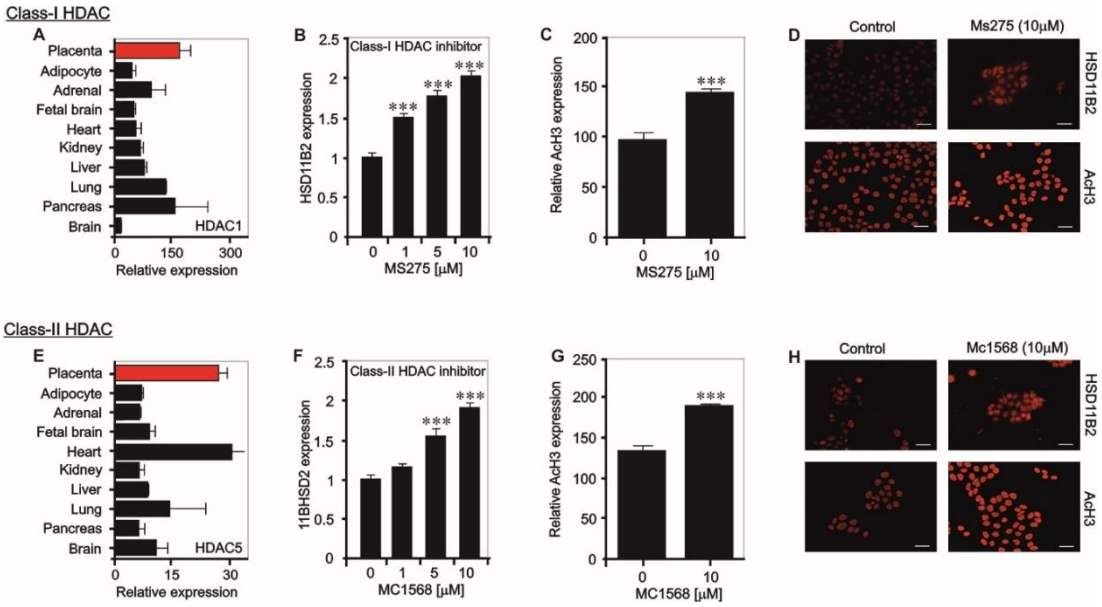

Figure 3: Class-specific HDACs on HSD11B2 regulation in the placenta. (A) Expression data from the BioGPS database showing the relative expression of a Class-I HDAC, HDAC 1 in the placenta relative to multiple human tissues. Graphical representation of (B) HSD11B2 and (C) AcH3 expression in JEG-3 cells treated with Class-I HDAC inhibitor MS275 for 24 h. (D) Representative photomicrographs of JEG-3 cells immunocytochemically stained for HSD11B2 and AcH3 after treatment with MS275 for 24 h. (E) Expression data from the BioGPS database showing the relative expression of a Class- II a HDAC, HDAC 5 in the placenta relative to multiple human tissues. Graphical representation of (F) HSD11B2 and (G) AcH3 expression in JEG-3 cells treated with Class- II a HDAC inhibitor MC1568 for 24 h. Data are expressed as mean ± SEM (H) Representative photomicrographs of JEG-3 cells immunocytochemically stained for HSD11B2 and AcH3 after treatment with MC1568 for 24 h. Data are expressed as mean ± SEM (*** $p < 0.001$ compared to 0μM; (B, F) one-way ANOVA with post hoc Tukey's and (C, G) Unpaired Student's *t*-test; 25 cells per group per experiment; N=3). Scale bar = 50μm.

Figure 4:

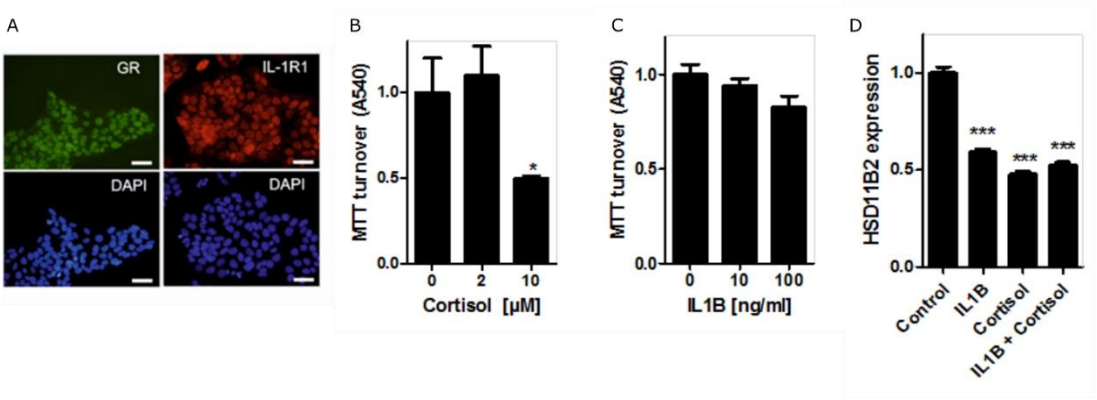

Figure 4: Cortisol and IL-1β response in JEG-3 cells. (A) Representative photomicrographs of JEG-3 cells immunocytochemically stained for the Glucocorticoid receptor (GR; green) and (C) the Interleukin 1 Receptor, Type I (IL-1R1; red). The second panel shows the corresponding DAPI stained image. (B, C) MTT assay examining the viability of JEG3 cells treated with either 0-10µM Cortisol (B) or 0-100ng/ml IL-1β (C) for 24 h *in vitro*. (D) Graphical representation showing the levels HSD11B2 in JEG-3 cells exposed to a vehicle (control), 10ng/ml IL-1β or 2µM cortisol for 24 h. Data are expressed as mean ± SEM (** $p < 0.05$* compared to 0µM, **** $p < 0.001$* compared to control ; one-way ANOVA with post hoc Tukey's; (D) 100 cells per group per experiment; N=3). Scale bar = 50µm.

Figure 5:

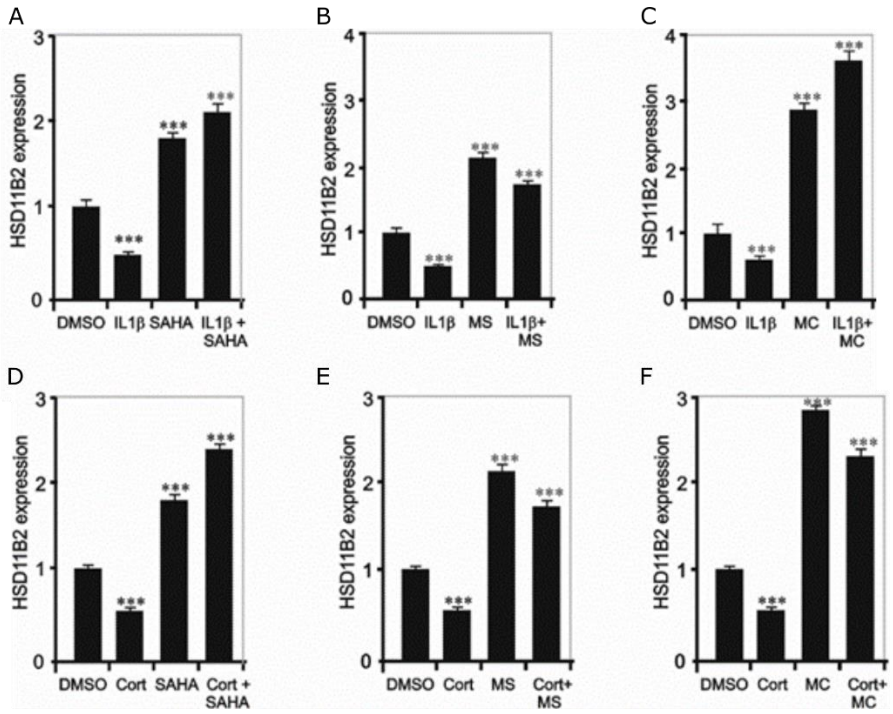

Figure 5: SAHA, MS275 and MC1568 prevent Cortisol and IL1β-induced decreases in HSD11B2 expression. Graphical representation and HSD11B2 expression in JEG-3 cells treated with 2µM Cort or 10ng/ml IL-1β in the presence or absence of 10µM SAHA (A, D), MS275 (B, E) or MC1568 (C, F) for 24 h. Data are expressed as mean ± SEM. (*** $p < 0.001$ compared to DMSO; one-way ANOVA with post hoc Tukey's; 25 cells per group per experiment; N=3).

3.6 Discussion:

The aim of this study was to examine the role of epigenetic regulators in the control of HSD11B2 protein expression in placental cells. We used the *in vitro* placental model JEG-3 cells, as despite their limitations, they are a well-established cell line commonly used to mimic placental trophoblast cells (Orendi et al., 2011). We employed pharmacological inhibitors of HDACs to modulate histone acetylation and examined the impact of this on HSD11B2 protein expression. Finally, to assess the potential of these compounds to regulate HSD11B2 expression under conditions of stress and inflammation, cells were exposed to biological mediators of these conditions, namely exogenous cortisol and IL-1β.

HSD11B2 has previously been shown to localise to trophoblast cells, with highest expression observed in the syncytiotrophoblast (Krozowski et al., 1989, Chen et al., 2015). In line with these studies we demonstrated HSD11B2 protein is strongly expressed in the term human placenta. To model trophoblast cells *in vitro*, we used the human choriocarcinoma cell line, JEG-3 cells. We found that these cells express *HSD11B2* mRNA making them a useful and convenient model to examine the molecular mechanisms that regulate HSD11B2 expression.

Using the BioGPS database we demonstrated high expression of class 1 HDACs 1, 2, 3 and 8 and class 2 HDACs 5, 4, 7 and 9, suggesting a role for HDAC proteins in the placenta. Based on these findings, we used a SAHA, a pan-HDAC inhibitor and demonstrated a dose dependant increase in HSD11B2 protein expression. To confirm the increased in HSD11B2 protein expression was paralleled by an increase in histone acetylation, we immunocytochemically stained the cells for AcH3 and showed a similar dose dependant increase AcH3. This is in contradiction to previous studies, where *HSD11B2* expression was reported to be unchanged in JEG-3 cells following treatment with broad spectrum class-I and class-II inhibitor trichostatin A (Alikhani-Koopaei et al., 2004). However the dose of TSA (300nm) used in these studies was much smaller than the dose at which we observed an effect (10uM) and we have identified that the effect of HDAC inhibition on HSD11B2 expression is dose dependant.

HDACs play a diverse role during fetal development (Haberland et al., 2009). Global knockdown of HDAC3 (Montgomery et al., 2008) HDAC1 (Montgomery et al.,

2007) and HDAC7 (Montgomery et al., 2007) results in fetal lethality, however mice lacking HDAC6 develop normally (Zhang et al., 2008b). HDACs have also been shown to be important regulators of placental development as inhibition of class-II HDACs has been shown to impair trophoblast differentiation through interactions with Hypoxia-inducible factor (Maltepe et al., 2005). Additionally interaction of HDACs with the STAT-1 TF may contribute to inhibition of IFN-γ-inducible gene expression in trophoblast cells thereby protecting the placenta cells from maternal immune rejection and contributing to a successful pregnancy (Choi et al., 2009). This broad range of functions of HDACs suggests that global inhibition could result in detrimental effects, therefore a more specific inhibition could represent an optimal method for modifying HSD11B2 expression. To determine if HDAC regulation of HSD11B2 protein expression is class specific we used class specific pharmacological HDAC inhibitors. We observed a similar increase in HSD11B2 protein expression with class specific inhibition of either class I or class IIa HDACs, suggesting many HDACS are likely involved in regulating HSD11B2 protein expression. Whilst this is the first study to examine the effects of class specific inhibitors on HSD11B2 expression, it is interesting to note that previous studies have demonstrated a class specific effect of HDACs on regulation of other placental genes. Specifically, matrix metalloproteinase 9 has been shown to be regulated by class-II but not class-I HDACs (Poljak et al., 2014).

Placental *HSD11B2* has been shown to be reduced in a number of adverse pregnancy conditions including anxiety, stress and infection (Jensen Pena et al., 2012, Conradt et al., 2013, Straley et al., 2014). As elevations in pro-inflammatory cytokines and steroids are observed in these conditions (Coussons-Read et al., 2007, Baibazarova et al., 2013), we used cortisol and IL-1β to mimic an environment of stress and inflammation. We have previously demonstrated a reduction in HSD11B2 protein expression in JEG-3 cells following administration of IL-1β (Straley et al., 2014). In this study we also report a decrease in HSD11B2 expression following cortisol administration. In contrast Ni and colleagues have previously shown an increase in HSD11B2 expression in primary human trophoblast cells exposed to Cort (Ni et al., 2009). However this study used a number of different methodological approaches to the work presented here. Firstly the work conducted

by Ni and colleagues used primary cell cultures and we used a cell line. This highlights the need for further study of these questions in primary trophoblast cells. Additionally the maximum dose of cortisol used was 1μM, whereby we observed a decrease at 2μM. It is possible that cortisol may act in an adaptive way to induce HSD11B2, thereby protecting the fetus from high maternal glucocorticoids, but at a certain threshold cortisol may begin to negatively impact on HSD11B2 expression. Finally, Ni and colleagues quantified HSD11B2 mRNA expression and we focused primarily on protein levels (Ni et al., 2009).Further examination of the role of HDACs and HSD11B2 gene expression will uncover if this regulation occurs at a transcriptional, translational and/or posttranslational level.

Interestingly broad or either class specific HDAC inhibitors were sufficient to prevent the cortisol and IL-1β-induced decreases in HSD11B2 expression. This raises the possibility of targeting key epigenetics modulators to protect the fetal glucocorticoid barrier and untimely fetal glucocorticoid overexposure. However given the critical role of epigenetic marks in fetal development, non-specific inhibition of HDACs, even at class level, could produce detrimental effects on fetal development, therefore identifying more specifically the precise epigenetic mechanism mediating HSD11B2 regulation using knockdown or overexpression of individual HDACs would allow the development of a more targeted approach. The advancement of targeted nanoparticles to deliver chemotherapeutic agents directly to the placenta represents an exciting new avenue to alter placental epigenetic mediators without inferring with the fetus (Kaitu'u-Lino et al., 2013). Notably we also observe a potentiation of the effects of SAHA on HSD11B2 expression when administered with cortisol. Once activated the GR can bind to many coactivator proteins with known HAT activity (Ito et al., 2005). The combined inhibition of HDACs by SAHA with the potential increase in HAT activity cause by GR activation from exogenous cortisol may explain this enhanced HSD11B2 protein expression. This relationship further highlights the complexity of HSD11B2 regulation and the epigenetic landscape and confirms the need for more studies examining how placental HSD11B2 protein is controlled under both basal and pathological conditions.

A limitation to this work is the use of commercially available antibodies, which have the potential to produce false positive results due to non-specific protein binding. The antibody we used in this study, acquired from Santa Cruz, has been frequently used to quantify HSD11B2 expression in placental cells and therefore we are confident that we have accurately measured HSD11B2 protein expression (Li et al., 2011, Ma et al., 2012, Ni et al., 2009). Although we acknowledge the sensitivity and specificity of this, and other commercially available antibodies for HSD11B2, warrants further investigation (Herrera et al., 2013). By quantifying protein expression via immunocytochemistry (versus western blotting) we were able to improve the specificity of our results by measuring protein levels in distinct cellular localizations (Kurien et al., 2011). For HSD11B2, cytoplasmic expression was quantified and for AcH3 nuclear levels were measured.

Here we provide evidence of a role for histone acetylation in the regulation of HSD11B2 in the placenta; a limitation is that the present study used JEG-3 cells. Although we confirmed HSD11B2 to be abundantly expressed in this cell line and that *HSD11B2* levels are comparable between JEG-3 cells and the human placenta, there are potential caveats associated with using JEG-3 cells (Sokolov et al., 2015). As such replicating the current study in primary trophoblasts will help to clarify the functional role of HDACs in the regulation of HSD11B2 protein expression in the placenta. However the present study demonstrates a role for HDACs in the regulation of a key enzyme that maintains the fetal glucocorticoid barrier under basal and pathological conditions. It is likely that combinations of different epigenetic modifiers including HDACs are involved in regulating HSD11B2 expression. As HDACs have a broad role in regulating fetal development, inhibition of all HDACs could be detrimental to the developing fetus. Therefore unravelling the role of individual HDACs in HSD11B2 regulation, using more specific pharmacological inhibitors or targeted knockdown of HDACs will be crucial to understand the epigenetic mechanisms that regulate HSD11B2 expression, and for developing novel protective pharmacotherapies for the human placenta.

Chapter 4:

Maternal distress in late pregnancy alters obstetric outcomes and the expression of genes important for placental glucocorticoid signalling.

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4.1 Abstract:

The experience of maternal distress in pregnancy is often linked with poorer obstetric outcomes for women as well as adverse short and long term outcomes for affected offspring. Alterations in placental glucocorticoid signalling and subsequent increased fetal exposure to cortisol during development have been suggested to underlie this relationship. In the current study 121 pregnant women receiving antenatal care at Cork University Maternity hospital completed the Perceived Stress Scale, State Trait Anxiety Inventory and Edinburgh Postnatal Depression Scale in the third trimester of pregnancy. Placental samples were collected immediately after delivery. Maternal history of psychiatric illness and miscarriage were significant predictors of poorer mental health in pregnancy. Higher levels of anxiety were associated with an increase in women delivering via elective Caesarean Section, and an increase in women bottle-feeding their infant. Birth temperature was mildly reduced among infants of women who reported high levels of depressive symptomology. Similarly babies of mothers who scored high in all stress measures (cumulative distress) had mildly reduced 5-minute Apgar scores. High cumulative distress reduced the expression of placental HSD11B2 and increased the expression of placental NR3C1 mRNA. This data supports a role for a relationship between prenatal distress and adverse obstetric outcomes. The alterations in placental gene expression support a potential role for altered placental glucocorticoid signalling in mediating this relationship. This highlights the importance of addressing mental health during pregnancy to promote healthier obstetric and infant outcomes.

4.2 Introduction:

There is now an extensive body of evidence showing that the *in utero* experience is a critical determinant of fetal outcome (Langley-Evans, 2006). One factor that has been extensively studied in this regard is the adverse effect of maternal prenatal distress on birth outcomes (Bussières et al., 2015). This is important, as understanding the relationship between prenatal distress and unfavourable births outcomes may allow for targeted maternal or fetal surveillance in high-risk pregnancies, or timely intervention to decrease the risk of an adverse outcome. The term prenatal distress is often used to collectively refer to negative psychological wellbeing and encompasses, stress, anxiety and depression. The prevalence of prenatal distress is estimated to be 31%, 28% and 12% for stress, anxiety and depression respectively (McDonald et al., 2013). Thus, a significant proportion of women experience clinically significant levels of maternal distress during pregnancy, highlighting the need to study its impact on birth outcomes.

A number of epidemiological studies have shown that fetal exposure to maternal prenatal distress can alter fetal development and increase short and long term disease risk. Prenatal stress and anxiety increases the risk of preterm birth (PTM) and low birth weight (LBW) (Ding et al., 2014b, Bussières et al., 2015). Prenatal depression has been found to increase the risk of operative deliveries (Hu et al., 2015), PTB and LBW (Grote et al., 2010). However in a more recent meta-analysis, Accortt and colleagues reported significant variability in existing studies (Accortt et al., 2015), highlighting the need for further studies examining the association between prenatal depression and birth outcomes.

While the clinical outcomes have been the subject of intensive investigation, the molecular and biological parallels of these changes in human population are not well known. Given the well-known effect of stress on glucocorticoid signalling, one proposed hypothesis is that alterations in placental glucocorticoid signalling, leads to overexposure of the fetus to maternal cortisol (Cottrell et al., 2013). This has been proposed as a key biological mechanism underpinning the programming effect of prenatal distress on poor outcomes (Cottrell et al., 2013). In particular prenatal distress has been shown to alter the expression of three important genes in the placenta; 11-beta hydroxysteroid dehydrogenase type 2 (HSD11B2) (Jensen

Pena et al., 2012, Seth et al., 2015, O'Donnell et al., 2012), the glucocorticoid receptor (NR3C1) (Palma-Gudiel et al., 2015) and FK506 binding protein (FKBP5) (Monk et al., 2016). Expression of these genes have been shown to correlate with infant birthweight (Green et al., 2015, Mulligan et al., 2012) and growth restriction (Zhao et al., 2014), suggesting an that altered placental glucocorticoid signalling may play a role in determining newborn outcome. Further alterations in the epigenetic expression of HSD11B2 (Appleton et al., 2015), NR3C1 (Sheinkopf et al., 2016), and FKBP5 (Paquette et al., 2014) may be predictive of neurobehavioral problems in infancy.

The primary objective of this study was to evaluate the link between prenatal stress, depression and/or anxiety in late pregnancy on neonatal and obstetric outcomes in pregnant women receiving antenatal care at Cork University Hospital. The secondary goal was to examine any changes in key genes involved in placental glucocorticoid signalling that have previously been linked to unfavourable birth outcomes and poorer neurodevelopment in infancy.

4.3 Methods:

4.3.0 Participant recruitment

This study was carried out with full ethical approval from the Research Ethics Committee of Cork Teaching Hospital. Participants attending antenatal care at Cork University Maternity Hospital (CUMH), Cork, Ireland between July 2015 and September 2016 were invited to participate in this study. The inclusion criteria were; 1. 18 years of age or older, 2. English speaking, 3. having a current singleton pregnancy and 4. plans to give birth in in the maternity hospital. The participants were recruited when they were greater than 28 weeks' gestation to time of delivery. Participants were recruited in late pregnancy as this time corresponds to a period where the prevalence of prenatal distress is heightened (Lee et al., 2007). Written informed consent was obtained from all women who agreed to take part and participants were asked to complete a combination of questionnaires used to assess maternal distress (Khashan et al., 2014) and donate a small biopsy of their placenta following delivery. Detailed clinical and demographic data were collected from the medical notes of patients once the entire cohort had given birth. This data included information on maternal age, body mass index (BMI), previous obstetric complications, previous psychiatric history, medical conditions, current obstetric complications, birthweight, gender, gestational age, Apgar score, birth temperature, neonatal resuscitation (if any), admissions to the Neonatal Intensive Care Unit (NICU) and mode of feeding on discharge from the hospital.

4.3.1 Questionnaires

This study used the 10-item Perceived Stress Scale (PSS). The PSS is a popular tool used to measure psychological stress and how individuals appraise stressful life events. Higher Scores on the PSS are indicative of a higher level of perceived stress. In this study, a score of greater than or equal to 20 was used as the cut-off for the 'high stress' group. Maternal anxiety was measured using the 6-item version of State Trait Anxiety Inventory (STAI). The 6 item STAI is a frequently used brief psychological measure of anxiety and the 6 item version which has been validated for use during pregnancy (Marteau and Bekker, 1992). As there are currently no

recommended cut off scores for the STAI during pregnancy, women were deemed anxious if they scored in the top 25% of the cohort. Depressive symptoms were assessed using the Edinburgh Postnatal Depression Scale (EPDS). Consistent with previous studies, we used a score of 13 or greater to indicate a high probability of depression (Rubertsson et al., 2011, Cohen et al., 1983). These self-reported questionnaires are marked by a 4-point Likert Scale. In this study, the Cronbach's alpha of the PSS, STAI and EPDS were 0.867, 0.838 and 0.894 respectively.

4.3.2 Placenta collection

Placental samples were collected within 3 hours of delivery. Placental weight was measured after the cord and membranes had been removed. 5 cross-sectional samples were randomly excised from each placenta to incorporate both the maternal and fetal sides. The samples were washed in dH_2O and immediately stored at -80 °C for further analysis.

4.3.3 RNA extraction, cDNA synthesis and PCR

RNA was extracted from placental samples using the Trizol method as per the manufacturers instructions. Briefly, placental sample was homogenised in 3 ml of Trizol reagent and left on ice for 10 minutes. Samples were centrifuges at 4°C from 5 minutes and 1 ml of the supernatant was transferred into a new Eppendorf. 200ul of chloroform was added, mixed and left at room temperature for 3 minutes. The sample was centrifuged at 4°C 12000g for 15 min. The upper clear aqueous phase was removed and placed in a new 1.5ml Eppendorf, 500ul of isopropanol was added and left at room temperature for 10 min. The sample was centrifuged at 4°C 12000g for 10 min. The supernatant was removed and the pellet was washed in 1 ml of 70% ethanol. The sample was centrifuged, supernatant removed and the pellet was left to air dry and re-suspended in 70ul of RNase free H_20. RNA concentration and quality was assessed using the Nanodrop 8000. 500 ng of placental RNA was reversed transcribed using the high capacity cDNA reverse transcription kit (Applied Biosystems) using the following parameters 25 °C for 10 min; 37 °C for 120 min; 85 °C for 5 min; and held at 4 °C until storage at 80 °C.

Real time PCR was performed for HSD11B2, FKBP5, NC3R1 under the following cycling parameters; 50 °C for 2 min; 95 °C for 10 min; 50 repetitions of 95 °C for 15 s and annealing/elongating at 60 °C for 1 min. All samples were run in duplicate, cycle threshold values were recorded and analysis was performed using the $2-\Delta\Delta$cycle threshold method (Livak and Schmittgen, 2002).

4.3.4 Data analysis

Data was collected and analysed on SPSS version 22 and Graphpad prism 5. Missing values were checked for and any questionnaire responses with greater than 3 missing values were removed. Where medical records were unavailable, participants' questionnaire scores were also removed from the cohort. Normality was tested for using the Kolmogorov-Smirnov tests prior to beginning inferential statistics. Birthweight, birthweight centiles and placental weight ratios were normally distributed and were analysed using an unpaired student t-tests or one-way ANOVA with Tukey's post hoc. Placental weight, Apgar scores and birth temperature displayed a non-normal distribution; therefore non parametric tests were used. For nominal data, binary logistic regression analyses with 95% confidence intervals were used. Multivariate data were evaluated with ordinal logistic regression analysis. Values of $P < 0.05$ were considered statistically significant. All analyses were adjusted for maternal age, body mass index (BMI) and social class.

In our analyses we grouped mode of delivery into 3 models. In model 1, mode of delivery was grouped as either spontaneous vaginal delivery (SVD) or operative. Model 2 included vaginal delivery versus Caesarean section (CS) delivery. In model 3, mode of delivery was grouped as SVD, emergency CS (EMCS), elective CS (ELCS), vacuum (VD) or Forceps delivery (FD). Additionally newborns were grouped into number of adverse birth outcomes based on six parameters; (a) Admission to the NICU (b) Newborn Resuscitation received (c) Delivered before 37 weeks gestation (d) 5 minute Apgar score ≤ 7 (e) Birth Temperature < 36.5°C and (f) Birth Centile ≤ 10 or ≥ 90.

4.4 Results:

4.4.0 Descriptive statistics

159 pregnant women were initially recruited into the study and completed the questionnaires. Participants were removed from the study if they were less than 28 weeks' gestation when they completed the questionnaires (13%), had incomplete survey information (3%) or if medical records were unavailable (7.5%) (Fig. 1). The final analysis included 121 participants. The mean age of the participants was 31.75 years (SD = 4.54, range 17 – 41). The average BMI was 26.33 (SD = 4.60, range 18.6 – 38.8). The mean gestational age at which the surveys were completed was 35.55 weeks' (SD 3.541, range 28 -41). The majority of the women in this study where multigravida (67.8%) or primigravida (26.4%). 2.5% of participants had delivered greater than 5 infants and 3.3% had greater than 7 previous deliveries. 23.1% of this population had a history of having one or more miscarriages (*n = 28*). Only 2 participants (1.7%) had a history of ectopic pregnancies and therefore no further analysis was conducted in relation to ectopic pregnancy history. 21.5% of the study population had a history of psychiatric illness (*n = 26*). Depression was the most common psychiatric illness in this population (9.9%, *n=12*), followed by anxiety (7.4%, *n=9*), postnatal depression (5%, *n=6*) and bipolar disorder (0.8%, n=1) (Table 1 & 2).

4.4.1 Perceived Stress Scale

The mean PSS score in the entire study cohort was 16.36 ± 0.591 (Appendix C, Fig. 1). This number is comparable to other studies that have used the 10-item PSS to assess prenatal stress in the third trimester (Liou et al., 2013). High levels of perceived psychological stress was determined by a PSS score of greater than or equal to 20 (33.1%, *n = 40*). Participants with a score of less than or equal to 19 were deemed 'low stress' (66.9%, *n=81*). Women with high levels of stress during pregnancy were 2 times more likely to have a history of psychiatric illness (OR 2.519; 95% CI=1.036–6.125). This relationship remained significant after controlling for maternal age, BMI and social class (aOR 2.591; 95% CI=1.033 – 6.499). When grouped based on type of psychiatric illness, maternal stress was not associated

with depression, anxiety or PND individually (Table 3). Prenatal stress had no effect neonatal outcome (Fig. 2), mode of delivery, gestational age at delivery or infant feeding (Table 3). Additionally scoring on the PSS was not related to neonatal resuscitations, admission to the NICU, birth centiles (Appendix C, Table 1) or number of neonatal adversities (Appendix C, Table 3).

4.4.2 State Trait Anxiety Inventory

The mean Score in the STAI was 6.17 ± 0.347. As there are currently no guidelines to indicate high anxiety in the perinatal period using the STAI, we defined highly anxious of as the top quartile of scores in the cohort (27.3%, *n=33*). Previous depression, anxiety or PND alone was not associated with scoring in the STAI. However, when grouped by any psychiatric illness there was an association with scoring high in the STAI and having a history of psychiatric illness (OR 3.020; 95% CI=1.215–7.509). This relationship remained significant after including maternal age and BMI into the model (aOR3.299; 95% CI=1.270-8.572) (Table 1). A history of miscarriage was associated with a 3 fold increased risk of scoring high in STAI (aOR 3.071; 95% CI=1.165-8.095) (Table 2), indicating previous pregnancy loss as a contributing factor to anxiety during pregnancy.

4.4.3 Edinburgh Postnatal Depression Scale

The mean EPDS was 9.07 ± 0.512 (Appendix C, Fig. 1 & Table 2). An EPDS score greater than or equal to 13 was used to mark participants as being 'highly likely depressed' (25.6%, *n=31*). Concordantly, participants who scored 12 or less were grouped in the 'not likely depressed' category (74.4%, *n = 90*). Participants who scored in the 'highly likely depressed' group were greater than 3 time more likely to have had a history of psychiatric illness (aOR 3.566; 95% CI=1.348-9.434). When stratified based on type of psychiatric illness (depression, stress or anxiety), women with a history of depression were 4 times more likely to be in the 'highly likely depressed' group after adjusting for maternal age, BMI and social class (aOR 4.451; 95% CI=1.198-16.538) (Table 1). Maternal history of miscarriage was associated with a three-fold increased risk of scoring high in the EPDS (aOR 3.878; 95% CI=1.420-10.590) (Table 5). Babies of women who were ranked in the 'highly likely

depressed' group on the EPDS had a reduced temperature at birth ($P < 0.05$ *) (Fig. 4D). Maternal depressive symptomology did not affect mode of delivery, gestational age at delivery or infant feeding (Table 5). Additionally scoring on the EPDS was related to neonatal resuscitations, admission to the NICU, birth centiles (Appendix C, Table 1) or number of neonatal adversities (Appendix C, Table 3).

4.4.4 Cumulative group

As prenatal stress, anxiety and depression are highly correlated (Appendix C, Fig. 2), a new group was created for participants who scored in the high category on all three questionnaires (14.9%, *n= 18*). Participants in this group were up to 3 times more likely to have a history of miscarriage (aOR = 3.903; 95% CI=1.224-12.446) (Table 6). The risk of having a history of psychiatric illness in this group rose to greater than 6 times (aOR = 6.229; 95% CI = 1.947-19.926). Similar to participants who ranked high in the EPDS, participants in this category were more likely to have a history of depression (aOR = 4.504; 95% CI=1.073-18.897) (Table 6). Infants born to women in the cumulative high stress group had a lower 5 minute Apgar score than other infants. Further, unlike babies from the low group, babies born to mothers in this group did not have a significant increase in their Apgar score from 1 to 5 minutes (Fig.4E). Maternal cumulative distress had no effect on mode of delivery, gestational age at delivery or infant feeding (Table 6).

4.4.7 Placental gene expression

To determine the relationship between prenatal distress and placental glucocorticoid gene expression, we performed real time PCR on placental samples for three key genes; HSD11B2, FKBP1, NR3C1. Placentae were available for 16 of the high cumulative distress participants and 9 of the low scoring participants. Scoring in the high group was associated with a significant decrease in HSD11B2 ($p = 0.0296$) and an increase in NR3C1 ($p=0.048$) mRNA in the placenta (Fig 6). Additionally placental NR3C1 was negatively correlated with placental weight ($r^2 = 0.159$; $p=0.025$) and birthweight centiles ($r^2 = 0.412$; $p=0.036$) (Appendix C, Table 5). There was no effect of prenatal cumulative distress on the expression of FKBP5 ($p=0.101$) (Fig 6). Placental FKBP5 expression positively correlated with maternal

BMI (r^2 = 0.185; p=0.025) (Appendix C, Table 6). Placental gene expression was not related to infant sex or obstetric outcomes (Appendix C, Fig. 3).

4.5 Figures and Figure Legends:

Figure 1:

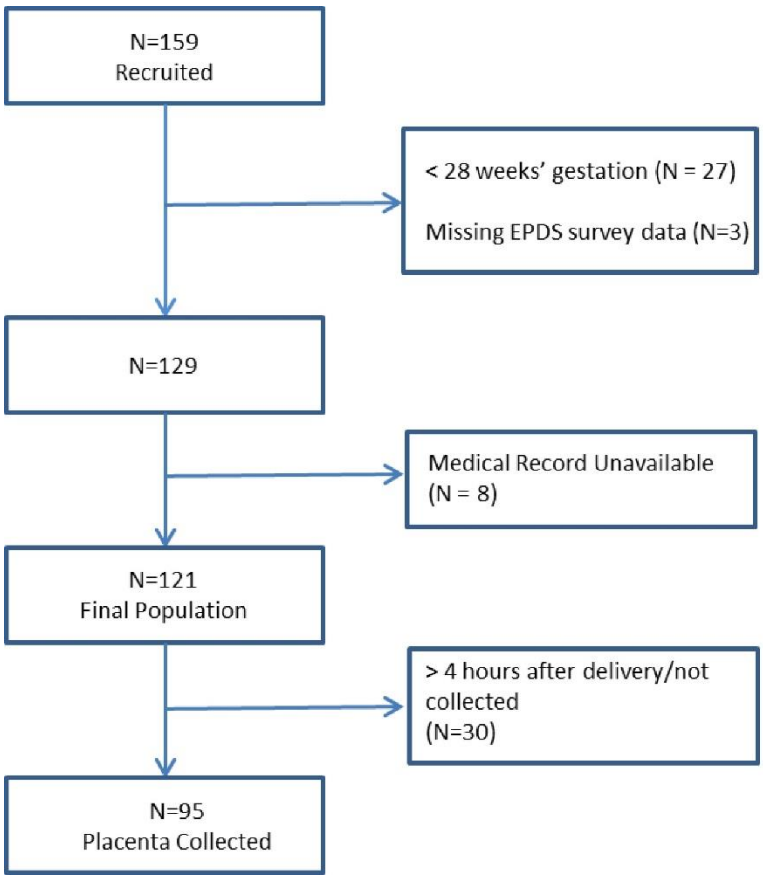

Figure 1: Flowchart of participants. 159 questionnaires were completed with 21 (13.2%) removed from the dataset for being less than 28 weeks gestation and a further 5 (3.1%) removed for incomplete filling of the questionnaire. Medical records were unavailable for 12 (7.5%) of the participants. Placental samples were collected, frozen and biobanked for 100 of the remaining participants (82.6%).

Table 1:

Demographics	*N (%)*
Age (years)	
≤ 18	1 (0.8%)
19 – 24	8 (6.7%)
25 – 29	26 (21.8%)
30 – 35	48 (40.3%)
≥ 35	36 (30.3%)
BMI (kg/m^2)	
Underweight (< 18.50)	0
Normal (18.5 – 24.99)	50 (41.7%)
Overweight (25 – 29.9)	44 (36.4%)
Obese (> 30.0)	26 (21.5%)
Social class	
1. Professional workers	14 (11.6%)
2. Managerial and technical	37 (30.6%)
3. Non-manual	8 (6.6%)
4. Skilled manual	28 (23.1%)
5. Semi-skilled	9 (7.4%)
6. Unskilled	8 (6.6%)
7. Unemployed	6 (5%)
Medical History	
History of miscarriage	
Yes	28 (23.1%)
No	93 (76.9%)
History of ectopic pregnancy	
Yes	2 (1.7%)
No	119 (98.3%)
Psychiatric history	
History of any psychiatric illness	
Yes	26 (21.5%)
No	95 (78.5%)
History of depression	
Yes	12 (9.9%)
No	109 (90.1%)
History of anxiety	
Yes	9 (7.4%)
No	112 (92.6%)

Table 1 continued:

History of postnatal depression	6 (5%)
Yes	115 (95%)
No	
Onset of delivery	
Spontaneous	51 (50.5%)
Induction	50 (49.5%)
Rupture of membranes	
SROM	35 (34%)
PROM	3 (2.9%)
AROM	65 (63.1%)
Mode of delivery	
Spontaneous vaginal delivery (SVD)	67 (55.8%)
Forceps delivery (FD)	4 (3.3%)
Vacuum delivery (KVD)	19 (15.8%)
Emergency Caesarean section (EMCS)	10 (8.3%)
Elective Caesarean section (ELCS)	20 (16.7%)
Resuscitation at birth	
Yes	17 (14.4%)
No	101 (85.6%)
Admission to the Neonatal Unit	
Yes	5 (4.2%)
No	115 (95.8%)
Mode of Feeding on discharge	
Exclusive breast fed	56 (46.3%)
Mixed feeding	7 (5.8%)
Bottle fed	58 (47.9%)

Table 1: Descriptive statistics of categorical variables.

Table 2:

Measure	*Mean (N)*	*SEM*	*SD*	*Range*
Maternal age	31.75	0.41	4.54	17 - 41
Maternal BMI	26.33	0.42	4.60	19 - 40
Birthweight	3654.67	40.66	445.49	2690 - 4800
Placental weight	558.33	10.84	106.29	300 - 900
Placental weight ratio	0.157	0.006	0.06	0.09 – 0.70
Birth Temperature	36.80	0.09	1.01	35 - 39
1 minute Apgar Score	8.54	0.10	1.19	3 - 10
5 minute Apgar Score	9.53	0.06	0.73	6 - 10
Gestational Age at delivery	39.58	0.09	1.04	37 - 41
Birthweight Centiles	47.58	2.46	26.87	1 - 98

Table 2: Descriptive statistics of continuous variables.

Figure 2:

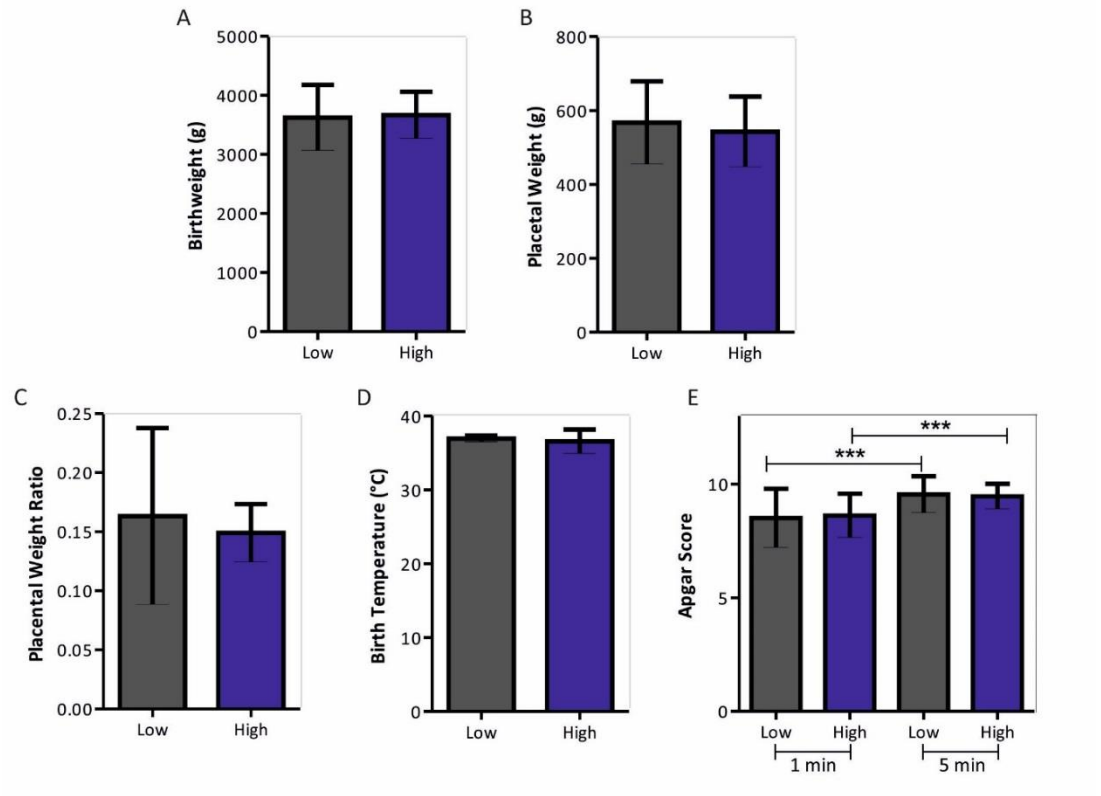

Figure 2: Prenatal stress and neonatal outcomes. Graphical representation of birthweight (A), placental weight (B), placental weight ratio (C), birth temperature (D) and Apgar score (E) in neonates whose mothers were ranked in the high and low stress category. Unpaired students t-test between low and high on birth weight (A) and placental weight ratio (C) show no significant difference between groups $P > 0.05$. Mann Whitney test between low and high on placental weight (B) and birth temperature (D) show no significant difference between groups $P > 0.05$. Mann Whitney test show increased Apgar score from 1 to 5 minute (E) in low and high stress group ($P < 0.0001$ ***).

Table 3:

Perceived Stress Scale			
		OR (CI; p-value)	aOR (CI; p-value)
Psychiatric history			
Psychiatric Illness		**2.519 (1.036 – 6.125 ; 0.042) ***	**2.591 (1.033 – 6.499 ; 0.042) ***
Depression		2.203 (0.663 – 7.338 ; 0.197)	2.648 (0.747 – 9.382 ; 0.131)
Anxiety		2.082 (0.696 – 10.867 – 0.149)	2.592 (0.622 – 10.796 ; 0.191)
Postnatal Depression		1.013 (0.178 – 5.780 ; 0.988)	0.837 (0.135 – 5.206 ; 0.849)
Miscarriage history			
Miscarriage		1.741 (0.730 – 4.155 ; 0.211)	2.278 (0.878 – 5.911 ; 0.091)
Mode of delivery			
Model 1	SVD	1 (Reference)	1 (Reference)
	Operative	1.023 (0.478 – 2.190 ; 0.954)	1.014 (0.466 – 2.204 ; 0.972)
Model 2	Vaginal	1 (Reference)	1 (Reference)
	CS	1.017 (0.423 – 2.441 ; 0.97)	1.178 (0.467 – 2.970 ; 0.729)
Model 3	SVD	1 (Reference)	1 (Reference)
	EMCS	0.511 (-2.302 – 0.961 ; 0.420)	0.503 (-2.345 – 0.969 ; 0.416)
	ELCS	1.364 (-0.720 – 1.340 ; 0.555)	1.706 (-0.556 – 1.625 ; 0.337)
	KVD	1.193 (-0.886 – 1.239 ; 0.745)	1.046 (-1.043 – 1.133 ; 0.935)
	FD	0.682 (-2.703 – 1.937 ; 0.746)	0.492 (-3.062 – 1.645 ; 0.555)
Gestational Age at delivery			
< 39 weeks gestation		0.673 (0.284 – 2.252 ; 0.673)	0.758 (0.266 – 2.157 ; 0.603)
Mode of Feeding			
Breastfed		1 (Reference)	1 (Reference)
Bottle-fed		1.111 (-0.674-0.885; 0.791)	1.059 (-0.737 – 0.852 ; 0.887)
Mixed		0.844 (-1.902-1.564; 0.848)	1.094 (-1.710 – 1.890 ; 0.922)

Table 3: Women with a history of psychiatric illness more likely to report prenatal stress. Odds ratios assessing the relationship between prenatal stress, history of psychiatric illness, history of miscarriage, mode of delivery, gestational age at delivery and mode of feeding. Binary logistic regression analysis revealed participants with a history of psychiatric illness were more likely to score high on the PSS ($P < 0.05$ *). Adjusted for maternal age, BMI and social class.

Figure 3:

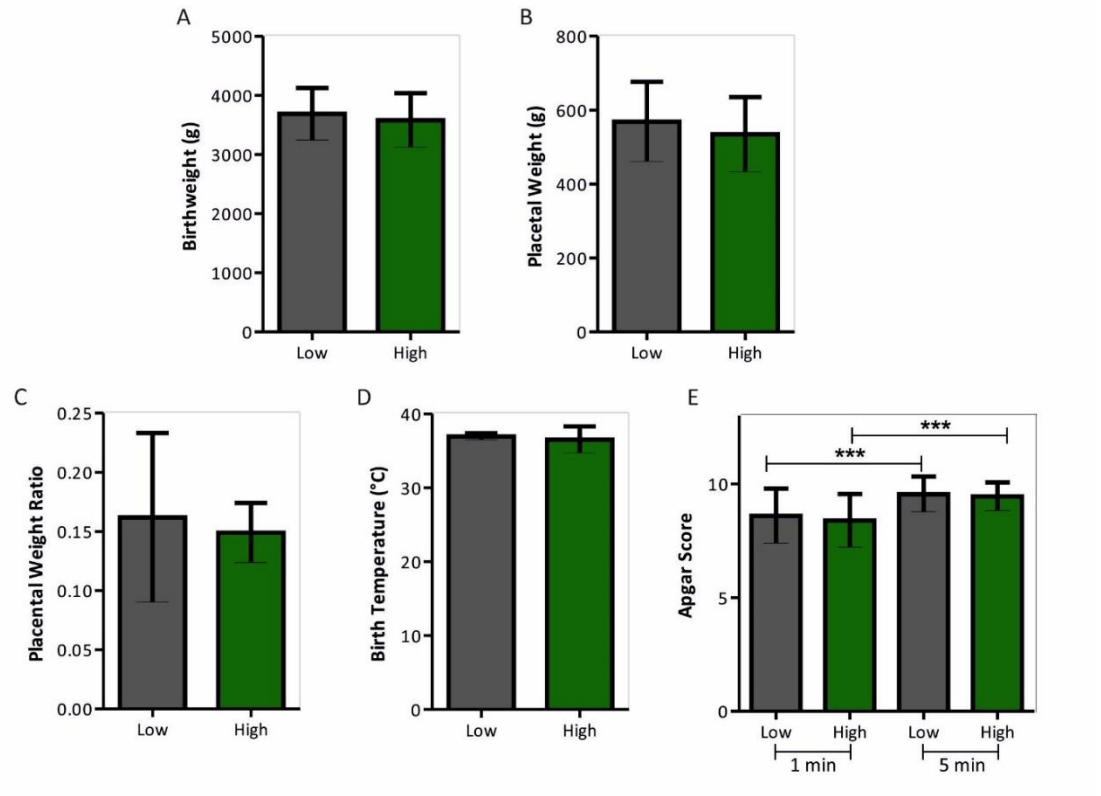

Figure 3: Prenatal anxiety and neonatal outcomes. Graphical representation of birthweight (A), placental weight (B), placental weight ratio (C), birth temperature (D) and Apgar score (E) in neonates whose mothers were ranked in the high and low anxiety category. Unpaired students t-test between low and high on birth weight (A) and placental weight ratio (C) show no significant difference between groups $P > 0.05$. Mann Whitney test between low and high on placental weight (B) and and birth temperature (D) show no significant difference between groups $P > 0.05$. Mann Whitney test show increased Apgar score from 1 to 5 minute in low and high anxiety group ($P < 0.0001$ ***).

Table 4:

State Trait Anxiety Inventory			
		OR (CI; p-value)	aOR (CI; p-value)
Psychiatric history			
Psychiatric Illness		**3.020 (1.215 – 7.509 ; 0.017) ***	**3.532 (1.322 – 9.389 ; 0.012) ***
Depression		2.066 (0.607 – 7.037 ; 0.246)	2.579 (0.699 – 9.515 ; 0.155)
Anxiety		1.367 (0.321 – 5.812 ; 0.672)	1.322 (0.275 – 6.358 ; 0.727)
Postnatal Depression		2.833 (0.542 – 14.806 ; 0.217)	2.509 (0.432 – 14.583 ; 0.306)
Miscarriage history			
Miscarriage		**3.163 (1.296 – 7.721 ; 0.011) ***	**3.807 (1.384 – 10.473 ; 0.010) ***
Mode of delivery			
Model 1	SVD	1 (Reference)	1 (Reference)
	Operative	**2.443 (1.077 – 5.545 ; 0.033) ***	2.264 (0.977 – 5.246 ; 0.057)
Model 2	Vaginal	1 (Reference)	1 (Reference)
	CS	2.222 (0.923 – 5.348 ; 0.075)	2.306 (0.895 – 5.937 ; 0.083)
Model 3	SVD	1 (Reference)	1 (Reference)
	EMCS	1.038 (-1.626 – 1.701 ; 0.965)	0.817 (-1.917 – 1.513 ; 0.817)
	ELCS	**4.154 (0.359 – 2.489 ; 0.009) ****	**5.411 (0.509 – 2.868 ; 0.005) ****
	KVD	2.423 (-0.227 – 1.997 ; 0.119)	2.235 (-0.345 – 1.954 ; 0.170)
	FD	1.385 (-2.017 – 2.668 ; 0.785)	1.013 (-2.424 – 2.451 ; 0.992)
Gestational Age at delivery			
< 39 weeks gestation		**3.182 (1.196 – 8.467 ; 0.020) ***	**3.235 (1.182 – 8.855 ; 0.022) ***
Mode of Feeding			
Breastfed		1 (Reference)	1 (Reference)
Bottle-fed		**2.964 (0.195 –1.978; 0.017)***	**2.710 (0.086 – 1.908 ; 0.032) ***
Mixed		3.917 (-0.293 – 3.023; 0.107)	5.189 (-0.148 – 3.441 ; 0.072)

Table 4: Prenatal anxiety, maternal history and obstetric outcomes. Odds ratios assessing the relationship between prenatal anxiety, history of psychiatric illness, history of miscarriage, mode of delivery, gestational age at delivery and mode of feeding. Binary logistic regression analysis revealed participants with a history of psychiatric illness or specifically depression and miscarriage were more likely to score high on the STAI ($P < 0.05$ *). High anxiety was associated with an increased risk of delivery by ELCS ($P < 0.01$ **). Prenatal anxiety related to increased risk of delivery before 39 weeks gestation ($P < 0.05$ *). Women in the high anxiety group

were more likely to be bottle feeding on hospital discharge ($P < 0.05$ *). Adjusted for maternal age, BMI and social class.

Figure 4:

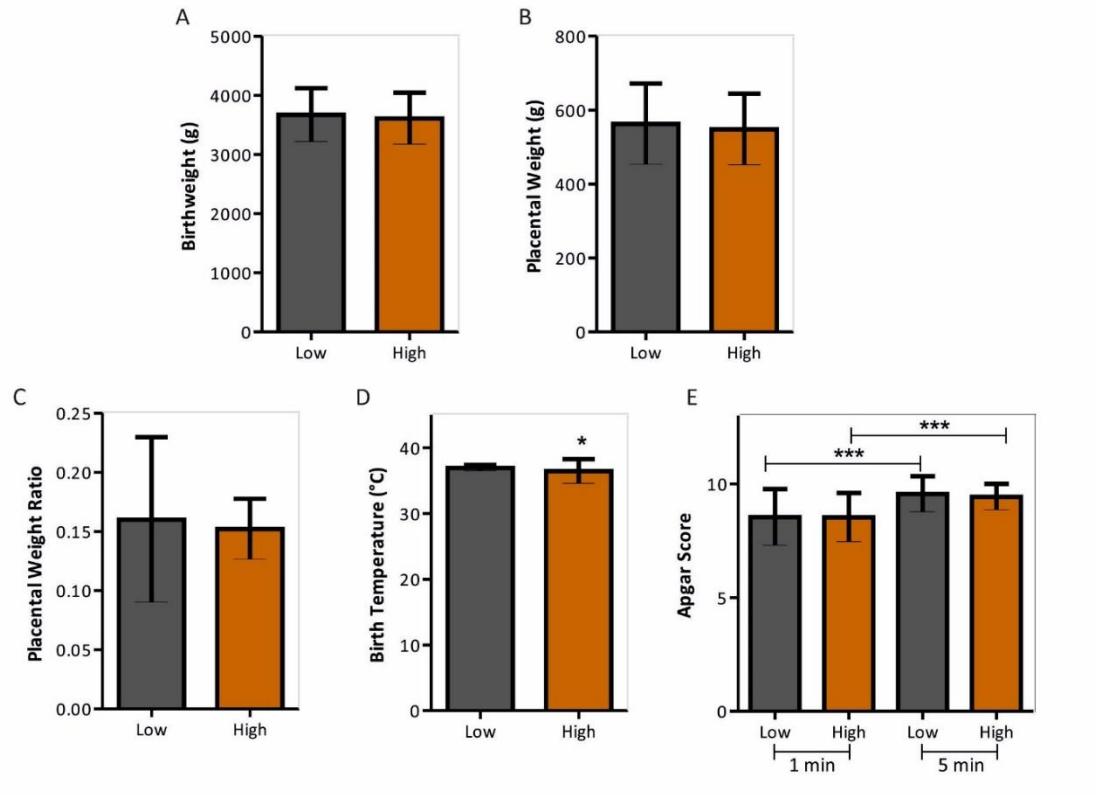

Figure 4: Prenatal depressive symptoms and neonatal outcomes. Graphical representation of birthweight (A), placental weight (B), placental weight ratio (C), birth temperature (D) and Apgar score (E) in neonates whose mothers were ranked in the high and low depressive category. Unpaired students t-test between low and high on birth weight (A) and placental weight ratio (C) show no significant difference between groups $P > 0.05$. Mann Whitney test between low and high on placental weight (B) show no significant difference between groups $P > 0.05$. Mann Whitney test shows decreased birth temperature (D) among the high depressive symptoms group ($P < 0.05$ *). Mann Whitney test show increased Apgar score from 1 to 5 minute in low and high depressive symptom group ($P < 0.0001$ ***).

Table 5:

Edinburgh Postnatal Depression Scale			
		OR (CI; p-value)	aOR (CI; p-value)
Psychiatric history			
Psychiatric Illness		**3.429 (1.366 – 8.607 ; 0.009) ****	**3.566 (1.348 – 9.434 ; 0.010) ***
Depression		3.360 (0.995 – 11.341 ; 0.051)	**4.451 (1.198 – 16.539 ; 0.026) ***
Anxiety		2.519 (0.631 – 10.053 ; 0.191)	2.042 (0.475 – 8.776 ; 0.337)
Postnatal Depression		1.483 (0.258 – 8.522 ; 0.659)	1.111 (0.149 – 8.312 ; 0.918)
Miscarriage history			
Miscarriage		**3.611 (1.463 – 8.913 ; 0.005) ****	**3.878 (1.420 – 10.590 ; 0.008) ****
Mode of delivery			
Model 1	SVD	1 (Reference)	1 (Reference)
	Operative	1.739 (0.764 – 3.960 ; 0.187)	1.751 (0.743 – 4.128 ; 0.200)
Model 2	Vaginal	1 (Reference)	1 (Reference)
	CS	1.344 (0.538 – 3.361 ; 0.527)	1.618 (0.592 – 4.419 ; 0.348)
Model 3	SVD	1 (Reference)	1 (Reference)
	EMCS	0.946 (-1.713 – 1.603 ; 0.948)	0.950 (-1.755 – 1.652 ; 0.953)
	ELCS	2.038 (-0.379 – 1.804 ; 0.201)	2.456 (-0.275 – 2.072 ; 0.133)
	KVD	1.747 (-0.574 – 1.691 ; 0.334)	1.521 (-0.777 – 1.617 ; 0.492)
	FD	3.786 (-0.715 – 3.378 ; 0.202)	3.485 (-0.869 – 3.366 ; 0.248)
Gestational Age at delivery			
< 39 weeks gestation		2.154 (0.792 – 5.855 ; 0.133)	2.391 (0.841 – 6.796 ; 0.102)
Mode of Feeding			
Breastfed		1 (Reference)	1 (Reference)
Bottle-fed		1.520 (-0.433-1.272; 0.336)	1.301 (-1.621 – 2.147 ; 0.784)
Mixed		1.467 (-1.377-2.143; 0.670)	1.337 (-0.594 – 1.175 ; 0.520)

Table 5: Prenatal depressive symptoms, maternal history and obstetric outcomes. Odds ratios assessing the relationship between prenatal depressive symptoms, history of psychiatric illness, history of miscarriage, mode of delivery, gestational age at delivery and mode of feeding. Binary logistic regression analysis revealed participants with a history of psychiatric illness or specifically depression and miscarriage were more likely to score high on the EPDS ($P < 0.05$ *, $P < 0.01$ **). Adjusted for maternal age, BMI and social class.

Figure 5:

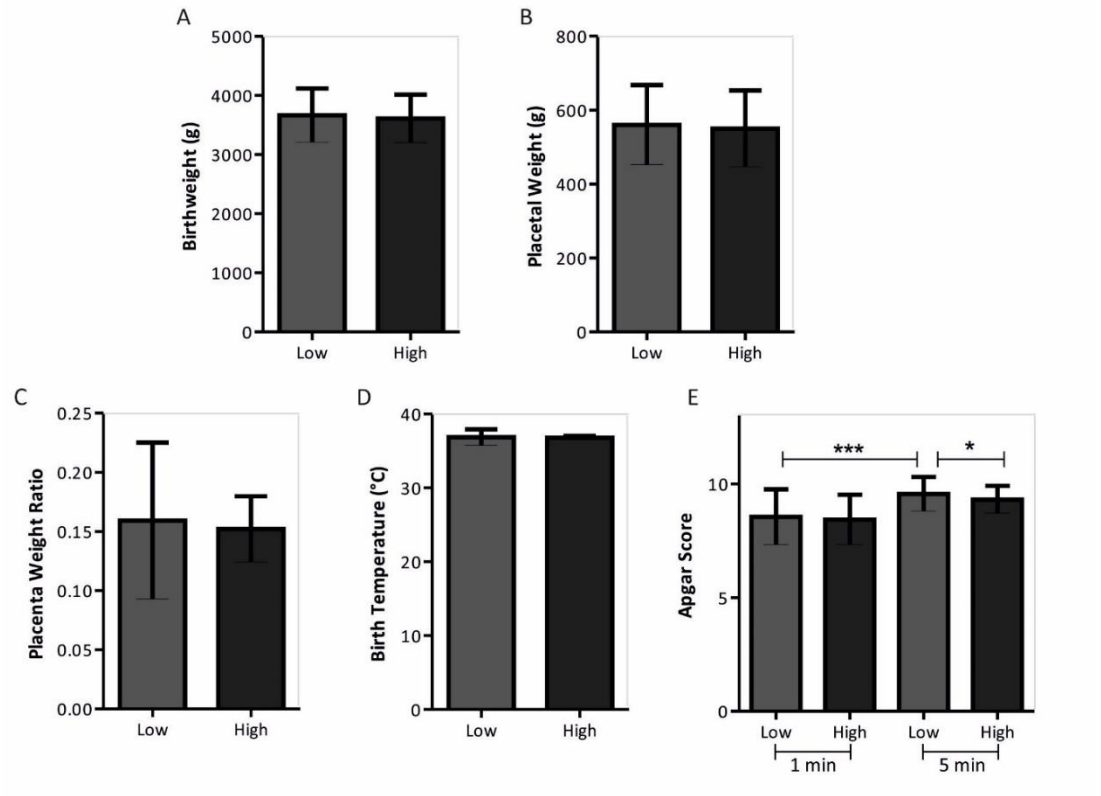

Figure 5: Prenatal cumulative distress and neonatal outcomes. Graphical representation of birthweight (A), placental weight (B), placental weight ratio (C), birth temperature (D) and Apgar score () in neonates whose mothers were ranked in the high and low cumulative distress category. Unpaired students t-test between low and high on birth weight (A) and placental weight ratio (C) show no significant difference between groups $P > 0.05$. Mann Whitney test between low and high on placental weight (B), and birth temperature (D) show no significant difference between groups $P > 0.05$. Mann Whitney test show increased Apgar score from 1 to 5 minute in low cumulative distress group ($P < 0.0001$ ***) but not in high group ($P > 0.05$). There was a significant reduction in 5 minute Apgar score among high cumulative distress group (F; Mann Whitney test; $P < 0.05$ *).

Table 6:

		Cumulative distress	
		OR (CI; p-value)	aOR (CI; p-value)
		Psychiatric history	
Psychiatric Illness		**5.059 (1.752 – 14.606 ; 0.003) ****	**6.229 (1.947 – 19.926 ; 0.002) ****
Depression		3.393 (0.902 – 12.763 ; 0.071)	**4.504 (1.073 – 18.897 ; 0.040) ***
Anxiety		3.233 (0.730 – 14.329 ; 0.122)	3.411 (0.649 – 17.916 ; 0.147)
Postnatal Depression		3.094 (0.523 – 18.302 ; 0.213)	3.136 (0.427 – 23.036 ; 0.261)
		Miscarriage history	
Miscarriage		**3.320 (1.162 – 9.488 ; 0.025) ***	**3.903 (1.224 – 12.446 ; 0.021) ***
		Mode of delivery	
Model 1	SVD	1 (Reference)	1 (Reference)
	Operative	2.193 (0.786 – 6.113 ; 0.133)	1.982 (0.684 – 5.739 ; 0.207)
Model 2	Vaginal	1 (Reference)	1 (Reference)
	CS	1.646 (0.558 – 4.852 ; 0.366)	1.758 (0.545 – 5.672 ; 0.345)
Model 3	SVD	1 (Reference)	1 (Reference)
	EMCS	0.952 (-2.258 – 2.161 ; 0.965)	0.704 (-2.647 – 1.945 ; 0.765)
	ELCS	2.857 (-0.230 – 2.329 ; 0.108)	**4.375 (0.005 – 2.947 ; 0.049) ***
	KVD	2.286 (-0.526 – 2.179 ; 0.231)	2.307 (-0.596 – 2.267 ; 0.252)
	FD	2.857 (-1.345 – 3.445 ; 0.390)	2.121 (-1.843 – 3.346 ; 0.570)
		Gestational Age at delivery	
< 39 weeks gestation		1.557 (0.452 – 5.355 ; 0.483)	1.548 (0.431 – 5.558 ; 0.503)
		Mode of Feeding	
Breastfed		1 (Reference)	1 (Reference)
Bottle-fed		1.638 (-0.535 – 1.522 ; 0.347)	1.409 (-0.730 – 1.415 ; 0.531)
Mixed		-	-

Table 6: Prenatal cumulative distress, maternal history and obstetric outcomes. Odds ratios assessing the relationship between prenatal cumulative distress, history of psychiatric illness, history of miscarriage, mode of delivery, gestational age at delivery and mode of feeding. Binary logistic regression analysis revealed participants with a history of psychiatric illness or specifically depression and miscarriage were more likely to be in high cumulative distress group ($P < 0.05$ *, $P < 0.01$ **). Prenatal cumulative distress was associated with increased delivery by ELCS (Ordinal regression; $P < 0.05$ *). Adjusted for maternal age, BMI and social class.

Figure 6:

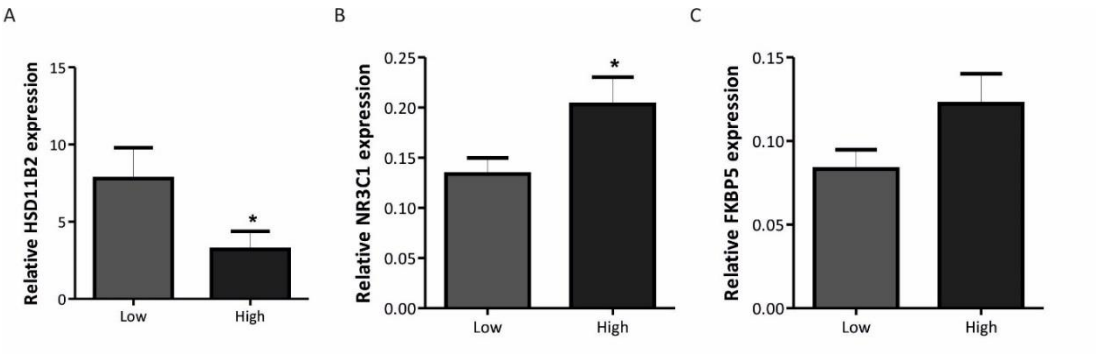

Figure 6: Placental gene expression. Real time PCR showing (A) HSD11B2, (B) NR3C1 and (C) FKBP5 expression in the term human placenta between participants in the low and high cumulative stress group using the 2-delta-Ct method (Low *n=9*, High *n=15*; Unpaired Student's t-test $P < 0.05$ *).

4.6 Discussion:

The present study adds to the existing literature highlighting a negative role for prenatal distress in mediating adverse pregnancy and birth outcomes. Prenatal stress is commonly reported during pregnancy and varies in its definition. Psychological wellbeing encompasses a broad area of health that expands beyond diagnosable mental health disorders such as depression, anxiety and posttraumatic stress disorder. There are a wide range of screening tools used to identify women who may be at risk of stress, anxiety and depression that are commonly used to measure psychological status in pregnancy. The extensive tools available means prenatal stress is commonly defined and assessed in multiple ways which has led to significant inconsistencies between studies. To characterise psychological distress in this study, we used the Perceived stress scale, State Trait Anxiety Inventory and Edinburgh Postnatal Depression. A large meta-analysis demonstrated these questionnaires to have excellent reliability and to currently be the best available tools to measure stress, anxiety and depressive symptomology during pregnancy (Nast et al., 2013).

The current study does not support a strong association between maternal prenatal distress and neonatal outcomes. Although we identified a significant reduction in birth temperature among neonates from women with high depressive symptoms, and reduced new-born temperature is related to increased infant mortality (Soll, 2008), we recommend this result be interpreted with caution as infants of mothers who report depression had an average temperature of 36.43 °C, and infants are only considered clinically hypothermic when temperature drops below 36°C. Additionally the reduced birth temperature observed here may be reflective of a slightly higher incidence of caesarean deliveries among the higher depressive group, as low operating room temperatures may contribute to reduced body temperatures at birth (Duryea et al., 2016). We also found a reduction in the five minute Apgar score among babies from the high cumulative stress group. Further these babies did not display an increase in Apgar scores from 1 to 5 minutes. A low 5-minute Apgar score is associated with infant mortality (Thorngren-Jerneck and Herbst, 2001) and is predictive of poor neurological function later in life (Lie et al., 2010) (Moster et al., 2001, Seidman et al., 1991).

We also found prenatal anxiety increased the risk of women delivering via elective C-Section and delivering before 39 weeks gestation. The reduced gestations observed among these women are likely a reflection of the increased rates of elective CS in this highly anxious group, which are often scheduled for 39 weeks' gestation (Laye and Dellinger, 2006). In line with previous studies (Fairlie et al., 2009), we show that women with high levels of anxiety in late pregnancy were more likely to bottle feed their infants prior to hospital discharge. This highlights a central role for anxiety in late pregnancy to mediate adverse obstetric outcomes, and may have implications for mode of feeding in the post-partum period.

Fetal over-exposure to maternal cortisol as a mediator of adverse fetal outcomes is well described in the literature (Reynolds, 2013). In the placenta, the fetus is protected from high levels of maternal cortisol by the actions of the enzyme HSD11B2. This enzyme cleaves cortisol into its inactive product cortisone and in doing so prevents fetal overexposure to maternal cortisol. Scientific enquires are now focusing on this enzyme to be a mediator of prenatal distress (Jensen Pena et al., 2012, Seth et al., 2015, Togher et al., 2014, Appleton et al., 2013). Here consistent with these studies we demonstrate a significant reduction in the expression of placental HSD11B2 in women who were in the high cumulative stress group. Additionally we find this group to have elevated levels of the NR3C1 receptor in the placenta. Maternal distress has previously been shown to increase the glucocorticoid receptor, NR3C1 in the placenta (Raikkonen et al., 2014, Reynolds et al., 2015). It is worth noting that increased placental NR3C1 may result in increased CRF production from the placenta (Bronson and Bale, 2015). Increased CRF entry into the fetal compartment may contribute to altered development (Cratty et al., 1995). Taken together this data suggests the placenta of women who experience distress may not only have a reduced capacity to break down cortisol but may also be increasingly sensitive to maternal glucocorticoids. Further the correlation that we observe between NR3C1 expression, placental weight and birthweight centiles suggests placental glucocorticoid signalling could lie at the core of mediating the adverse effects of maternal distress on birth outcomes. Additionally the well described association between these placental genes with infant neurodevelopment (Conradt et al., 2013, Sheinkopf et al., 2016) suggests

altered placental HSD11B2 and/or NR3C1 expression may be a biomarker for, or indicative of, altered neurodevelopment in infancy.

We identified maternal history of psychiatric illness or miscarriage as significant predictors of poorer mental health in pregnancy. This is in line with previous studies where women with a history of early pregnancy loss have elevated levels of stress, anxiety and depression in subsequent pregnancies (Woods-Giscombé et al., 2010, McCarthy et al., 2015). Identifying these women and offering support measures to reduce the risk of mood disturbance in their current pregnancy may be beneficial in reducing neonatal and obstetric complications. Of interest prenatal yoga has been shown to enhance positive mood, reduce depressive and anxiety symptoms (Davis et al., 2015), and decrease salivary cortisol and alpha amylase levels (Kusaka et al., 2016) in pregnancy. Additionally, a preliminary trial showed significant improvement in anxiety and depressive symptoms in women receiving an antenatal mindfulness intervention (Woolhouse et al., 2014). The 'Centering Pregnancy' trial which promotes *group* prenatal care has been shown to reduce rates of preterm birth and enhance breastfeeding initiation and is widely implemented across the United States (Ickovics et al., 2007). Examining the effectiveness of such strategies in a country specific manner will be crucial in progressing clinical practise and promoting interventions to manage maternal distress in pregnancy to ultimately improve maternal and birth outcomes in this high risk population.

Study limitations

The current study has several limitations. First the relatively limited sample size in this cohort may explain why we do not find an effect with prenatal distress and birth and placental weight. Additionally enrolling women after 28 weeks gestation precludes our ability to measure any association with extremely PTB. Further the earliest age at delivery in this cohort was 37 weeks' gestation which prevented us from investigating an association with pre-term delivery which has a background incidence in our population of 4%, lower than the global estimated of 5-10% (Zeitlin et al., 2013). The limited ethnicity of our cohort did not allow us to examine any ethnic differences, which is an important factor when considering prenatal distress

(Borders et al., 2015), however this limitation resulted in a relatively homogenous study cohort that reduced the influence of confounding. Measuring maternal distress in late pregnancy only, underscores the importance of other vulnerable periods during gestation. It is worth noting however, that the third trimester represents a particularity vulnerable developmental period whereby the fetus can be affected by maternal perturbations. Finally we were unable to assess or control for maternal antidepressant use or lifestyle factors such as exercise, diet, smoking or alcohol intake which may mediate the relationship between prenatal distress and adverse outcomes (Shapiro et al., 2013).

Conclusions:

Despite these limitations the current study is important as it identifies prenatal anxiety in late pregnancy as a significant factor leading to increased opting for C-section delivery and increased bottle feeding of infants. Additionally the changes in placental HSD11B2 and NR3C1 expression further confirms a role for placental glucocorticoid signalling at the core of the programming effects of prenatal distress. Further studies conducted longitudinally throughout pregnancy and including both psychological and physiological measures of distress in larger and more ethnically diverse populations will help to clarify specifically at risk vulnerable individuals. Translating these results into prenatal clinics and establishing interventions to manage distress in pregnancy will be crucial in reducing morbidities in pregnancy and neonates.

Chapter 5:

Placental FKBP51 mediates a link between second trimester maternal anxiety and birthweight in female infants

Katie L. Togher, Gerard W. O' Keeffe, Ali S. Khashan, Gerard Clarke, Louise C. Kenny,

Submitted to *Scientific Reports*

5.1 Abstract:

Prenatal distress is associated with adverse short and long-term outcomes in offspring. Alterations in placental glucocorticoid signalling and fetal overexposure to excessive glucocorticoids have been implicated as an underlying mechanism. Infant sex is emerging as an important factor in disease susceptibility following exposure to prenatal distress and the placental responses of one sex over the other is suggested to underlie these differences. This study aimed to examine the effects of maternal distress across pregnancy on birth outcomes and placental glucocorticoid genes in a sex dependant manner. Nulliparous pregnant women completed the Perceived Stress Scale, State Trait Anxiety Inventory and Edinburgh Postnatal Depression Scale throughout pregnancy. Glucocorticoid regulating genes in the placenta, HSD11B2, NR3C1 and FKBP51 were analysed by real time PCR and cortisol was measured in newborn hair as an indicator of fetal exposure. Second trimester stress was negatively correlated with birthweight in males and positively correlated with NR3C1 in female placenta. Second trimester anxiety was negatively correlated with birthweight and FKBP51 in females only. In mediation analysis, placental FKBP51 expression was found to mediate the link between prenatal anxiety and birthweight. Newborn cortisol was negatively correlated with second trimester anxiety and positively correlated with female placental FKBP51. Again FKBP51 mediated the link between anxiety and newborn cortisol. These results highlight an important role for FKBP51 in the placental response to prenatal distress in females. The precise role that placental FKBP51 has in infant development has not been extensively studied and warrants further investigations.

5.2 Introduction:

There is now a large body of evidence showing that the *in utero* experience is a critical determinant of future health (Langley-Evans, 2006, Cao-Lei et al., 2016, Todd et al., 2017). One factor that has been extensively studied in this regard is the adverse effects of prenatal maternal psychological distress, which we define as the experience of significant levels of psychological stress, depression, and/or anxiety during pregnancy (Togher et al., 2017) (Khashan et al., 2014). We have previously reported the incidence of this in pregnancy using the SCOPE (Screening for Pregnancy Endpoints) pregnancy cohort of nulliparous healthy pregnant women (Kenny et al., 2014, Larsen et al., 2013). All participants completed a combination of validated questionnaires used to assess maternal psychological distress (Khashan et al., 2014). These included the 10-item Perceived Stress Scale (PSS) to measure psychological stress (Cohen et al., 1983), the 6-item version of State Trait Anxiety Inventory (STAI) to measure maternal anxiety (Marteau and Bekker, 1992), and the Edinburgh Postnatal Depression Scale (EPDS) to measure maternal depressive symptoms in pregnancy (Rubertsson et al., 2011, Cohen et al., 1983). We found that 15% of women experienced 'very high levels of perceived psychological stress (≥90th percentile score), 18% were classified as being as 'very highly anxious' (≥90th percentile score), while 15% were classified as being 'highly likely depressed' (EPDS score >9) (Khashan et al., 2014). Collectively these data have shown that approximately one in seven women experience clinically significant levels of prenatal maternal psychological distress during pregnancy.

This is important as numerous epidemiological studies have reported that exposure to prenatal maternal psychological distress is a risk factor for a range of adverse short and long-term outcomes in affected offspring. These include an increased risk of adverse obstetric outcomes including Caesarean delivery, preterm birth, low birth weight and babies who are small for gestational age (Liou et al., 2016, Grote et al., 2010, Ding et al., 2014a, Rose et al., 2016, Togher et al., 2017, Khashan et al., 2014). Moreover prenatal maternal psychological distress has been proposed to be a risk factor for the development of immune (Flanigan et al., 2016, Khashan et al., 2012), metabolic (Entringer, 2013, Entringer et al., 2008) and neuropsychiatric disorders (Class et al., 2014, Khashan et al., 2011, Khashan et al.,

2008a) later in life, with the relative risk varying by offspring sex (Khashan et al., 2011, Quarini et al., 2016, Mueller and Bale, 2008, Weinstock, 2007). These studies highlight the importance of prenatal maternal psychological distress as a risk factor for adverse outcomes in exposed offspring; however the causal pathways mediating these associations are unclear.

The glucocorticoid hypothesis is the most widely studied biological mechanism proposed to mediate the association between prenatal maternal psychological distress and adverse outcomes (Reynolds, 2013). During pregnancy, changes in the maternal hypothalamic-pituitary-adrenal (HPA) axis, leads to an exponential rise in cortisol in the maternal circulation (Nolten et al., 1980, Goland et al., 1988). This cortisol stimulates the release of corticotrophin releasing hormone (CRH) from the placenta that enters the maternal circulation and further increases the production of cortisol forming a feed forward loop. As a result maternal cortisol levels are up to ten-fold higher than fetal levels (Riley and Challis, 1991). This progressive increase in maternal cortisol is necessary for fetal organogenesis, however excessive fetal exposure may alter developmental trajectories (Togher et al., 2014). The maternal-fetal cortisol gradient is maintained by the expression of 11β-hydroxysteroid dehydrogenase type 2 (HSD11B2) in the placental trophoblast which converts active cortisol into inactive cortisone (Togher et al., 2014). Additionally, the glucocorticoid receptor (NR3C1) and FKPB51, a chaperone protein which regulates nuclear transport of NR3C1 (Zhang et al., 2008a), play an important role in the fetal response to cortisol. We and others have shown that maternal distress particularly in late pregnancy reduces placental HSD11B2 expression (Togher et al., 2017, Seth et al., 2015, O'Donnell et al., 2012). We also found that glucocorticoid receptor NR3C1 is upregulated by third trimester distress (Togher et al., 2017). Increased methylation of placental FKBP51 has been reported following early third trimester stress (Monk et al., 2016), however we previously observed no change in FKBP51 expression following distress in the third trimester (Togher KL, 2017), indicating the need to examine other trimesters. Collectively these data suggest that prenatal maternal psychological distress may alter molecular mechanisms that regulate fetal exposure to maternal cortisol. Importantly alterations in the expression and regulation of HSD11B2, NR3C1 and/or

FKBP51 has been linked to poor birth outcomes (Dy et al., 2008, Causevic and Mohaupt, 2007, Filiberto et al., 2011) as well as neurobehavioral problems in infants (Conradt et al., 2013, Appleton et al., 2015, Marsit et al., 2012, Paquette et al., 2014), suggesting that these may play a causal role in mediating the association between maternal distress and adverse outcomes.

In this study we sought to examine the relationships between psychological prenatal distress across pregnancy with birth outcomes and placental HSD11B2, NR3C1 and FKBP51 expression, as three key mediators of placental cortisol signalling. Moreover we undertook causal mediation analysis to determine whether any changes in the placental expression of these genes were associated with birth outcomes using gender-sensitive methodology.

5.3 Methods:

5.3.0 Participants

This study received full ethical approval from the Clinical Research Ethics Committee of Cork Teaching Hospitals. Nulliparous pregnant women enrolled in the IMPROvED study (Navaratnam et al., 2013) at Cork University Maternity Hospital were invited to participate in this study. Participants completed the PSS, STAI, and EPDS in the second and/or third trimesters of pregnancy. Detailed demographic and medical information was acquired from the participants' medical records.

5.3.1 Newborn Hair Collection and Processing

Newborn hair was acquired from the posterior vortex of the newborns head within 24h of birth and stored at room temperature until processing. 1mg of hair was incubated in 1 ml of methanol at 50°C for 24 h. Samples were sonicated for 30 min at 37 °C followed by another incubation for 24 h 50°C. The supernatant was removed and evaporated under nitrogen and the pellet was resuspended in Phosphate Buffered Solution. Cortisol concentration was determined by ELISA as per the manufacturer's instructions (Enzo life Sciences).

5.3.2 Placental collection and real-time PCR

Placenta biopsies were collected from 56 participants within 2h of delivery, washed in dH_2O and immediately stored at -80°C. RNA was extracted from placental samples using Trizol reagent as previously described (Togher KL, 2017). Briefly, placental samples were homogenised in Trizol and left on ice for 10 min. Samples were centrifuged and the supernatant was incubated in chloroform at room temperature for 5min followed by centrifugation for 15min at 4°C to remove the aqueous phase. RNA was isolated by incubation of the aqueous phase with propanol at room temperature for 10min. Samples were centrifuged and the pellet washed in 70% ethanol before resuspension in RNAse free H_2O (Sigma). RNA quality and quantity were determined by the Nanodrop 1000. RNA was reverse transcribed into cDNA (400 ng/ml) using the high capacity cDNA reverse transcription kit (Applied Biosystems) under the following parameters: 25 °C for 10 min; 37 °C for

120 min; 85 °C for 5 min; 4 °C for at least 10 min. Real time PCR was performed for using the following targets; GAPDH, YWAZ, HSD11B2, NR3C1, FKBP51 and IL1B (Integrated DNA Technologies; IDT) using the following parameters 50 °C for 2 min; 95 °C for 10 min; 50 repetitions of 95 °C for 15 s and annealing/elongating at 60 °C, as previously described (Togher KL, 2017). All samples were run in triplicate and gene expression determined the 2−ΔΔcycle threshold (2dCT) method (Livak and Schmittgen, 2002).

5.3.3 Statistical Analysis

Data analysis was performed on SPSS v22. Normality of predictor and outcome variables was tested for using Kolmogorov-Smirnov tests. All questionnaire scores were normally distributed. Relationships were determined using linear regression analysis. Outliers were determined using a Grubbs test and removed if $p < 0.05$.

5.4: Results:

5.4.0 Exposure to second trimester maternal anxiety negatively affects female birth weight

We first sought to determine whether maternal psychological distress scores affected infant birth weight. To do this we examined the associations between PSS, STAI and EPDS scores in the second and third trimester with birth weight (mean = 3623 ± 460.3 g) (n=55). We found that PSS scores in the second trimester were negatively correlated with male ($p < 0.05$), but not female birth weight (Figure 1a, b). We found no associations between PSS scores in the third trimester and male or female birth weight (Table 1). In contrast, second trimester STAI scores were negatively correlated with female ($p < 0.05$) but not male birth weight (Figure 1c, d). We found no associations between STAI scores in the third trimester and birth weight in male or female infants (Table 1). Similarly, we found no association between EPDS scores in the second or third trimester with birth-weight of infants of either sex (Table 1). As infant birth weight was significantly altered by maternal BMI ($\beta = .31$, $t(54) = 2.41$, $p = 0.019$) we adjusted our regression model to examine the potential confounding effects of maternal BMI. When BMI was included in the analyses, the relationship between second trimester PSS scores and male birth weight disappeared ($a\beta = -.32$, $t(23) = -1.72$, $p = 0.099$). In contrast, second trimester anxiety remained correlated with female birth weight when BMI was included in the regression model ($a\beta = -.46$, $t(26) = -2.71$, $p = 0.012$). These data revealed a gender specific effect of maternal anxiety on birth weight in female infants.

5.4.1 Placental FKBP51 mediates the association between second trimester maternal anxiety and female birth weight

As second trimester anxiety was associated with female birth weight, we next examined the relationship between second trimester STAI scores and three key genes involved in glucocorticoid signalling in the placenta, HSD11B2, NR3C1 and FKBP51. In agreement with our findings on female birth weight (Figure 1), we found a significant negative correlation between second, but not third, trimester STAI

scores and placental FKBP51 expression in females (β= -.64, t (25) = -4.10, p < 0.0001), but not males (β= -.53, t(23) = -1.78, p = 0.09). We found no significant associations between STAI scores in the second or third trimester and HSD11B2 or NR3C1 expression in males or females. We also found no associations between PSS and EPDS scores in the second or third trimester and placental expression of HSD11B2, NR3C1 and FKBP51, indicating that this effect appears specific to heightened anxiety levels. We subsequently examined if placental FKBP51 expression was an independent predictor of infant birth weight. There was a significant association between placental FKBP51 with birth weight in female (β= .54, t (29) = 3.38, p = 0.002) but not male (β= -.16, t (24) = -0.78, p = 0.44) infants. These data show that second trimester anxiety (STAI scores) negatively correlated with both birth weight and placental FKBP51 in females, and that FKBP51 positively correlates with birth weight in females. Given these findings, we hypothesised that placental FKBP51 may be mediating the relationship between maternal anxiety and female birth weight. In support of this hypothesis when FKBP51 was included into the regression model the association between maternal anxiety and female birth weight disappeared (β = .19, t (25) = -0.816, p = 0.423) (Figure 2a). These data show that the association between second trimester maternal anxiety and female birth weight is mediated by placental FKBP51.

5.4.2 Alterations in second trimester maternal anxiety and placental FKBP51 are associated with newborn cortisol levels

The placental and fetal response to glucocorticoids is crucial in determining fetal growth outcomes. This is highlighted by studies showing that eexposure to synthetic glucocorticoids during pregnancy is associated with reductions in birth weight (Khan et al., 2011), with some sex-specific outcomes also observed (Stevenson et al., 2000). As FKBP51, negative regulates nuclear transport of NR3C1 (Zhang et al., 2008a), we hypothesized that this may result in alterations in cortisol levels in infants. In an exploratory technique we measured cortisol levels in newborn hair as a potential retrospective measure of cortisol exposure *in utero.* We next examined the relationship between maternal distress and infant cortisol levels. Maternal PSS or EPDS scores in the second or third trimester did not correlate with

infant cortisol levels (Table 4). Surprisingly however, second trimester maternal anxiety (STAI) was negatively associated with infant hair cortisol levels (β= -.43, t (25) = -2.33, p = 0.028). We next went on to examine the relationship between placental genes and birth outcomes with infant cortisol. Placental HSD11B2 (β= -.02, t (28) = -0.10, p = 0.920) and NR3C1 (β= -.19, t (28) = -1.00, p = 0.325) were not related to newborn cortisol. Intriguingly however, FKBP51 expression was positively correlated with infant cortisol levels in females only (β= .54, t (13) = 2.23, p = 0.045). As both maternal anxiety and FKBP51 were related to newborn cortisol levels, when we included both in the mediation, the relationship between second trimester anxiety (aβ= -.27, t (11) = -0.62, p = 0.550) and FKBP51 (aβ= .30, t (11) = 0.68, p = 0.510) with newborn cortisol levels disappeared. These data suggest that FKBP51 mediates the relationship between second trimester anxiety and newborn cortisol levels (Figure 2b). However, there was no correlation observed between newborn cortisol levels and infant birth weight.

5.5 Tables and Figures:

Figure 1:

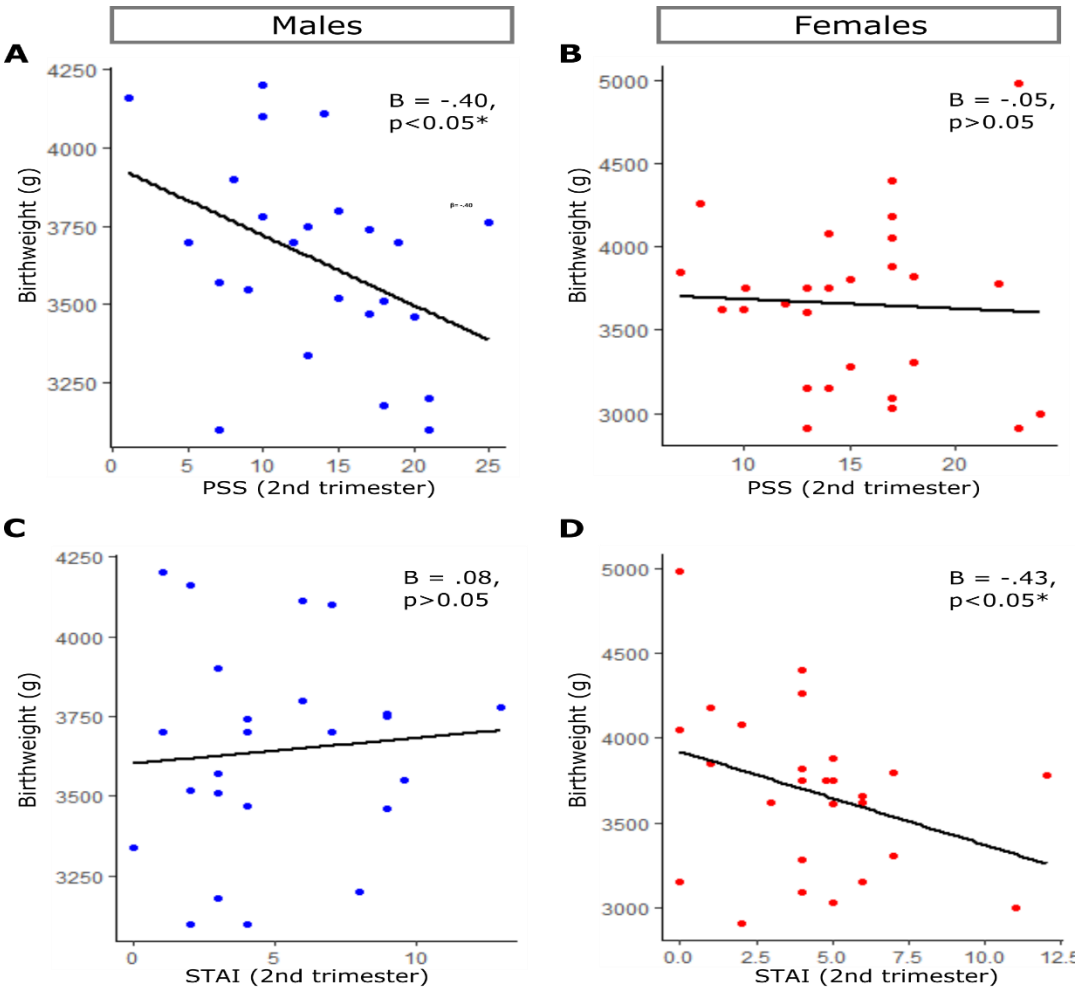

Figure 1: Scatter plots of birthweight and (A-B) second trimester stress (PSS) and (C-D) second trimester anxiety (STAI) in males (blue) and females (red). Statistical analysis: Univariate linear regression *p < 0.05.

Table 1:

Linear Regression Analysis			
PSS (2nd trimester)	**Both**	**Males**	**Females**
Birthweight	β=-.18, t_{50}=-1.32, p=0.19	**β=-.40, t_{23}=-2.08, p=0.04**	β=-.05, t_{26}=-0.25, p=0.79
Birthweight Centiles	β=-.18, t_{50} =-1.29, p=0.20	β=-.30, t_{23}=-1.50, p=0.14	β=-.16, t_{26}=-0.82, p=0.41
Gestational Age	β=-.18, t_{50}=-1.31, p=0.19	β=-.18, t_{23}=-0.90, p=0.37	β=-.16, t_{26}=-0.16, p=0.41
PSS (3rd trimester)			
Birthweight	β=-.10, t_{45}=-0.71, p=0.47	β=-.24, t_{20}= -1.08, p=0.29	β=-.05, t_{24}=0.25, p=0.80
Birthweight Centiles	β=-.05, t_{45}=-0.37, p=0.70	β=-.15, t_{20}=-0.67, p=0.50	β=.00, t_{24}=0.01, p=0.99
Gestational Age	β=-.15, t_{45}=-1.00, p=0.32	β= -.18, t_{20}=-0.82, p=0.42	β=-.23, t_{26}=-0.85, p=0.39
STAI (2nd trimester)			
Birthweight	*β=-.25, t_{50}=-1.85, p=0.07*	β=.08, t_{23}=0.39, p=0.69	**β=-.43, t_{26}=-2.42, p=0.02**
Birthweight Centiles	β=-.19, t_{50}=-1.41, p=0.16	β=-.00, t_{23}=-0.02, p=0.97	*β=-.34, t_{26}=-1.80, p=0.08*
Gestational Age	β=-.13, t_{50}=-0.91, p=0.36	β=.06, t_{23}=0.30, p=0.764	β=-.23, t_{26}=-1.22, p=0.23
STAI (3rd trimester)			
Birthweight	*β=-.28, t_{45}=-1.95, p=0.05*	β=-.18, t_{20}=-0.81, p=0.42	β=-.33, t_{24}=-1.70, p=0.10
Birthweight Centiles	β=-.19, t_{45}=-1.33, p=0.18	β=-.19, t_{20}=-0.84, p=0.40	β=-.23, t_{24}=-1.17, p=0.25
Gestational Age	**β=-.29, t_{45}=-2.02, p=0.04**	β=-.13, t_{20}=-0.59, p=0.56	β=*-.36, t_{24}=-1.88, p=0.07*
EPDS (2nd trimester)			
Birthweight	β=-.13, t_{50}=-0.95, p=0.34	β=-.15, t_{23}=-0.72, p=0.47	β=-.13, t_{26}=-0.69, p=0.49
Birthweight Centiles	β=-.09, t_{50}=-0.69, p=0.49	β=-.22, t_{23}=-1.06, p=0.29	β=-.10, t_{26}=-0.51, p=0.60
Gestational Age	β=-.20, t_{50}=-1.46, p=0.15	β=-.13, t_{23}=-0.65, p=0.51	β=-.20, t_{26}=-1.04, p=0.30
EPDS (3rd trimester)			
Birthweight	β=-.12, t_{45}=-0.85, p=0.39	β=-.08, t_{20}=-0.37, p=0.70	β=-.14, t_{24}=-0.72, p=0.47
Birthweight Centiles	β=-.06, t_{45}=-0.39, p=0.69	β=-.08, t_{20}=-0.35, p=0.73	β=-.03, t_{24}=-0.15, p=0.87
Gestational Age	β=-16, t_{45}=-1.13, p=0.26	β=-.22, t_{20}=-0.99, p=0.33	β=-.18, t_{24}=-0.90, p=0.37

Table 1: Prenatal distress and birth outcomes. Linear regression analysis of maternal distress across pregnancy and birth outcomes.

Table 2:

Linear Regression Analysis			
HSD11B2	**Both**	**Males**	**Females**
2nd trimester			
PSS	*β=.26, t_{49}=1.88, p=0.06*	*β=.39, t_{22}=1.99, p=0.06*	β=.09, t_{26}=0.47, *p*=0.63
STAI	β=-.11, t_{49}=-0.80, *p*=0.42	β=-.11, t_{22}=-0.54, *p*=0.59	β=-.11, t_{26}=-0.55, *p*=0.58
EPDS	β=.09, t_{49}=0.67, *p*=0.50	β=.05, t_{22}= 0.27, *p*=0.78	β=.07, t_{26}=0.39, *p*=0.69
3rd trimester			
PSS	β=-.10, t_{45}=-0.70, *p*=0.48	β=-.20, t_{20}=-0.90, p=0.37	β=-.02 t_{24}=-0.10, *p*=0.91
STAI	β=-.00, t_{45}=-0.02, *p*=0.98	β=-.00, t_{20}=-0.02, *p*=0.97	β=-.01, t_{24}=-0.06, *p*=0.95
EPDS	β=-.05, t_{45}=-0.34, *p*=0.73	β=.07, t_{20}=0.33, *p*=0.74	β=-.14, t_{24}=-0.71, *p*=0.48
NR3C1			
2nd trimester			
PSS	β=.11, t_{50}=0.80, *p*=0.42	β=-.14, t_{23}=-0.14, p=0.48	***β=.42, t_{26}=2.32, p=0.02***
STAI	β=.14, t_{50}=1.03, *p*=0.30	β=.16, t_{23}=0.78, p=0.44	β=.13, t_{26}=0.66, p=0.51
EPDS	β=.02, t_{50}=0.19, *p*=0.84	β=-.13, t_{23}=-0.63, p=0.53	β=.16, t_{26}=0.16, p=0.42
3rd trimester			
PSS	β=-.20, t_{45}=-1.39, *p*=0.17	β=-.31, t_{20}=-1.44, p=0.16	β=-.12, t_{24}=-0.59, p=0.55
STAI	β=-.02, t_{45}=-0.14, *p*=0.89	β=-.01, t_{20}=-0.07, p=0.94	β=-.02, t_{24}=-0.09, p=0.92
EPDS	β=-.18, t_{45}=-1.25, *p*=0.21	β=-.24, t_{20}=-1.07, p=0.29	β=-.15, t_{24}=-0.75, p=0.45
FKBP51			
2nd trimester			
PSS	β=-.04, t_{49}=-0.32, *p*=0.75	β=-.03, t_{23}=-0.17, p=0.86	β=-.08, t_{25}=-0.42, p=0.67
STAI	**β=-.46, t_{49}=-3.59, *p*=0.001**	*β=-.53, t_{23}=-1.78, p=0.088*	***β=-.64, t_{25}=-4.10, p=0.000***
EPDS	β=-.25, t_{49}=-1.82, *p*=0.07	β=-.24, t_{23}=-1.15, p=0.25	*β=-.33, t_{25}=-1.74, p=0.09*
3rd trimester			
PSS	β=-.15, t_{44}=-1.05, *p*=0.29	β=-.12, t_{20}=-0.53, p=0.60	β=-.21, t_{23}=-1.03, p=0.31
STAI	β=-.21, t_{44}=-1.40, *p*=0.16	β=-.13, t_{20}=-0.61, p=0.54	β=-.30, t_{23}=-1.50, p=0.14
EPDS	β=-.13, t_{44}=-0.92, *p*=0.36	β=.07, t_{20}=0.34, p=0.73	*B=-.40, t_{23}=-2.06, p=0.05*

Table 2: Maternal distress across pregnancy and placental gene expression. Linear regression analysis of maternal distress across pregnancy and placental HSD11B2, NR3C1 and FKBP51 expression.

Table 3:

Linear Regression Analysis			
HSD11B2	**Both**	**Males**	**Females**
Birthweight	*β*=.05, t_{53}=0.36, *p*=0.72	*β*=-.17, t_{23}=-0.81, *p*=0.42	*β*=.14, t_{29}=0.76, *p*=0.45
Birthweight Centiles	*β*=.14, t_{53}=1.04, *p*=0.30	*β*=-.07, t_{23}=-0.36, *p*=0.71	*β*=.21, t_{29}=1.17, *p*=0.24
Gestational Age	*β*=-.01, t_{53}=-0.07, *p*=0.94	*β*=-.05, t_{23}=-0.25, *p*=0.79	*β*=.05, t_{29}=0.27, *p*=0.78
NR3C1			
Birthweight	*β*=-.07, t_{54}=-0.55, *p*=0.58	*β*=-.05, t_{24}=-0.24, *p*=0.80	*β*=.09, t_{29}=-0.47, *p*=0.63
Birthweight Centiles	*β*=-.16, t_{54}=-1.24, *p*=0.21	*β*=-.03, t_{24}=-0.15, *p*=0.87	*β*=-.26, t_{29}=-1.43, *p*=0.16
Gestational Age	***β*=.27, t_{54}=2.09, *p*=0.04**	*β*=.27, t_{24}=1.36, *p*=0.18	*β*=.29, t_{29}=1.64, *p*=0.11
FKBP51			
Birthweight	*β*=.21, t_{53}=1.58, *p*=0.11	*β*=-.16, t_{24}=-0.78, *p*=0.44	***β*=.54, t_{29}=3.38, *p*=0.002**
Birthweight Centiles	*β*=.21, t_{53}=1.57, *p*=0.122	*β*=-.09, $_{t24}$=-0.43, *p*=0.67	***β*=.56, t_{29}=3.51, *p*=0.002**
Gestational Age	*β*=.02, t_{53}=0.16, *p*=0.87	*β*=-.22, t_{24}=-1.11, *p*=0.27	*β*=.20, t_{29}=1.06, *p*=0.29

Table 3: Placental gene expression and birth outcomes. Linear regression analysis of placental HSD11B2, NR3C1 and FKBP51 expression with infant gestational age and birthweight.

Figure 2:

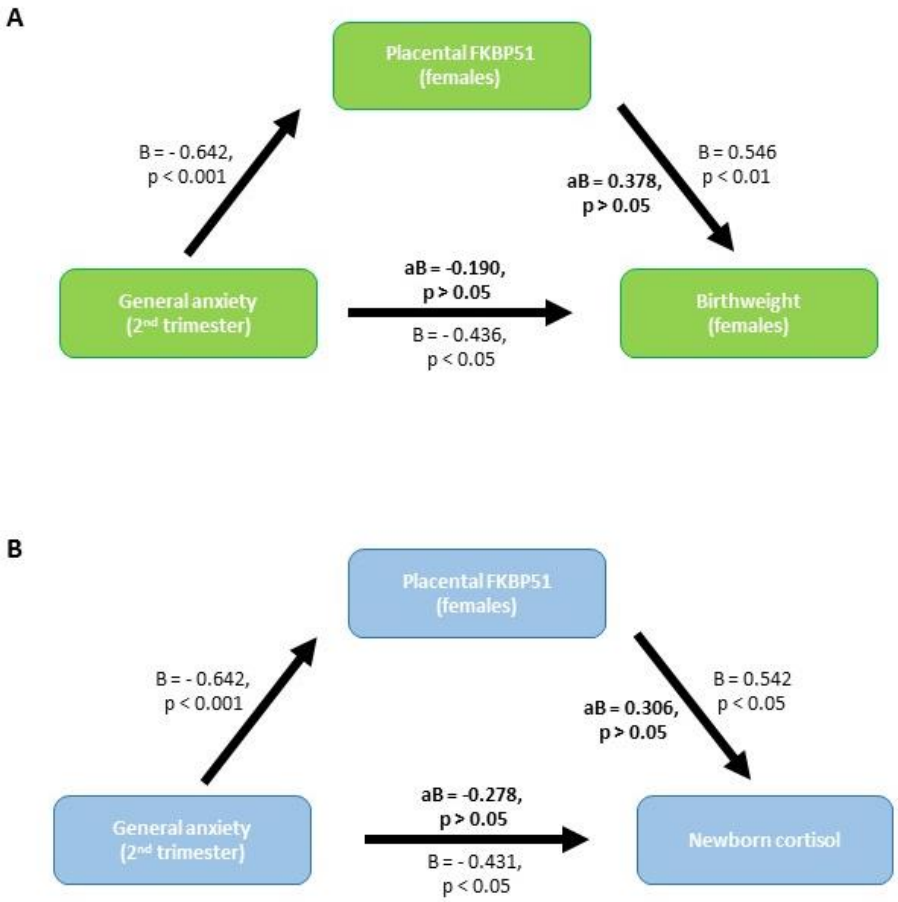

Figure 2: Placental FKBP51 mediates the relationship between prenatal anxiety and birthweight in females. Mediation Plots (A) Placental FKBP51 mediates the relationship between second trimester maternal anxiety and infant birthweight in females. (B) Placental FKBP51 mediates the relationship between second trimester maternal anxiety and newborn hair cortisol in females. Statistical Analysis: Linear regression analysis.

Table 4:

Linear Regression Analysis			
Newborn Cortisol	**Both**	**Males**	**Females**
PSS (2^{nd} trimester)	β=-.13, t_{25}= -0.68, p=0.49	β=.12, t_{13}= 0.42, p=0.68	β=-.41, t_{25}= -1.44, p=0.17
PSS (3^{rd} trimester)	β=-.10, t_{26}= -0.53, p=0.59	β=.01, t_{13}= 0.03, p=0.97	β=-.17, t_{12}= -0.60, p=0.55
STAI (2^{nd} trimester)	**β=-.43, t_{25}= -2.33, p=0.028**	*β=-.51, t_{13}= -2.06, p=0.06*	*β=-.51, t_{11}= -1.91, p=0.08*
STAI (3^{rd} trimester)	β=-.16, t_{26}= -0.81, p=0.42	β=-.23, t_{13}= -0.83, p=0.42	β=-.10, t_{12}= -0.36, p=0.72
EPDS (2^{nd} trimester)	*β=-.36, t_{25}= -1.91, p=0.067*	β=-.15, t_{13}= -0.54, p=0.59	β=-.42, t_{11}= -1.46, p=0.17
EPDS (3^{rd} trimester)	β=-.15, t_{26}= -0.80, p=0.42	β=-.04, t_{13}= -0.15, p=0.87	β=-.24, t_{12}= -0.84, p=0.41

Table 4: Maternal stress across pregnancy and newborn cortisol. Linear regression analysis of maternal distress across pregnancy and newborn cortisol levels.

Figure 3:

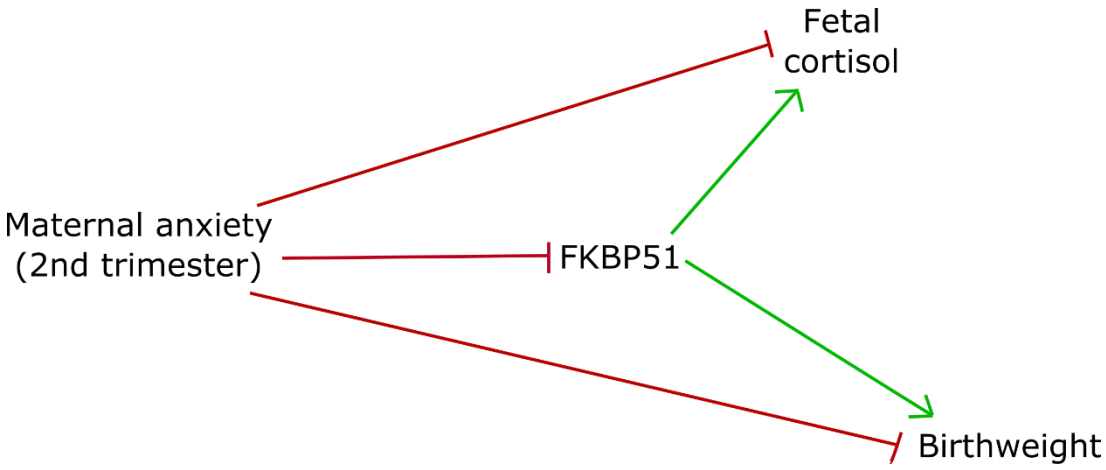

Figure 3: Summary figure. Second trimester maternal anxiety decreases infant birthweight in female offspring by inhibiting placental FKBP51. Similarly second trimester maternal anxiety reduced fetal cortisol exposure by inhibiting FKBP51 in female offspring.

5.6: Discussion:

A large number of women report experiencing psychological distress in their pregnancy (Khashan et al., 2014, O'Donnell et al., 2012). This is important as prenatal distress has been linked to a wide range of poor obstetric and neonatal outcomes as well as an increased risk of disease in childhood and adulthood. Of particular importance prenatal distress is commonly linked to birthweight and birth size (Bussières et al., 2015). Reduced birthweight remains a significant clinical challenge as it is often associated with increased mortality and morbidity (Jeschke et al., 2016). Additionally infants of lower birthweights are at an increased risk of developmental impairments in childhood, particularity in relation to neurodevelopment (Walhovd et al., 2012, Howe et al., 2016). Whilst the poor outcomes associated with being born low birthweight are well documented, the prenatal determinants linking psychological distress and birthweight are not very well understood.

Here we find the effect of maternal distress on birth outcomes to depend on the type of distress, timing of distress and sex of the infant. We initially observed a significant relationship between second trimester stress and reduced infant birthweight in males. However this association disappeared after adjustment for maternal BMI. Most notably we observe a significant negative correlation between birthweight and second trimester anxiety specifically, consistent with a recent report (Pinto et al., 2017). When stratified based on sex, this relationship was only observed in females. This sex difference in relation to birthweight and prenatal anxiety has previously being reported where males born from anxious pregnancies has increased birthweight compared to male controls, and females born from anxious mothers had reduced birthweights compared to female controls (Kaitz et al., 2015). Additionally anxious mothers of females are also more likely to develop obstetric complications, whereas anxious mothers of males are not (Kaitz et al., 2014). Male fetuses are generally more vulnerable to the effects of maternal distress (Stormer, 2011). It has been postulated that under conditions of adversities the male fetus favours growth at the expense of other developmental processes, whereas the female fetus conserves growth, thus being born at lower weights but with fewer morbidities in late life (Kaitz et al., 2014). In support of this, mid

pregnancy exposure to dexamethasone, a synthetic glucocorticoid, was found to decrease maternal blood in sinusoids of the female but not male placenta, restricted blood flow may mechanistically explain restricted growth in female fetuses (O'Connell et al., 2017).

At a biological level, sex specific responses in the placenta to maternal perturbations may explain why one sex is more vulnerable over the other (Clifton, 2010). In particular sex specific responses to maternal glucocorticoids, or more specifically how the placenta regulates glucocorticoids differentially may play a role (Hodyl et al., 2010, Cuffe et al., 2017). In this study we focused on three genes in the placenta involved in glucocorticoid regulation; HSD11B2, NR3C1 and FKBP51. Inconsistent with our previous work (Togher et al., 2017) and work of others (Seth et al., 2015), we do not observe a reduction in HSD11B2 following prenatal distress, however the mean stress score in this population was relatively low and the effect of maternal stress on HSD11B2 expression has been shown to be dependent on severity (Welberg et al., 2005). Second trimester maternal stress increased NR3C1 expression in female placentae. Additionally we find increased placental NR3C1 to be related to increased gestational age irrespective of sex. This increase in NR3C1 we observe among females but not males could again represent an adaptive response of the female placenta in response to maternal distress.

FKBP51 is a chaperone protein that stimulates translocation of the glucocorticoid receptor into the nucleus, resulting is less available receptor for glucocorticoid binding and subsequently reduced glucocorticoid response (Zhang et al., 2008a). Consistent with previous reports (Monk et al., 2016, Kertes et al., 2016) we find prenatal distress to reduce placental FKBP51 expression. Importantly we find placental FKBP51 to mediate a relationship between second trimester anxiety and infant birth weight in females only, suggesting a critical role for this chaperone protein in the female fetal response to maternal anxiety. As FKBP51 regulates the glucocorticoid receptor, reduced expression my result in increased receptor availability for cortisol binding and subsequently increased placental production of Cortisol releasing factor (CRF). Increased CRF may in turn alter fetal developmental trajectories (Glover, 2015). As such, a retrospective examination of maternal, placental and/or fetal CRF would be of interest.

We hypothesised that placental changes in FKBP51 would result in alterations in fetal cortisol. Indeed we observed a positive correlation between placental FKBP51 and newborn cortisol in females only. Of particular interested second trimester anxiety was the only distress variable that influences newborn cortisol levels. Following our mediation we show second trimester maternal anxiety decreases newborn cortisol levels by reducing FKBP51 in female placenta. Although newborn cortisol exposure was not related to birth outcomes this may be another mechanism by which the placenta protects the female fetus from maternal distress.

The current study has a number of limitations. We first acknowledge the small sample size and suggest this research be carried out in a larger scale, although appreciate the difficulties in running large scale placental collection studies (Burton et al., 2014). However even with this limited sample we observe clinically significant observations. Although we report significant associations with prenatal distress and birthweight we would like to highlight that only one (1.8%) infant in this cohort was born <2500g, the WHO estimate for clinically defined low birthweight (World Health Organisation, 2004).

Overall this study is important as it identifies a crucial role for the timing of distress, the type of distress and fetal sex in the relationship between prenatal distress and placental gene expression. This adds to the existing literature supporting a role for alterations in placental glucocorticoid signalling following prenatal distress. To our knowledge this is the first study to identify an association between placental FKBP51 and infant birthweight. Importantly we identify this gene to be a key mediator underlying a relationship between prenatal anxiety and birthweight in females, which highlights the crucial role placental signalling, has, in terms of exposure to maternal distress and infant development. The identification of this relationship warrants further investigation into the precise role that FKBP51 has in fetal development.

Chapter 6:

Prenatal distress exposure remodels the maternal gut microbiome: Implications for offspring gut microbiome assembly

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6.1 Abstract:

The experience of prenatal maternal stress is associated with high rates of adverse obstetric outcomes and altered infant development. The mechanisms underpinning this may be linked to inappropriate remodelling of the maternal microbiome during pregnancy and subsequent vertical transmission of a suboptimal microbiome at birth. The current study aimed to assess the association between prenatal maternal stress and the maternal gut, vaginal and infant microbiome in a clinical population. Nulliparous pregnant women completed stress, anxiety and depression questionnaires and provided saliva and fecal samples in the second and third trimester of pregnancy. Vaginal swabs were collected at the time of delivery. Infant fecal samples were obtained at 1wk, 2wks, 3wks, 3mo and 5mo. Microbial community structure was analysed by 16S rRNA gene sequencing. Second trimester stress and depressive symptoms were associated with a reduced diversity of the second trimester maternal gut microbiota. Second trimester cortisol was associated with increased diversity of the third trimester gut microbiota and reduced diversity of the vaginal microbiota. Infants born from pregnancies experiencing higher stress in the second trimester had increased alpha diversity and reduced relative abundance of fecal *Bifidobacteria* and *Lactobacilli.* Conversely, third trimester depressive symptomology was related to reduced alpha diversity in the infant gut microbiota. Infant gut colonization was also influenced by newborn cortisol levels. Further studies are required to clarify the implications of these stress-induced microbial alterations for obstetric outcomes and infant development.

6.2: Introduction:

The experience of maternal psychological stress (stress, anxiety and/or depressive symptomology) during pregnancy is associated with the development of a number of obstetric complications including preterm birth (Shapiro et al., 2013, Staneva et al., 2015, Khashan et al., 2009), growth restriction (Khashan et al., 2014) and low birth weight (Khashan et al., 2008b, Hasanjanzadeh and Faramarzi, 2017). Infants exposed prenatally to high maternal psychological distress are at an increased risk of developing diseases of metabolic (Ingstrup et al., 2012, Hohwu et al., 2014, Li et al., 2013a), immune (Khashan et al., 2012, Chiu et al., 2012, Suh et al., 2017) and nervous systems (Khashan et al., 2011, Class et al., 2014) in childhood and adulthood. The underlying biological mechanisms linking maternal distress to adverse outcomes particularly in humans are unclear, but fetal overexposures to stress-induced elevations in maternal glucocorticoids have been a primary focus (Reynolds, 2013). However, it is now known that stress and the associated activity of the hypothalamus–pituitary–adrenal axis can influence the composition of the gut microbiota in a bidirectional fashion, however the functional consequences of this have only begun to emerge (Clarke et al., 2013, Clarke et al., 2014b). Most recently, pre-clinical studies have proposed that stress-related alterations in maternal microbiota during pregnancy and subsequently vertical transmission of this stressed microbiota to the infant during parturition is a potential mechanism linking prenatal maternal stress to infant neurodevelopment (Jasarevic et al., 2014).

Bacterial colonization of the infant gastrointestinal tract largely begins at birth as the baby ingests its mother's vaginal microbes during passage through the birth canal (Milani et al., 2015). Over the first weeks/months of a newborn's life, the microbial populations continue to be established with influences from birth mode and environmental sources playing a major role in the developmental trajectory of the infant gut microbiota. This establishment of the infant gut microbiota in early life overlaps with critical periods of immune (Dimmitt et al., 2010), metabolic (Backhed, 2011) and nervous system (Borre et al., 2014, Sudo et al., 2004) development and as such, alterations to the developmental trajectory of the infant microbiota may increase disease risk. Gestational age (Cong et al., 2016),

antibiotic use (Azad et al., 2016), mode of delivery (Hill et al., 2017) and mode of feeding (Thompson et al., 2015) are critical determinants of the infant microbiome.

Recently prenatal stress has been associated with altered seeding and development of the infant gut microbiota. In rodents, a chronic stress paradigm in early pregnancy was found to disrupt the vaginal and infant gut microbiome, notably a reduction in lactobacilli in both, suggesting direct transmission during birth (Jasarevic et al., 2015a). Additionally, stressed-induced microbial changes were related to alterations in gut metabolic profiles and amino acid availability in the hypothalamus (Jasarevic et al., 2015a). Most recently, pregnancy stress has been shown to disrupt the maternal gut, in addition to vaginal and infant microbial communities in rodents (Jasarevic et al., 2017, Golubeva et al., 2015). In rhesus monkeys, stress in late pregnancy was found to reduce the abundance of bifidobacteria and lactobacilli in the infants gut (Bailey et al., 2004). Only one study, to our knowledge, has examined this relationship in a clinical cohort, whereby maternal anxiety and cortisol levels in late pregnancy were related to reduced lactobacilli and bifidobacteria and increased pathogenic bacteria in infants gut. Furthermore, this stress-associated microbial signature was related to allergic symptoms at 3 months of age. However, in this study maternal distress was only assessed in late pregnancy and focused primarily on measures of anxiety without concurrent maternal microbiota assessments (Zijlmans et al., 2015b).

To our knowledge, no previous clinical study, has prospectively examined the impact of pregnancy stress on the prenatal maternal gut and vaginal microbiota and subsequently infant gut microbiota seeding and assembly. Additionally, whether a trimester specific effects exists has not yet been explored. The current study aims to address this limitation using a well phenotyped clinical sample population, assessing both psychological and physiological measures of maternal stress in the second and third trimesters of pregnancy and using high throughput 16S gene sequencing to analyse microbial community structure in the maternal gut, vagina and infant gut microbiota.

6.3: Methods:

6.3.0 Subject Recruitment, samples and data collection

This study received full ethical approval from the Clinical Research Ethics Committee of Cork Teaching Hospitals. For detailed protocol see Appendix A. Nulliparous healthy pregnant women enrolled in the IMPROvED study at Cork University Maternity hospital (CUMH) between September 2015 and August 2016 were invited to participate in this study (Navaratnam et al., 2013). 111 pregnant mothers and subsequently their offspring participated in this study. Inclusion criteria for this study included being enrolled in the IMPROvED study (Navaratnam et al., 2013) Provide details of inclusion and exclusion criteria. Participants were asked to complete the Perceived stress Scale (PSS; stress), State Trait Anxiety Inventory (STAI; anxiety) and Edinburgh Postnatal Depression Scale (EPDS; depressive) in the second and/or third trimesters of pregnancy. In both trimesters, participants were asked to collect saliva and fecal samples at home and store at 4°C until transport to CUMH within 24h of collection. Saliva samples were collected using Salivettes (Sarstedt) at four time points; upon waking, 30min after waking, 60 min after waking and 180min after waking. Fecal samples were collected in plastic containers containing Anaerobic Sachets (Fisher Scientific). Vaginal swabs were acquired when participants where admitted to CUMH just prior to delivery. Parents collected fecal samples from their infants at home and stored them at 4°C until transport to CUMH in coolers within 24h. All samples were stored at -80°C until processing.

6.3.1 Maternal cortisol concentration

Saliva samples were thawed on ice and cortisol concentration was determined by Enzyme Linked Immunosorbent Assay (ELISA; Enzo life Sciences) according to the manufacturer's instructions. The cortisol awakening response (CAR) was determined by calculating the area under the curve with respect to ground (AUC_G) (Pruessner et al., 2003).

6.3.2 Newborn cortisol concentration

A hair sample was collected from the newborn infant's head within 24h of birth. 1mg of hair was incubated in 1 ml of methanol at 50°C for 24 h. Samples were sonicated for 30 min at 37 °C followed by another incubation for 24 h at 50°C. The supernatant was removed and evaporated under nitrogen and the pellet was resuspended in phosphate buffered solution. Cortisol concentration was determined by ELISA as per the manufacturer's instructions (Enzo life Sciences).

6.3.3 DNA extraction from fecal samples

Prior to extraction, fecal samples were thawed on ice. Maternal fecal samples were added to a tube containing Qiagen Lysis Buffer and a mix of sterile zirconia beads (Thistle Scientific). The sample was homogenised for 90 sec using the MagnaLyser Instrument (Roche) and incubated at 90 °C for 5 min. Samples were centrifuged and the supernatant transferred to a clean tube. DNA extraction proceeded according to the manufacturer's instructions (Qiagen DNA stool Mini Kit). DNA was extracted from vaginal swabs and infant fecal samples in a slightly adapted method to improve DNA yield as previously described (Yu and Morrison, 2004).

6.3.4 Sequencing

Preparation of DNA samples for 16S sequencing was performed according to the manufacturer's instructions (Illumina). DNA was amplified to the V4 region of the bacterial 16S rRNA under the following parameters; 95°C for 3 min, 25 cycles of 95°C for 30 sec, 55°C for 30 sec and 72°C for 30 sec and finally 72°C for 5 min before being held at 4°C. The PCR product was cleaned using AMPure XP beads and 5µl of supernatant was transferred to a new PCR plate where PCR was performed with Nextera XT index primers under the same conditions as mentioned above. The product was cleaned again using AMPure XP beads and the final product was analysed on a Bioanalyser DNA 1000 chip for quality assurance (size ~630bp). Samples were diluted and sequenced by the Miseq Illumina System.

6.3.5 Outcome measures

We analysed the maternal gut microbiome in the second and third trimester of pregnancy, the vaginal microbiome prior to delivery and the infant gut microbiome at 1, 2 and 3 weeks and 3 and 5 months. Chao1 Index, Shannon Index, Simpson Index, Phylogenetic diversity and number of species were used as indicators of alpha diversity. Bacterial OTUs were analysed at phylum, family and genus levels.

6.3.6 Statistical Analysis

For the EPDS, we ranked women scoring equal to and above 9 as exhibiting high depressive symptoms and those scoring below 9 as exhibiting low depressive symptoms as previously described (Navaratne et al., 2016). For the PSS, STAI and CAR women were ranked as scoring high or low if they scored above or below the mean respectively. As scoring in maternal distress questionnaires was not significantly correlated with salivary cortisol levels we treated maternal cortisol concentration as an independent variable. As the infant gut differs based on mode of delivery (Hill et al., 2017), we examined the infant gut in vaginally and caesarean delivered infants separately. Here we report the results for vaginally delivered infants only. Distribution was tested using Kolmogorov-Smirnov tests; $p < 0.05$ non-normal distribution and $p > 0.05$ normal distribution. Following normality testing, unpaired student t-tests were used to calculate differences between alpha diversity indices. Phylum, family and genus levels were analysed by Mann Whitney U-tests. Results were deemed significant if $p < 0.05$. Statistical analysis was performed using R and SPSS.

6.4: Results:

6.4.0 Maternal Demographics and descriptive statistics

The final cohort consisted of 111 nulliparous pregnant women. 95 (85.5%) women completed the questionnaires in the second trimester (Mean Gestational Age (GA): 20 ± 0.92) and 79 (71.1%) women completed the questionnaires in the third trimester (Mean GA: 32 ± 3.62). The cortisol awakening response was available for 79 (71.1%) and 75 (67.5%) women in the second and third trimesters, respectively. The mean (± SD) age and BMI of this cohort were 30.6 ± 4.4 and 25.4 ± 3.8 respectively. Maternal demographics and descriptive statistics can be found in (Appendix D, Table 1 & 2).The mean PSS, STAI, and EPDS were 13.88 ± 5.32, 4.61 ± 3.63 5.77 ± 4.40 in the second trimester and 11.22 ± 5.87, 4.94 ± 3.40 and 5.66 ± 4.64 in the third trimester, respectively.

6.4.1 Correlations between psychological and physiological stress

We first sought to identify any correlations between the four measures of maternal stress. Maternal stress (β=.03, t_{76}=.25, p=0.79), anxiety (β=.00, t_{76}=.06, p=0.94) or depressive (β=-.09, t_{76}=-.08, p=0.41), scores in the second trimester did not correlate with second trimester maternal cortisol (AUCg). Similarly maternal stress (β=.17, t_{64}=1.39, p=0.16), anxiety (β=.15, t_{64}=1.27, p=0.20) or depressive (β=.15, t_{76}=1.23, p=0.22) scores in the third trimester did not correlate with third trimester maternal cortisol (AUCg). There was a positive correlation between second trimester anxiety and third trimester maternal cortisol (β=.28, t_{64}=2.36, p=0.02). As there were no strong correlations observed between maternal psychological and physiological stress, we treated these as four independent distress variables.

6.4.2 Second trimester stress and depressive symptomology shapes the diversity and composition of the bacterial communities in the maternal gut

We next examined the effect of maternal distress in the second trimester on the maternal gut microbiome in the second trimester. High stress was associated with reduced species richness of the maternal gut in the second trimester ($p < 0.05$). At family level, women with high stress had a reduction in the dominant

Ruminococcaceae ($p < 0.05$). High depressive symptoms was associated with reductions in species richness and diversity (Figure 1a-c), increased *Faecalibacterium* ($p < 0.05$) and reduced *Ruminococcaceae uncultured* (Figure 1j). Second trimester anxiety or cortisol had little effect on the second trimester maternal gut microbiome with only a few alterations observed in non-dominant genus (Appendix D). We examined if second trimester distress altered the maternal gut microbiome in the third trimester. Second trimester stress, anxiety and depressive symptoms did not impact the maternal gut in the third trimester (Figure 1d-f, k; Appendix D). High cortisol in the second trimester was associated with increased alpha diversity ($p < 0.05$), *Erysipelotrichaceae* and *Peptostreptococcaceae uncultured* and decreased Alcaligenaceae ($p < 0.05$) in the third trimester gut (Appendix D). Third trimester distress measures had minimal effect on the maternal gut microbiome (Figure 1g-i, l, Appendix D). This suggests that second, but not third trimester maternal distress, markedly disrupts the composition of the maternal gut microbiota.

6.4.3 Second trimester maternal cortisol correlated with reduced diversity of the vaginal microbiome

Research from rodents suggests that stress induced changes in the maternal vaginal microbiome are vertically transmitted to the infant during parturition (Jasarevic et al., 2015a). To test this, we acquired vaginal samples from women just prior to giving birth. We found a significant association between second trimester anxiety and reduced abundance of the dominant *Streptococcaceae* ($p < 0.01$) in the maternal vagina. Second trimester stress and depressive symptoms had no effect on the vaginal microbiota ((Figure 2a-i).). The vaginal microbiome of women who had elevated cortisol in the second trimester displayed reduced species richness and diversity compared to non-stressed controls (Figure 2j-l). Third trimester stress did not alter the diversity or composition of dominant bacteria in the maternal vagina. Of note, there was a trend towards reduced richness of vaginal communities by both second and third trimester maternal stress ($p < 0.1$) (Appendix D).

6.4.4 The development of the infant gut microbiota is influenced by the timing and nature of the prenatal distress exposure

Second trimester distress:

The diversity of the infant gut microbiota was increased when women were exposed to high second trimester stress at 3wk, 3mo and 5mo (Figure 3a-e). At a compositional level, prenatal stress was associated with a number of alterations: increased Firmicutes and *Veillonellaceae* and reduced *Bifidobacteriaceae, Bacteroidaceae* and *Porphyromonadaceae* at 3wks ($p < 0.05$), increased Firmicutes ($p < 0.05$) and *Erysipelotrichaceae* ($p < 0.01$) and decreased *Bifidobacteriaceae* ($p < 0.05$) and *Lactobacillaceae* ($p < 0.01$) at 3mo and increased *Clostridiaceae* and decreased *Lactobacillaceae* at 5mo ($p < 0.05$) (Figure 3f, g). Maternal anxiety was associated with reduced Actinobacteria ($p < 0.01$) and *Nocardiaceae* and increased *Lactobacillaceae* at 1wk ($p < 0.05$), reduced *Nocardiaceae* and decreased *Clostridiaceae* 1 at 3mo ($p < 0.05$) and decreased *Clostridiaceae* 1 at 5mo ($p < 0.05$). *Veillonellaceae* was increased at 1wk in infants of mothers who had high depressive symptoms ($p < 0.05$). The diversity of the infant gut at 2wks was reduced in the group with high maternal cortisol (Shannon & Simpson Index; $p < 0.05$). High cortisol was also related to reduced Bacteroidetes ($p < 0.05$), *Coriobacteriaceae* ($p < 0.001$) and *Porphyromonadaceae* ($p < 0.05$) at 2wk, reduced *Enterococcaceae* at 3wks ($p < 0.05$), reduced *Coriobacteriaceae* at 3mo ($p < 0.05$) and reduced *Coriobacteriaceae* and *Veillonellaceae* at 5mo ($p < 0.05$) (Appendix D).

Third trimester distress:

Coriobacteriaceae was reduced in infant gut microbiota at 2wks by high third trimester stress ($p < 0.05$). At 3wk and 3mo, *Nocardiaceae* was reduced by high maternal stress ($p < 0.05$). At 1wk old *Clostridiaceae* was elevated in the high maternal anxiety group ($p < 0.05$) while at 2wks, *Nocardiaceae* was reduced by high anxiety ($p < 0.05$) and at 5mo of age, *Lachnospiraceae* was reduced ($p < 0.05$) by maternal anxiety. The diversity of the infant gut was reduced at 1wk and 2wks following high depressive symptomology in the third trimester ($p < 0.05$). At 1wk, *Clostridiacea*e 1 was increased ($p < 0.05$), at 2wks *Nocardiaceae* was reduced and at

5mo *Erysipelotrichaceae* was reduced ($p < 0.05$) by maternal depressive symptoms. High maternal cortisol in the third trimester was associated with reduced Coriobacteriaceae at 2wks and increased Actinobacteria and *Bifidobacteriaceae* at 3mo ($p < 0.05$) (Appendix D).

6.4.5 Infant cortisol

We finally examined if the infant gut microbiome development could be influenced by *in utero* stress exposure. In an exploratory analysis we used new born hair as a retrospective indicator of fetal exposure to cortisol in the last month of pregnancy. New born hair cortisol was available for 49 infants with a mean concentration of 2579.28 ± 1072.32 ng/ml. There was a positive correlation between infant hair cortisol levels and the diversity of the infant gut at 3wks ((β= .27, t (62) = 2.23, p =0.029) and 5mo (PD; β= .37, t (32) = 2.24, p =0.032 & Species; β= .36, t (32) = 2.17, p =0.037) (Figure 4). For compositional analysis, infants were grouped as having high or low hair cortisol levels if they scored above (high) or below (low) the mean. At 2wks, high cortisol was related to reduced abundance of *Collinsella* ($p < 0.05$). At 3mo old, there was a reduction in *Coriobacteriaceae* and *Enterococcaceae* ($p < 0.05$) with high cortisol, whilst *Collinsella* and *Lachnospiraceae uncultured* were reduced at 5mo ($p < 0.05$).

6.4.6 Potential Confounders

There was a positive correlation between maternal age and Chao1 Index at 1wk ($p < 0.05$). As none of our predictor variables correlated with Chao1 Index at 1wk, no further adjustments were performed including maternal age. Maternal BMI across pregnancy was negatively correlated with maternal cortisol in the third trimester ($p < 0.01$) and the diversity of the infant gut at 1wk ($p < 0.01$). BMI in the third trimester also positively correlated with the diversity of the vaginal microbiome. We subsequently adjusted our analysis of the vaginal microbiome and second trimester cortisol for maternal BMI, which did not influence this relationship (aβ=-.40, p =0.021), suggesting the effect of maternal cortisol on the vaginal microbiome is independent of BMI.

6.5: Figures and Figure Legends:

Figure 1:

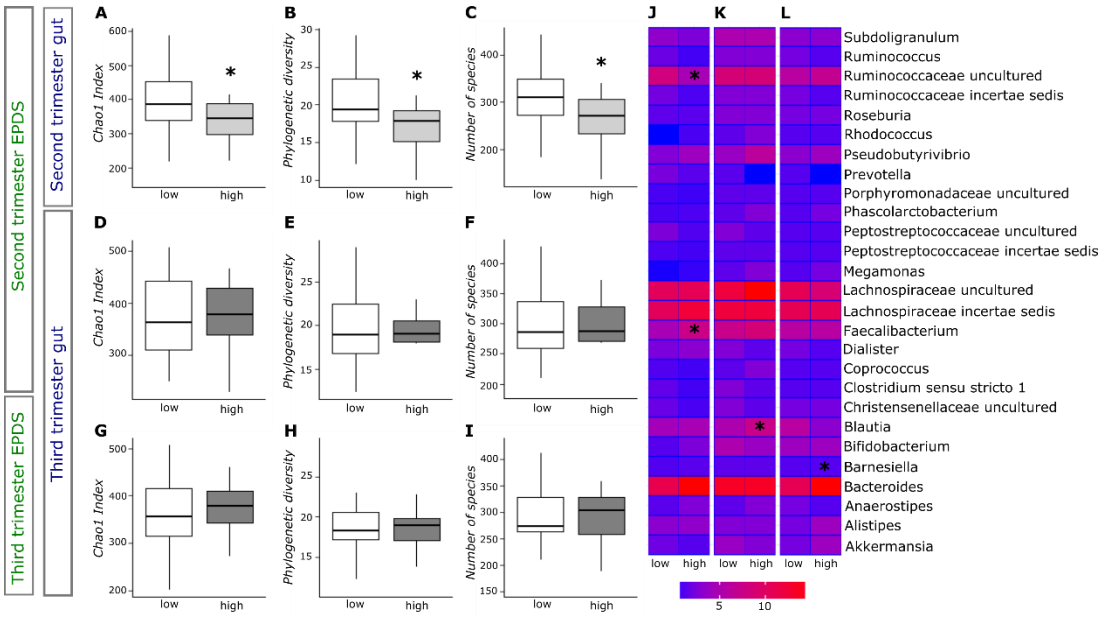

Figure 1: Maternal depressive symptoms in the second trimester alters the diversity and composition of the second trimester maternal gut: (A-I) Box-whisker plots showing the alpha diversity of the maternal gut by depressive symptoms. The top and bottom of each box are the 25th and 75th percentiles of the samples, respectively. The line in the middle of each box is the sample median. Whiskers extend to the upper and lower 95% confidence interval of the median. (J-K) Heat maps show the relative abundance of dominant genus in the maternal gut by maternal depressive symptoms. (A-C, J) maternal second trimester gut by second trimester depressive symptoms (EPDS < 8 low N=36, EPDS > 9 high N=10), (D-F, K) maternal third trimester gut by second trimester depressive symptoms (EPDS < 8 low N=23, EPDS > 9 high N=8), (G-I, L) maternal third trimester gut by third trimester depressive symptoms (EPDS < 8 low N=25, EPDS > 9 high N=8). (A-I) Unpaired Student t-test *p < 0.05, (J-L) Mann Whitney U-tests *p < 0.05. Abbreviations: Edinburgh Postnatal Depression Scale (EPDS).

Figure 2:

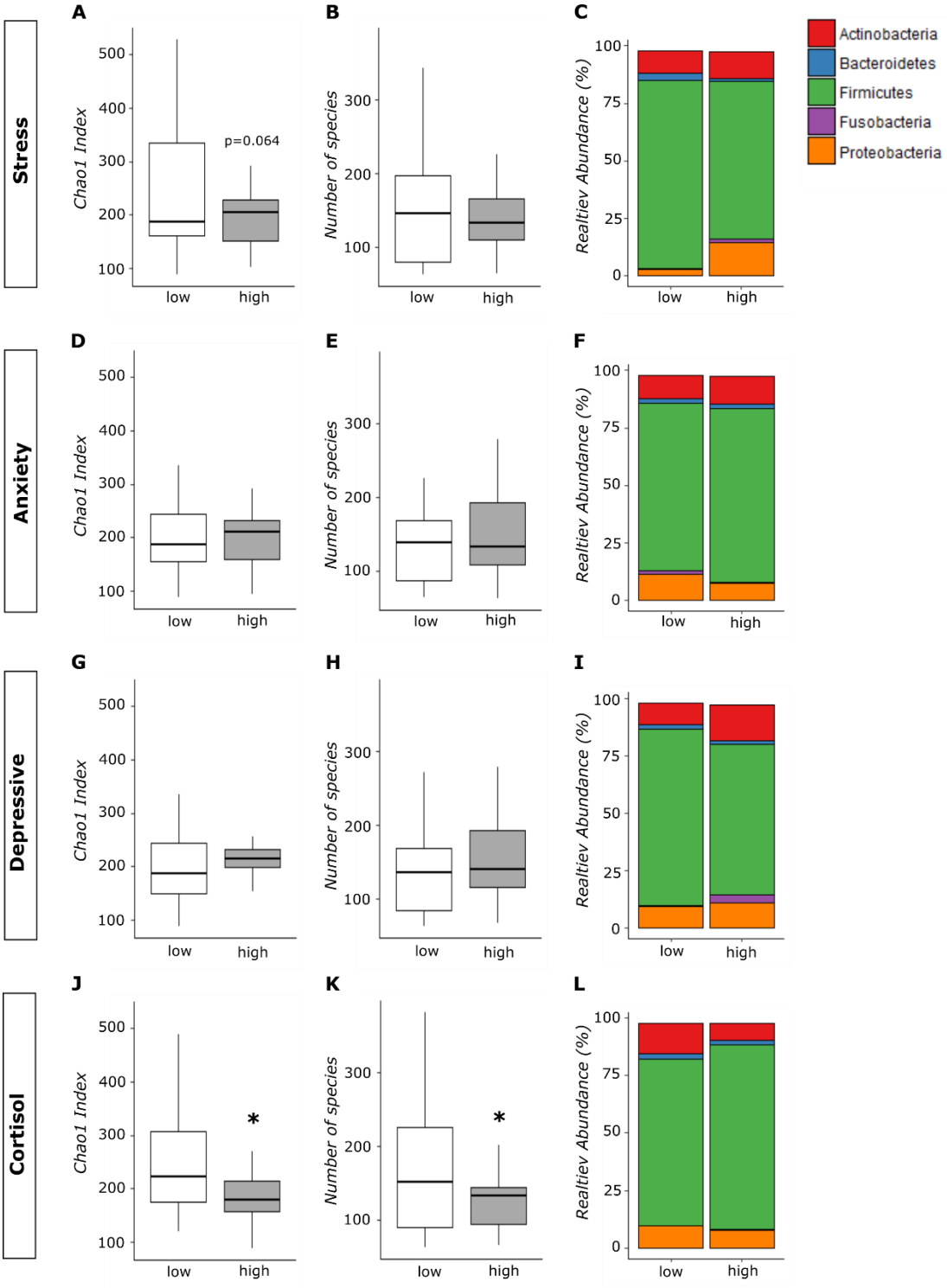
A
B
C
D
E
F
G
H
I
J
K
L
Stress
Anxiety
Depressive
Cortisol
Chao1 Index
Number of species
Realtiev Abundance (%)
p=0.064
*
low
high
Actinobacteria
Bacteroidetes
Firmicutes
Fusobacteria
Proteobacteria

Figure 2: Second trimester maternal cortisol reduces the diversity and richness of the vaginal microbiome. Box-whisker plots showing the diversity of the maternal vaginal microbiome by second trimester maternal stress. The top and bottom of each box are the 25th and 75th percentiles of the samples, respectively. The line in the middle of each box is the sample median. Whiskers extend to the upper and lower 95% confidence interval of the median. Bar graph demonstrating the relative abundance of dominant phyla (abundance greater than 1%) in the maternal vagina by second trimester distress. (A-C) Vaginal microbiome by second trimester stress (PSS < 13 low N=21, PSS > 14 high N=31), (D-F) vaginal microbiome by second trimester anxiety (STAI < 4 low N=31, STAI > 5 high N=21), (G-I) vaginal microbiome by second trimester depressive symptoms (EPDS < 8 low N=39, EPDS > 9 high N=13), (J-L) vaginal microbiome by second trimester cortisol (CAR < 545131 low N=29, CAR > 545132 high N=19). Unpaired Student's *t*-test; * $p < 0.05$.

Figure 3:

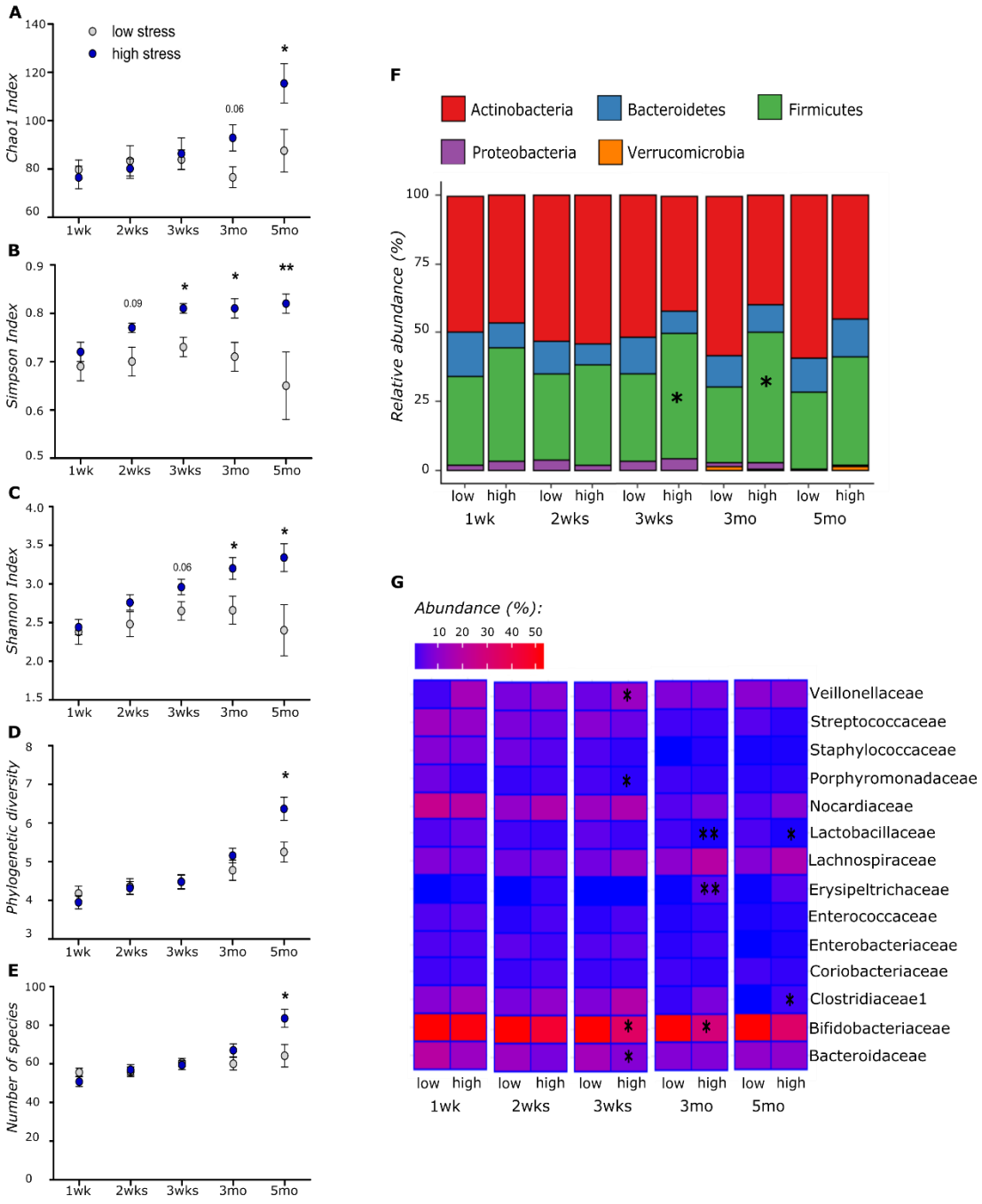
A
low stress
high stress
Chao1 Index
0.06
B
Simpson Index
0.09
C
Shannon Index
0.06
D
Phylogenetic diversity
E
Number of species
1wk
2wks
3wks
3mo
5mo
F
Actinobacteria
Bacteroidetes
Firmicutes
Proteobacteria
Verrucomicrobia
Relative abundance (%)
low high
G
Abundance (%):
10 20 30 40 50
Veillonellaceae
Streptococcaceae
Staphylococcaceae
Porphyromonadaceae
Nocardiaceae
Lactobacillaceae
Lachnospiraceae
Erysipeltrichaceae
Enterococcaceae
Enterobacteriaceae
Coriobacteriaceae
Clostridiaceae1
Bifidobacteriaceae
Bacteroidaceae

Figure 3: Infant gut colonization is altered by second trimester maternal stress: Diversity and composition of the infant gut at 1wk, 2wks, 3wks, 3mo and 5mo by the experience of maternal stress (PSS) in the second trimester. (A) Chao1 index, unpaired students *t*-test $p < 0.05$ *, (B) Simpson index, unpaired students *t*-test < 0.01 **, $p < 0.05$ * (C) Shannon index, unpaired students *t*-test $p < 0.05$ *, (D) Phylogenetic diversity, unpaired students *t*-test $p < 0.05$ *, (E) Number of species, unpaired students *t*-test $p < 0.05$ *, (F) Bar charts of dominant phyla in the infant gut by maternal stress in the second trimester. Each colour represent a different phyla. Mann Whitney U-tests $p < 0.05$ *. (G) Heat map showing the relative abundance of dominant families in the infant gut by second trimester maternal stress. Lowest abundance blue and highest abundance red. Mann Whitney U-tests; $p < 0.01$ **; $p < 0.05$ *.

Figure 4:

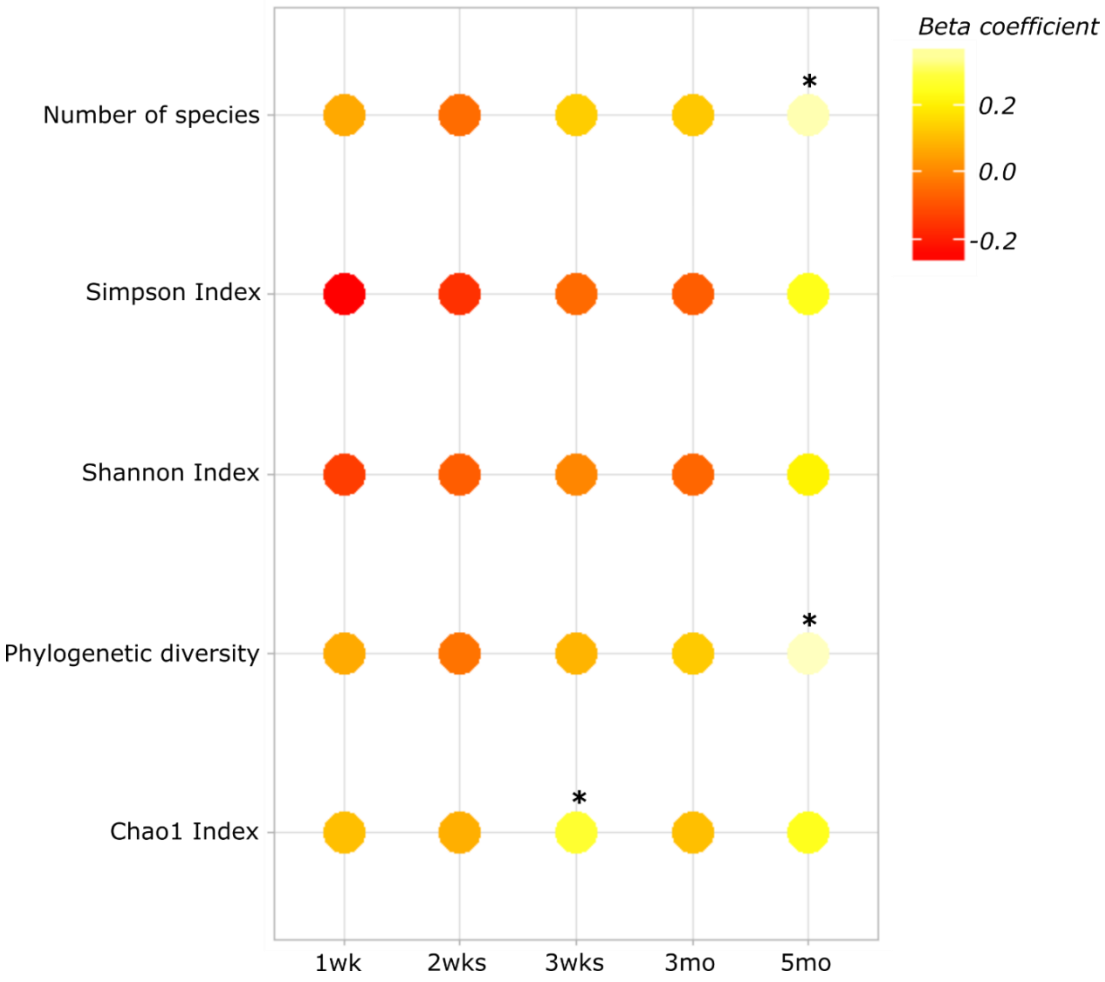

Figure 4: Diversity of the infant gut differs based on newborn hair cortisol levels: alters the Correlation bubble plot of newborn cortisol levels with alpha diversity of the infant gut. Bubble colour corresponds to standardized beta coefficient. Size of bubble is equal for all variables. Univariate linear regression analysis; * $p < 0.05$.

6.6: Discussion:

The establishment of the infant gut microbiome is a key developmental process that contributes to postnatal maturation of the immune, metabolic and nervous systems (Borre et al., 2014, Mueller et al., 2015a). This is evident by a number of studies showing disruption to microbiome development in early life precedes the onset of a number of disorders in childhood including diabetes, eczema, and asthma (Abrahamsson et al., 2014, Fujimura et al., 2016, Kostic et al., 2015, Wang et al., 2008). Understanding factors that might influence early bacterial colonization of an infant's gut is therefore important. Indeed delivery mode (caesarean versus vaginal), gestational age at delivery, pre-/post-natal antibiotic exposure and mode of feeding (formulae versus breast) have emerged as key regulators of infant microbiome development (Hill et al., 2017, Fallani et al., 2010, Mueller et al., 2015a, Yassour et al., 2016, Groer et al., 2014, Smith and Morin, 2005, Zijlmans et al., 2015a, Jasarevic et al., 2015b). More recently prenatal maternal stress is emerging an important contributor influencing the development of infant microbiome (Jasarevic et al., 2017, Jasarevic et al., 2015a), however this has only been examined once in a human cohort, with limited measures of prenatal maternal stress (Zijlmans et al., 2015b).

To explore the impact of prenatal stress on infant microbiome development we collected fecal samples form infants a 1wk, 2wks, 3wks, 3mo and 5mo old. As mode of delivery is considered an important contributor to microbiome development (Hill et al., 2017), we examined vaginal delivered infants only. We found a main effect for maternal stress in the second trimester, infants whose mothers had high PSS scores had increased diversity of the infant gut at 3wks, 3mo and 5mo. This is consistent with one other study in a human population where prenatal distress was also found to be associated with increased overall diversity of the infant gut across the first 115 days of life (Zijlmans et al., 2015a), although distress here was measured in very late pregnancy and we find this effect specific to second trimester stress. The consequence of increased bacterial diversity this early on in life is unclear in contrast to during adulthood where higher diversity is associated with health. Infants born by C-section typically have a microbial signature characterised by reduced diversity (Hill et al., 2017). Conversely, formula

feeding is associated with increased diversity, in comparison to exclusive breastfeeding (O'Sullivan et al., 2015, Gritz and Bhandari, 2015). Typically reduced alpha diversity in infancy has been suggestive of an adverse microbial environment as reduced diversity has been linked to the development of eczema, asthma and type-1 diabetes in childhood (Abrahamsson et al., 2014, Abrahamsson et al., 2012, Kostic et al., 2015). However, most recently increased alpha diversity measured at 1 year of age was found to correlate with poorer cognition and changes to grey matter volumes in infants by the age of 2 (Carlson et al., 2017), suggesting physiological systems may respond differently to the degree of diversity of the infant gut that is likely dependant on infant age. Indeed the increased diversity we observed here among the infants from high stressed pregnancies corresponds to a period of increased *Clostridiaceae* and decreased *Bifidobacteriaceae* and *Lactobacillaceae*, which could be suggestive of an adverse microbial ecosystem. *Bifidobacteria* are known to be master colonizers of the infant gut, which feed on human milk oligosaccharides in breastmilk, have anti-inflammatory properties and decrease intestinal permeability (Underwood et al., 2015). Reduced abundance of *Lactobacilli* and *Bifidobacteria* have been observed in infants with colic (de Weerth et al., 2013b, de Weerth et al., 2013a) and enrichment of *Bifidobacteria* is generally associated with healthier infants (Zheng et al., 2016). The importance of *Bifidobacteria* is highlighted by its use as a probiotic to treat disorders in neonates including colic and necrotizing enterocolitis (Di Gioia et al., 2014). The reductions of these beneficial bacteria appears to be a hallmark of prenatal stress as a number of preclinical and clinical studies have reported similar reductions in *Bifidobacteria* and *Lactobacilli* (Zijlmans et al., 2015a, Bailey et al., 2004). Interestingly, Zijlmans and colleagues show that a reduction in lactic acid bacteria, including *Lactobacilli,* was associated with the development of allergic and gastrointestinal symptoms at 3 months of age (Zijlmans et al., 2015a). Although we did not measure physiological parameters of disease in these infants, the stressed microbial signature we observed suggests these infants may be at an increased risk of developing certain disorders. Indeed a further follow-up of these infants to examine immune and nervous system function would be of interest.

It is largely accepted that the fetus develops in a sterile environment *in utero* and receive their first microbes at birth when the amniotic membranes rupture and as they pass through the birth canal. During this process the microbes of the maternal vagina are vertically transmitted to the infant (Milani et al., 2015, Asnicar et al., 2017b). In rodents it has been shown that a stress-induced microbial signature is vertically transmitted from mother to infant at birth (Jasarevic et al., 2015a). Although we were unable to directly correlate stress-induced vaginal microbe changes with that of the infant gut, our results do not strongly support the transmission of a stressed microbiome at birth. Indeed, we found that the vaginal microbiome was most notably influenced by maternal cortisol levels in pregnancy, whereas the infant gut was not. Similarly, second trimester perceived stress (and third trimester depressive symptoms) which we have linked to alterations in the infant gut microbiome, did not impact the vaginal microbiome. This suggests there may be other mechanisms by which maternal stress could influence the development of the infant gut microbiome.

Fetal overexposure to glucocorticoids during development may be one such mechanism. Examining fetal stress exposures *in utero* remains a methodological challenge. Analysis of newborn hair after birth is a novel approach to explore fetal exposures in the final months of gestation (Kapoor et al., 2014a). Here, we measured cortisol in hair samples from newborns acquired within 24h of birth. We found newborn cortisol levels to positively correlate with the diversity of the infant gut at 3wks and 5mo old and segregate infants into a number of compositional changes at varying ages. To our knowledge, this is the first attempt to examine the impact of *in utero* cortisol exposure of infant microbiome development. The alterations we observe suggest that fetal exposure to glucocorticoids during development may also play a role in establishment of the infant microbiome, suggesting another mechanism independent of vertical transmission, which may be important to microbial succession.

To date, most studies have examined the role of environmental factors that primarily alter infant gut colonization, with very little focus being placed on maternal bacterial communities. To characterize the impact of prenatal distress on the maternal gut microbiome throughout pregnancy, we collected fecal samples

from healthy nulliparous pregnant women in the second and/or third trimester of pregnancy. We observed a number of changes in the maternal gut of women with high perceived stress in the second trimester including reduced species richness and reduced abundance of the dominant family *Ruminococcaceae*, a family with an important role in energy metabolism (Gosalbes et al., 2011). The maternal gut was most profoundly influenced by depressive symptoms in the second trimester with reduced species richness and diversity of the second, but not third trimester maternal gut microbiome. This decrease in alpha diversity indices of the gut microbiome has recently been demonstrated in a cohort of clinically depressed patients (Kelly et al., 2016a). The consequence of this reduced microbial diversity in the maternal gut warrants further investigations. Among the general population reduced diversity has been link to metabolic type symptoms including insulin resistance, increased adiposity and increased inflammation (Le Chatelier et al., 2013). Further reduced alpha diversity of the gut in first trimester of pregnancy has been linked to high serum zonulin, a marker for intestinal permeability (Mokkala et al., 2016). Of interest increased intestinal permeability has been observed among pregnant women with intrahepatic cholestasis (Reyes et al., 2006). At the compositional level, maternal depressive symptoms were associated with reduced *Ruminococcaceae uncultured* and increased abundance of *Faecalibacterium* of the *Clostridiaceae* family. Whether the changes we observed here have implications for obstetric or neonatal outcomes remains to be determined.

The current study has a number of limitations. Firstly the cut-off scores we used to define high and low stress, anxiety and cortisol were based on group means which may or may-not be clinically meaningful. Further we grouped women as having high depressive symptomology with EPDS scores greater than 9. Typically, an EPDS score greater than 13 is used to detect depressive symptomology in pregnancy (Su et al., 2007) although generating more accurate and specific scoring of the EPDS is currently a topic of scientific enquiry (Thombs et al., 2015). None the less we observed significant alterations in the maternal microbiome even with this low EPDS cut-off. We did not measure physiological parameters in the infants therefore we do not yet know the consequences, if any, of these microbial changes for infant health. As the current study had a number of predictor variables we

acknowledge the limitation of using multiple testing in this cohort. However we highlight this research as exploratory and descriptive but an important starting point in examining the impact of prenatal distress on the maternal and infant microbiome.

Overall, this study supports a role for maternal stress as an important contributor to the development of the infant gut microbiome, although most likely independent from direct vertical transmission from the mother at birth. The concept that maternal mental health may influence infant microbial succession is relatively new and the consequences that these changes could have for infant health needs to be further investigated. Indeed alterations in early gut colonization may be another biological mechanism by which prenatal maternal stress programmes adverse infant development. Additionally, we observed substantial alterations in the maternal gut microbiome by second trimester stress, particularly depressive symptomology. Such alterations could have an impact on maternal prenatal health, and as such, the role that these changes may have on pregnancy and neonatal outcomes should be further examined. Our study may have implications for both the mother and the infant with second trimester prenatal distress in particular associated with marked alterations in both the maternal and infant microbiome. Lower prenatal distress is associated with benefits for both the maternal microbiome, in terms of increased diversity, and the infant microbiome in terms of increased relative abundance of beneficial bacteria such as *Bifidobacteria*. Further studies are required to tease apart the mechanisms underpinning these effects and to provide an evidence base for beneficial preventative stress-reduction or microbiota-based interventions during pregnancy that will improve both maternal and offspring health.

Chapter 7:

General Discussion

Summary of results:

The present thesis focused on exploring the biological factors that may underlie the relationship between maternal prenatal distress and adverse pregnancy and infant outcomes. The current literature positions fetal overexposure to glucocorticoids during critical periods of development as the primary link. More recently, alterations in the infant gut microbiome are emerging as another potential component in this complex relationship. Though this is a topic that is being extensively studied in preclinical environments, its translation into clinical cohorts has not been well examined. Understanding the mechanisms that underlie the link between maternal exposure to prenatal distress and the risk of adverse offspring outcomes is a major healthcare objective. Not only will this further our understanding of this relationship at a biological level, but it will enable the development and implementation of appropriate therapeutic strategies to manage or reduce the impact of maternal distress in pregnancy, with an ultimate objective to reduce adverse pregnancy and infant outcomes.

In the first phase of this thesis I undertook an *in vitro* experiment to explore the impact of potential biological stress mediators on the placental expression of HSD11B2, a critical regulator of fetal glucocorticoid exposure (Togher et al., 2014). This study found that high dose cortisol and the pro-inflammatory cytokine, IL-1β downregulated the expression of placental HSD11B2 following 24h of exposure. Previous work has shown HSD11B2 to be downregulated in the placenta of women who report high distress in pregnancy, particularly in the third trimester (O'Donnell et al., 2012, Seth et al., 2015). As cortisol and IL-1β are elevated in the plasma of stressed women (Coussons-Read et al., 2007, Davis and Sandman, 2010, Seth et al., 2015), our results suggest psychological stress-induced increases in these compounds may be the biological mechanism by which prenatal distress alters placental HSD11B2 expression. Epigenetic regulation is emerging as the key molecular mechanism through which prenatal distress alters HSD11B2 expression, with many studies focusing primarily on DNA methylation (Monk et al., 2016). Using an *in vitro* approach, my work confirms a role for DNA methylation in HSD11B2 regulation (Appendix B, Supplementary Fig. 2), but more specifically identifies HDACs as important directors of HSD11B2 expression. I found that pharmacological

inhibition of these HDACs prevented cortisol and IL-1β induced reductions in HSD11B2 expression, highlighting the potential of targeting epigenetic modifiers to prevent HSD11B2 alterations in pregnancy. Of particular interest for interventions in the prenatal period, the epigenome can be influenced and modified by dietary components as well as the gut microbiome (Pham and Lee, 2012, Stilling et al., 2014).

In my next study (Chapter 4), I moved to a clinical cohort to explore the influence of prenatal distress on pregnancy and birth outcomes and further examine the interaction between prenatal distress and placental gene expression. Although the role that maternal prenatal distress plays in contributing to an increased risk of adverse outcomes has been extensively studied (Ding et al., 2014a, Grote et al., 2010, Accortt et al., 2015), significant variation exists largely due to the extensive range of tools used to assess and define maternal distress, which incorporates environmental exposures, ranging from subjective reports of daily life stress using questionnaires to major life events such as exposure to bereavement, war and famine. In this study, I focused on three well-validated psychological screening tools that measure different aspects of distress; the Perceived Stress Scale (PSS; stress), the State Trait Anxiety Inventory (STAI; anxiety) and the Edinburgh Postnatal Depression Scale (EPDS; depressive symptoms). These well-validated questionnaires have previously been used to capture the maternal experience of distress in a similar cohort in Cork (Khashan et al., 2014, Larsen et al., 2013). A recent systematic review identified these questionnaires as the most accurate psychometric instruments to examine distress in pregnancy (Nast et al., 2013). Using these questionnaires (in the late third trimester), I identified that women with a history of psychiatric illness and/or miscarriage are at an increased risk of experiencing distress in their pregnancy. Identifying at risk individuals is important as it will allow increased surveillance of these women in the prenatal period. Moreover, I found that women who report high anxiety were up to five times more likely to deliver via elective caesarean section (CS) and two times less likely to breastfeed their infant. Formulae feeding is linked with an increased risk of illness in childhood (Martin et al., 2016b). The long-term effects of delivery by CS are the subject of intense scrutiny (Stevens et al., 2009, Curran et al., 2016, Curran

et al., 2015). I analysed the placentae of the women who scored high in all three distress questionnaires and those who scored low in all three distress questionnaires to create a cumulative distress group. I found, consistent with previous reports (Seth et al., 2015, O'Donnell et al., 2012), a reduction in placental HSD11B2 mRNA expression among the high distress group. Additionally, I observed an increase in the expression of the glucocorticoid receptor (NR3C1) in the placentae from the high distress women. The decrease in HSD11B2 with a corresponding increase in NR3C1 suggests that the fetus may have been exposed to increased levels of cortisol throughout their pregnancy, although an assessment of fetal cortisol would be required to confirm this assumption.

For the next part of this thesis I recruited a second cohort of pregnant women (SMArTI cohort) from the IMPROvED study (Navaratnam et al., 2013) at Cork University Maternity Hospital. This cohort yielded a final population of 111 healthy nulliparous pregnant women who completed the PSS, STAI, EPDS, Pregnancy Distress Questionnaire (PDQ), Pittsburgh Sleep Quality Index (PSQI), Childhood Trauma Questionnaire (CTQ), Food Frequency Questionnaire (FFQ) and questionnaires relating to gastrointestinal function in the second and/or third trimesters of pregnancy. Women provided corresponding saliva samples for cortisol analysis and fecal samples for 16S rRNA gene sequencing. At the time of delivery, vaginal swabs for 16S rRNA sequencing, newborn hair for cortisol analysis and placental samples were acquired. Infant fecal samples were subsequently collected over the first 5 months of life for 16S rRNA sequencing. Biological samples were collected in excess and bio-banked for future studies (subject to ethical approval). Final numbers of data and samples collected can be found in Appendix A.

From this cohort, I went on to further study the relationship between prenatal distress and placental genes involved in glucocorticoid signalling. This cohort allowed us to assess distress in both the second and third trimester of pregnancy, where I found an association between second trimester anxiety and placental gene expression that was dependant on fetal sex. Most notably, I found anxiety in the second trimester to negatively correlate with infant birthweight and birthweight centiles in female infants only. I also observed a positive correlation between second trimester maternal anxiety and the expression of FKBP51 in the

placentae of female infants. Of particular interest, the relationship between second trimester maternal anxiety and infant birthweight in females was found to be mediated by placental FKBP51. FKBP51 is a chaperone protein that regulates NR3C1 (Stechschulte and Sanchez, 2011). The precise role of FKBP51 in the placenta remains understudied; however, FKBP5 methylation levels have recently been linked to poorer neurodevelopment (Paquette et al., 2014). In a novel and exploratory technique, I used newborn hair to measure newborn cortisol levels as a potential indicator of fetal cortisol exposure *in utero* (Kapoor et al., 2016). I found second trimester maternal anxiety to negatively correlate with newborn cortisol levels, a relationship again found to be mediated by placental FKBP51 in female infants. Although newborn cortisol levels were not related to infant birthweight, it may be possible that cortisol may influence developmental processes that could influence disease risk later in life. This study highlights an important role for placental FKBP51 in the placental response to prenatal distress in female infants.

In the final stage of this thesis I explored the microbiome as an alternative biological mechanism potentially linking prenatal distress to adverse outcomes. I first examined if the maternal *gut* microbiome in the second and third trimester differed based on maternal distress (PSS, STAI, EPDS and cortisol). An association between second trimester depressive symptoms and the second trimester maternal gut microbiome was observed. Women who were ranked as having higher depressive symptoms in the second trimester had gut microbiomes characterised by reduced species richness and diversity. This microbial phenotype has previously been observed among a cohort of depressed patients (Kelly et al., 2016b). The consequence that this may have for pregnancy and neonatal outcomes warrants further investigation.

Preclinical studies suggest that stress induced alterations in the vaginal microbiome are transmitted to the infant at birth (Jasarevic et al., 2015b, Jasarevic et al., 2015a); therefore I examined the vaginal microbiome just prior to delivery. The vaginal microbiome remained largely unchanged by second and third trimester psychological distress (PSS, STAI and EPDS), however the diversity of the vaginal microbiome was reduced in women with a high second trimester cortisol awakening response. I subsequently analysed the infant gut microbiome of

vaginally delivered infants at 1wk, 2wks, 3wks, 3mo and 5mo old. I found that vaginally delivered infants born to women who reported high second trimester perceived stress had an increased alpha diversity of their gut microbiome at 3wk, 3mo and 5mo along with corresponding decreases in the beneficial *Bifidobacteriaceae* and *Lactobacillaceae* and increased *Clostridiaceae,* suggestive of an adverse microbial environment. As the maternal vaginal microbiome remained unchanged by second trimester stress and the emergence of the infant microbial phenotype was evident from 3wks and not at earlier ages, this work does not support vertical transmission of a stressed microbiome during birth. Conversely infants born to women with high depressive symptomology in the third trimester displayed reduced alpha diversity at 1wk and 2wks but not at later ages.

Placental HSD11B2

The most commonly proposed biological mechanism to be mediating the effects of prenatal distress on adverse pregnancy outcome is the glucocorticoid hypothesis. This hypothesis proposes that fetal overexpose to glucocorticoids alters fetal developmental trajectories that confers disease risk in later life (Reynolds, 2013). The glucocorticoid hypothesis is supported by many studies that have linked maternal cortisol levels in pregnancy to adverse outcomes in affected children (Rondo et al., 2010, Rondó et al., 2010, Bolten et al., 2011, Baibazarova et al., 2013, Buss et al., 2012a, Davis and Sandman, 2010). However, some studies do not support this relationship (Bergman et al., 2010a, Baibazarova et al., 2013) and the work presented in this thesis, consistent with other reports, demonstrates maternal psychological distress is not always accompanied by elevations in maternal cortisol (Baibazarova et al., 2013, Hompes et al., 2012, Davis and Sandman, 2010, Bergman et al., 2010b). Failure to find a correlation between psychological and physiological stress in pregnancy could be reflective of the difficulties in accurately assessing cortisol levels as described in the introduction of this thesis. In light of this, placental HSD11B2 is an integral part of the glucocorticoid hypothesis. In the placenta HSD11B2 converts cortisol into its inactive cortisone, thereby controlling the amount of cortisol the fetus is exposed to (Togher et al., 2014). Anything that

therefore reduces placental HSD11B2 has the potential to allow glucocorticoid overexposure. Of note, prenatal distress is often accompanied by elevations in maternal cytokines and noradrenaline, both of which have been shown to downregulate placental HSD11B2 (supported by the work presented in Chapter 3 of this thesis) (Kossintseva et al., 2006, Sarkar et al., 2001). Therefore, stress induced increase in maternal cortisol is not the only mechanism by which maternal stress may induce increased fetal glucocorticoid exposure and examination of placental HSD11B2 levels may be a more accurate barometer of the glucocorticoid hypothesis.

In chapter 4 of this thesis I found that maternal distress in late pregnancy was associated with reduced placental expression of HSD11B2. This is consistent with previous reports (Seth et al., 2015, O'Donnell et al., 2012). However, in our second cohort, described in chapter 5, maternal distress was not associated with the same decrease. Of note, the mean stress score in the SMArTI cohort was slightly lower than the cohort from chapter 4 and the effect of maternal stress on HSD11B2 expression has been shown to be dependent on the severity of the stress exposure, with acute stress upregulating its expression and chronic stress causing a downregulation (Welberg et al., 2005). Notably in the SMArTI cohort, I observed a trend towards a significant increase in HSD11B2 mRNA expression in placenta from male infants with high second trimester stress. This result is consistent with another study demonstrating socioeconomic adversity in pregnant women to be related to reduced methylation, and thus increased expression of placental HSD11B2 (Appleton et al., 2013) and when stratified by sex this effect was only seen in males (Appleton et al., 2013). This upregulation of HSD11B2 in the male placenta could result in the fetus not being exposed to sufficient amounts of cortisol at a critical window of organ development. Alternatively, this stress induced increase could be an adaptive mechanism whereby the placenta is attempting to protect the fetus from maternal cortisol. Indeed fetal exposure to elevated maternal cortisol in mid-pregnancy decreases infant physical and neuromuscular maturation in male offspring only (Ellman et al., 2008), suggesting males may be particularity vulnerable to fluctuations in maternal hormones at this time.

FKBP51: a novel player in fetal programming

Mutations in FKBP51 are commonly associated with stress reliance, depressive behaviour and anxiety type disorders (O'Leary et al., 2013) and in mice, pharmacological inhibition of FKBP51 can reduce anxiety like behaviours (Hartmann et al., 2015). The relationship I observe here between maternal anxiety and placental FKBP51 is striking and the increase in FKBP51 could be reflective of a genetic increase in the mother which then appears in the placenta, it would be of interest to examine FKBP51 in maternal circulation. FKBP51 interacts with steroid hormones receptors through chaperone heat shock protein 90 (HsP90), inhibiting the activation of the glucocorticoid receptor and the progesterone receptor (PR) and increasing the activation of androgen receptor (Stechschulte and Sanchez, 2011). The precise role of FKBP51 in the placenta and fetal development has not yet been examined; therefore it is difficult to frame the biology underlying the association between maternal anxiety, placental FKBP51 and birthweight. It would be plausible to speculate a role for fetal cortisol exposure, as I found placental FKBP51 to positively correlate with newborn cortisol levels; however I identified no association between newborn cortisol and birthweight. Alternatively, the inhibitory action of FKBP51 on the progesterone receptor may underlie this relationship. Progesterone supplementation is commonly administered to women at risk of PTB and women who receive progesterone are less likely to deliver a preterm or LBW infant (Dodd et al., 2005). As the relationship I observed between placental FKBP51 and birthweight was specific to females, it is of particular interest that increased maternal serum placental progesterone in the first trimester was found to be associated with increased birthweight in females, with no significant effect on males (Hartwig et al., 2013). Therefore, it may be possible that the inhibitory actions of FKBP51 on the PR may underlie the link between maternal anxiety and female birthweight. None the less our finding, together with the previously reported relationship between placental FKBP51 methylation and neurobehavioral problems in infants (Paquette et al., 2014), suggests placental FKBP51 as a novel player in fetal programming.

Prenatal depressive symptoms influence the maternal gut microbiome.

The prenatal maternal microbiome is emerging as an important contributor to maternal and infant health (Dunlop et al., 2015, Solt, 2015). In particular, alterations to the vaginal microbiome in pregnancy is now commonly linked to adverse pregnancy outcomes (Hyman et al., 2014, Jayaprakash et al., 2016, Kwak et al., 2014, Dunlop et al., 2015). Very little research has focused on the impact that the maternal gut microbiome may have for pregnancy health despite studies that have shown gut microbial communities to be important in physiological parameters in pregnancy (Koren et al., 2012). One study that examined the effect of the maternal gut and vaginal microbiome in relation to PTB found the maternal gut but not vaginal microbiome was predictive of PTB. In particular, women that went on to deliver preterm had a great abundance of *Clostridium, Lactobacillus* and *Bacteroides* (Shiozaki et al., 2014). The maternal gut microbiome has been shown to be influenced by maternal factors such as gestational diabetes, weight gain and BMI (Singh et al., 2017). This thesis identifies maternal depressive symptoms to be another potential contributor to maternal gut bacterial communities. This may have important consequences for pregnancy health. When the microbiome from depressed patients was transplanted into germ free animals, the depressed phenotype also appeared to be transplanted suggesting the microbiome is a driving force contributing to depressive symptoms (Kelly et al., 2016b). This is exciting as it highlights the potential of targeting the microbiome to improve depressive symptoms, which would be of particular importance in the pregnant population to move away from or avoid pharmacotherapies. The impact that the depressive-associated microbial changes I observed in this thesis have for obstetric and neonatal outcomes remains to be determined. The extensive database collected as part of the SMArTI cohort will allow us to examine such associations in the future.

Questioning vertical transmission?

According to the sterile womb hypothesis, a fetus develops in a sterile environment and is only exposed to its first microbes at birth through vertical transmission, when the baby ingests its mothers' vaginal microbes as it travels through the birth canal

(Mueller et al., 2015a, Asnicar et al., 2017a). Preclinical studies have shown that maternal prenatal stress alters the composition of the maternal microbiome, and this stressed microbiome is then vertically transmitted to the infant at birth (Jasarevic et al., 2015a, Jasarevic et al., 2017). The work presented in this thesis is the first time, to my knowledge; this has been examined in a clinical population and does not directly support this hypothesis. Here I find the vaginal microbiome, but not the infant microbiome, is altered by second trimester maternal cortisol. Conversely the infant microbiome, but not the vaginal microbiome is altered by second trimester maternal psychological stress. This suggests an alternative mechanism of action may play a role; potential mechanisms are proposed below.

Maternal prenatal distress can impair the development of the fetal HPA axis, which may result in permanent changes in HPA functioning in the postnatal period. This has been shown in both human and animal models (Emack et al., 2008, Kapoor and Matthews, 2005, Diego et al., 2004, Oberlander et al., 2008, Brennan et al., 2008, Grant et al., 2009, Yehuda et al., 2005). The microbiome can influence the HPA axis, likewise the HPA axis can influence the microbiome, and this is one of the mechanisms behind the bidirectional communication between gut microbes with the brain (Foster et al., 2017). Therefore alterations to the developing HPA by PNMD might impact the microbial communities in the infant gut postnatally. In this thesis, I measured cortisol in newborn hair which may be more reflective of cortisol exposure *in utero* as opposed to HPA axis functioning in the newborn (Kapoor et al., 2014b). It would have been beneficial to examine HPA axis functioning in these infants through blood or salivary cortisol measures at corresponding time points to fecal sample acquisition.

Although I did not examine maternal care in the postpartum, it may be possible that maternal behaviours in the postnatal period could contribute to the alterations I observed in the infant gut in relation to the second trimester maternal stress. Indeed the experience of stress in pregnancy is associated with an increased risk of depression in the postpartum period as well as poor mother-infant bonding (Robertson et al., 2004, Rossen et al., 2016) and maternal cortisol levels in the postpartum have been shown to influence maternal behaviours (Fleming et al., 1987, Fleming et al., 1997). Although the influence that maternal care may have on

infant microbiome development has not been examined in clinical populations, in rodents maternal separation has been shown to be associated with perturbations in the early gut microbiota (O'Mahony et al., 2009). Additionally in preterm infants, maternal skin-to-skin care has been shown to influence oral microbial communities in the neonate (Hendricks-Munoz et al., 2015) suggesting the potential of maternal behaviours in the early postnatal period to influence the infant microbiome.

Infant feeding practises is a major determinant of the composition of the gut microbiome (O'Sullivan et al., 2015, Guaraldi and Salvatori, 2012). *Bifidobacteria* and *Lactobacilli* are abundantly expressed in breast milk and are directly transmitted to the infant during feeding (Soto et al., 2014, Solis et al., 2010). Additionally human milk oligosaccharides found in breast milk provide a food source which allows *Bifidobacteria* to flourish (Wickramasinghe et al., 2015, Underwood et al., 2015). As a result, breastfed infants are dominant in *Bifidobacteria,* whereas bottle fed infants have higher amounts of *Bacteroides* and *Clostridium* (Fallani et al., 2010, Penders et al., 2006). Additionally, breastfed infants typically have reduced alpha diversity compared to infants that are formulae fed (O'Sullivan et al., 2015, Backhed et al., 2015). In this thesis, I show infants born from high stressed pregnancies have increased alpha diversity with reduced abundance of *Bifidobacteria* and *Lactobacilli,* similar to the profile of bottle fed infants. Indeed in our cohort infants born from women with high second trimester stress have a higher percentage of bottle-feeding than breastfeeding (albeit not statistically significant) (Appendix D). Therefore, an increased preference of formulae feeding in the high stressed pregnancies may contribute to the alterations I observe in the infant microbiome. Although this cohort was not designed to and does not have the power to analyse such an association, it would be of interest to examine any potential mediating effect of feeding mode on the relationship between maternal stress and the infant microbiome in future studies.

Another mechanism by which prenatal stress might impact infant microbiome development could be through altering the composition of breast milk. Although to our knowledge the composition of the milk microbiome in stressed women has not been examined, the milk microbiome is susceptible to modification by maternal factors as both maternal weight in pregnancy and mode of delivery has

been shown to influence the microbial composition of breastmilk (Cabrera-Rubio et al., 2012, Gomez-Gallego et al., 2016). As well as having its own microbiome, human breastmilk contains many bioactive substances including immune cells/molecules and steroids which are transferred to the infant by feeding (Gomez-Gallego et al., 2016). Of particular interest, cortisol has been detected in breastmilk and levels of cortisol in breastmilk have been shown to be related to temperament in infants (Grey et al., 2013, Sullivan et al., 2011). Additionally, maternal distress in the postpartum was found to correlate with immune markers in breastmilk (Kawano and Emori, 2015). Collectively, this data suggests the potential for a stressed microbiome to be vertically transmitted from mother to infant through breastmilk as oppose to, or in addition to, vaginally during parturition.

In rodents, prenatal and early life stress have been associated with impaired intestinal barrier function (Soderholm et al., 2002, Gareau et al., 2006, Golubeva et al., 2015) and the microbiome plays a role in modulating the integrity and structure of the gastrointestinal tract (Jandhyala et al., 2015). It may therefore be possible that maternal perturbations in this period could influence the development of the gastrointestinal tract that may have consequences for microbial assembly in the postnatal period. Of interest, I found an association between second, but not third, trimester stress and the infant gut microbiome and the second trimester is a period whereby the fetal gastrointestinal system undergoes substantial growth (Marnerides et al., 2012, Zalel et al., 2003).

Second trimester window of vulnerability:

This work presented from the SMArTI cohort in chapters 5 and 6 identifies the second trimester as a critical developmental window whereby the fetus may be most vulnerable to the effects of maternal experience of distress. Indeed, the importance of second trimester adversities is well documented in the literature. Maternal use of cocaine primarily in the second trimester is associated with poorer infant development (Richardson et al., 2008). Second trimester maternal distress using the same psychological assessments I used increased the risk of SGA (Khashan et al., 2014). Similarly, Class and colleagues identified mid-gestation exposure to

severe life events, particularly in months 5 and 6 of pregnancy, heightened the risk for PTB, LBW and SGA (Class et al., 2011). The vulnerability of the second trimester is further evident by a number of studies identify second trimester stress specifically to predict poorer neurodevelopment in infants (King and Laplante, 2005, Glynn et al., 2001, Buss et al., 2010, Buss et al., 2011). The second trimester is a period of rapid fetal growth, particularly for the fetal brain (Buss et al., 2012b). Further, the fetal HPA response becomes active from 20 weeks of pregnancy (Gitau et al., 2001), therefore maternal stress arising in this period may have a more detrimental impact on fetal HPA axis functioning. Additionally, it has been suggested that by the third trimester of pregnancy the maternal HPA no longer responds to stressors, due to the natural surge of maternal cortisol and it has been postulated that as pregnancy advances women become more resistant to the effect of psychological stress (Kammerer et al., 2002, Glynn et al., 2008). In line with this the natural increases in placental HSD11B2 with advancing pregnancy may further protect the fetus from the effects of maternal stress in the third trimester (Schoof et al., 2001).

Therapeutic Implications:

This thesis adds further support to the adverse role that prenatal distress can have for pregnancy and infant outcomes. Whilst there are no standardized estimates for the prevalence of distress in pregnancy, there is sufficient evidence to conclude a significant proportion of women will experience some form of distress throughout their pregnancy (Khashan et al., 2014). Therefore, it is important to understand more about the biology of prenatal distress to optimize intervention strategies, in the hope of improving psychological symptoms among pregnant women and reducing the risk of adverse outcomes for both mother and baby.

Placental HSD11B2 is the first line of defence protecting the developing fetus from excessive glucocorticoid exposure. I, along with others (Seth et al., 2015, Appleton et al., 2013) have shown this enzyme to be downregulated by third trimester maternal distress which could suggest fetal overexposure to maternal cortisol. Therefore, preventing downregulation of HSD11B2 by maternal distress

would be of interest for the fetus. This thesis has identified HDACs as important regulators of HSD11B2, and found HDAC inhibition could prevent decreases in HSD11B2 under condition of stress and inflammation, therefor targeting HDACs to increase HSD11B2 expression may be of therapeutic benefit. Excitingly, HDACs can be modified by diet which represents an optimal intervention strategy for pregnant women (Togher et al., 2014).

In chapter 4 I identified that maternal anxiety in late pregnancy was associated with increased risk of delivery by elective CS. CS without medical indication is associated with an increased risk of adverse outcomes for both the mother and infant (Souza et al., 2010, Steer and Modi, 2009, Finn et al., 2016). The prevalence of caesarean delivery is on the rise (Betrán et al., 2016). The WHO estimates indicate that in the year 2008, 3.18 million CS were necessary whilst 6.20 were performed with a total excess cost of 2.32 billion (Gibbons et al., 2010) therefore elective CS delivery not only represents a significant health problem for mom and child but also a global economic burden. Based on our results reducing anxiety among pregnant women may be one potential strategy to reduce the rates of CS deliveries.

In chapter 6, I found that the maternal gut microbiome was altered by maternal depressive symptoms. The consequence of this alteration for pregnancy outcomes needs to be determined; however it highlights the potential of targeting the gut microbiome to potentially reduce depressive symptoms in pregnancy. Indeed probiotics have been suggested to be safe for use in pregnancy (Elias et al., 2011) and a recent meta-analysis among the general population concluded probiotics to be effective at alleviating depressive symptoms (Wallace and Milev, 2017). Therefore probiotics and/or prebiotics have the potential to be an exciting new therapeutic avenue used to managing mental health in pregnancy. Exploring new means to manage depression in pregnancy is particularly important given the potential adverse effects antidepressants may have for infant development (Oberlander et al., 2006).

Strengths and limitations:

The main strength of this work is the prospective longitudinal cohort study design employed in the SMArTI cohort with high quality phenotyping and accompanying bio-samples. Recently the first human study to examine the impact on PNMD on infant microbiome development was published (Zijlmans et al., 2015a). This first limitation to this study was the measurement of PNMD in the third trimester only and using questionnaires very specific to anxiety as oppose to stress and depression. I have overcome this in the SMArTI cohort by prospectively examining multiple aspect of PNMD in both the second and third trimester of pregnancy. The cohort would have been further strengthened if I were also able to capture these parameters in the first trimester, however this was not a possibility for my work having recruited from the IMPROvED cohort where compulsory visits only began in the early second trimester (Navaratnam et al., 2013).The second, and largest restriction to the study published by Zijlmans and colleagues was not assessing the maternal microbiome and focusing primarily on the microbiome of infants (Zijlmans et al., 2015a). This means the SMArTI cohort, that I have collected here, is the very first time the microbiome has been looked at in pregnancy in relation to maternal mental health. This study will therefore have important implications for novel approaches to how we manage mental health in pregnancy. The final advantage of the SMArTI study, over that of the study conducted by Zijlmans, is the longer assessment of the infant microbiome. The final infant sample time point I collected was at 5 months of age, as compared to 3 months in the Zijlmans study (Zijlmans et al., 2015a).

The next advantage of the SMArTI cohort is the clinical phenotyping of PNMD. In addition to the in-depth analysis of trimester specific effects, I employed both *psychological* and *physiological* measurements of maternal distress. I measured maternal HPA activity by collecting saliva samples from the participants over 4 time-points across the morning, allowing me to assess the cortisol awakening response (CAR). Measuring the CAR overcomes many of the methodological challenges and downfalls of other studies utilizing single time point measures (Stewart et al., 2015) and/or measuring cortisol levels in serum samples (Aardal-Eriksson et al., 1998, LeWinn et al., 2009, Baibazarova et al., 2013).

Additionally the psychometric instruments I used to assess psychological distress (PSS, STAI and EPDS) have been shown to be the most reliable questionnaire based approach to examine PNMD in pregnancy (Nast et al., 2013).

The quality of the biobank I produced from the SMArTI study will allow for many more investigations that will further our knowledge of the biological underpinnings linking PNMD to adverse outcomes. Every biological sample acquired for SMArTI was collected with very strict Standard Operating Procedures (SOPs) that I created before commencing recruitment for this study. These SOPs allowed every sample to be collected and bio-banked in the exact same manner, thereby limiting any collection error. Most importantly, for infant fecal samples I arranged collection from the participants' home within the first few hours of the sample being acquired. The infant microbiome is less stable than that of children and adults and therefore storing fecal samples for longer periods disrupts the microbiome composition (Guo et al., 2016). The collection method I used therefore allowed for accurate analysis of the infant gut microbiome. Additionally every placental sample collected in this thesis was done so within two hours of delivery, a tiresome task that ensured accuracy of our gene expression analysis (Wolfe et al., 2014). Additionally to determine microbial composition, I used the 16S gene rRNA sequencing approach as opposed to shotgun metagenomics. Both technologies are much superior to culture based techniques employed in the past. Whilst the 16S approach is subject to greater bias, it allows for a deeper analysis than that of shotgun metagenomics with a greater ability to detect less abundant and rare bacterial species (Shah et al., 2011).

By limiting the inclusion of SMArTI participants to women enrolled in the IMPROvED study (Navaratnam et al., 2013), SMArTI was able to produce a homogenous cohort of healthy nulliparous pregnant women which reduces the influence of confounding. Additionally the array of maternal data collected, which included measurements of early childhood trauma, sleep quality, pregnancy specific anxiety, food intake and gastrointestinal function will allow us to further examine how the maternal environment can alter the microbiome in pregnancy and any potential contributing effects to the depressive microbial phenotype I presented in

this thesis. Lastly, this cohort provides an excellent database to continue to unravel the complex biological underpinnings of PNMD in a clinical population.

The present thesis also has a number of limitations. The primary constraint to the cohort I recruited in chapter 4 was analysis of distress in the third trimester only with no physiological measurements of distress. However, I overcame this limitation in the SMArTI cohort. In both cohorts, I did not measure maternal behaviour in the postpartum period. Psychological disturbances in the prenatal period is a significant predictor of developing postpartum depression (O'Hara and Swain, 1996). It is possible that alterations in maternal behaviours in the postpartum period may have an impact on infant microbiome composition and infant development. Indeed, in preclinical models prenatal stress has been shown to influence infant development by altering postpartum maternal care (Champagne and Meaney, 2006). As this was an exploratory and descriptive cohort, I kept the experience of stress, anxiety, depression and maternal cortisol as independent predictor variables. As a result, a limitation to the present work is the use of multiple testing. However, analysing the data in this way allowed us to examine specifically which psychological experience was most detrimental to outcomes and whilst stress, anxiety and depression are often comorbid with some overlaps in their pathophysiology their clinical presentation and thus management often differ (Hirschfeld, 2001, Itoi and Sugimoto, 2010). As discussed in the introduction of this thesis there is currently a large amount of variability in relation to scoring on questionnaires to define PNMD. In particular, I used a cut-off of greater than or equal to 9 to indicate high depressive symptomology in pregnancy and I acknowledge that this is a relatively low score to imply clinically relevant depressive symptoms. However, it is important to note that even at this subclinical level of depressive symptoms I observed significant alterations in the maternal gut microbiota. The current work did not identify any strong association between maternal psychological distresses with maternal salivary cortisol; however this is not uncommon in the prenatal population (Davis and Sandman, 2010). Another limitation to this work was the complexity and demanding nature of this study resulted in a relatively high percentage of loss-to-follow-up. Finally, I were unable to measure physiological parameters in these infants, therefore the consequence, if

any, that the stressed induced microbial changes may have for infant physiology has yet to be determined.

Future perspectives:

The work presented in the current thesis has identified several possibilities for future research. Firstly we have shown that HSD11B2 is epigenetically regulated by histone acetylation in a relatively broad manner. As HDACs are critical for normal fetal and placental development, understanding specific individual HDACs that can regulate HSD11B2 would be of interest when devising therapeutic strategies. A targeted knock down approach using short interfering RNAs of individuals HDACs *in vitro* would allow the identification of specific HDACs involved in HSD11B2 regulation. Additionally the ability of HDACs in regulating NR3C1 and particularly FKBP51 should be examined using a similar approach. Once individual HDAC have been identified, examining there expression in our placental samples and correlating the expression with HSD11B2, NR3C1 and FKBP51 should be performed.

In chapter 6 of this thesis I report that the second trimester maternal gut microbiome undergoes is altered by maternal depressive symptoms. It will be important to determine the functional consequence that these changes may have for pregnancy health and subsequently outcomes. As part of the SMArTI cohort I have acquired a detailed account of medical data from each participant's pregnancy as well as detailed information pertaining to pregnancy and neonatal outcomes, including onset of labour, mode of delivery, gestational age at delivery, birthweight, gestational size and Apgar scores. A thorough analysis of the influence of the maternal gut microbiome on these outcomes should be examined. Subsequently if any maternal bacteria may influence or mediate the relationship between PNMD and birth outcomes warrants investigation.

I have recruited a comprehensive cohort of mother infant-dyads where I have demonstrated that second trimester maternal stress has significant impacts on the establishment of the infant gut microbiome. It is critical that the infants in this cohort be further studied to examine developmental outcomes particularity in relation to immune system dysfunction and neurodevelopment. The recent report

demonstrating increased diversity of the infant gut to be related to poorer cognition highlights the need to do neurodevelopmental assessments on these infants (Carlson et al., 2017). The microbial profile of infants born from high stress pregnancies also displayed increased diversity, highlighting the importance for neurodevelopmental assessment, in particular, of these infants. The first infant in the SMArTI cohort turned two years old in January 2017, and the neurodevelopmental alteration observed by Carlson and colleagues was evident from two years old, therefore following up these infants in the coming year will be of utmost importance.

Production of metabolites by the microbes is one of the primary mechanisms by which the microbiome interacts with host physiology (Al-Asmakh et al., 2012). Of particular interest are short chain fatty acids butyrate, propionate and acetate which due to their ability to readily cross the blood brain barrier could have consequences for infant neurodevelopment (Al-Asmakh et al., 2012). Therefore, examining the corresponding metabolomics profiles accompanying the microbiome alterations I observe would help elucidate potential microbial consequences. I have extracted fecal water from maternal and infant fecal samples that will allow me to examine the impact of the microbial changes on the metabolome in the future.

I have observed significant alterations in both the maternal and infant gut by PNMD, albeit by different aspects of distress (maternal gut influenced by depressive symptoms and the infant gut by stress). Potential strategies to reverse these microbial changes should be examined. Firstly, can the depressed microbial signature in pregnant women be reversed by probiotics, prebiotics and/or diet? And if so can this improve the experience of depressive symptoms in pregnant women? Probiotics have been shown to reduce stress in the non-pregnant population (Allen et al., 2016) and a randomized controlled trial found a Mediterranean diet intervention to be effective at reducing depressive symptoms in a cohort of patients with major depression (Jacka et al., 2017), showing the potential of microbiome targeted interventions to improve psychological wellbeing. Based on the work presented in this thesis such interventions should start to be considered for the prenatal population in an effort to move away from pharmacotherapies in pregnancy.

Finally, it appears that changes in the infant gut microbiome as a result of PNMD are independent of the maternal gut microbiome. The functional consequences of this alteration in the infant gut microbiome need further investigation but if they are shown to be linked with adverse neonatal development or other health consequences, future work should focus on microbial interventions applied directly to infants.

Conclusion:

Maternal prenatal stress leaves an imprint across pregnancy, the signs of which are often visible in the developing infant. This thesis suggests these effects appear to be mediated via a number of different mechanisms and confirms that prenatal stress produces a number of effects over and above overexposure to maternal cortisol alone. Regulation of glucocorticoids in the placenta and subsequent fetal exposure is an incredibly complex system. I have attempted to unravel this process by examining three key placental genes that have been linked to poorer infant outcomes. Our results highlight the intricacy of placental glucocorticoid signalling, with differential responses based on timing of maternal distress, type of distress, severity of distress and infant sex. Most notably this thesis identifies an important role for placental FKBP51 in mediating the effect of maternal anxiety on infant birthweight in female infants. This highlights the need for future studies to examine the precise role of FKBP51 in placental and infant development. This is the first study to show the maternal gut microbiome is altered by maternal mental health. Furthermore, I found evidence to support a role for maternal prenatal distress in altering the development of the infant gut microbiome. Examining the consequences of these changes for maternal and infant health is warranted to devise future therapeutic strategies targeting placental gene expression and microbial changes in the gastrointestinal tract to counteract the adverse impacts of prenatal stress.

Figure 1:

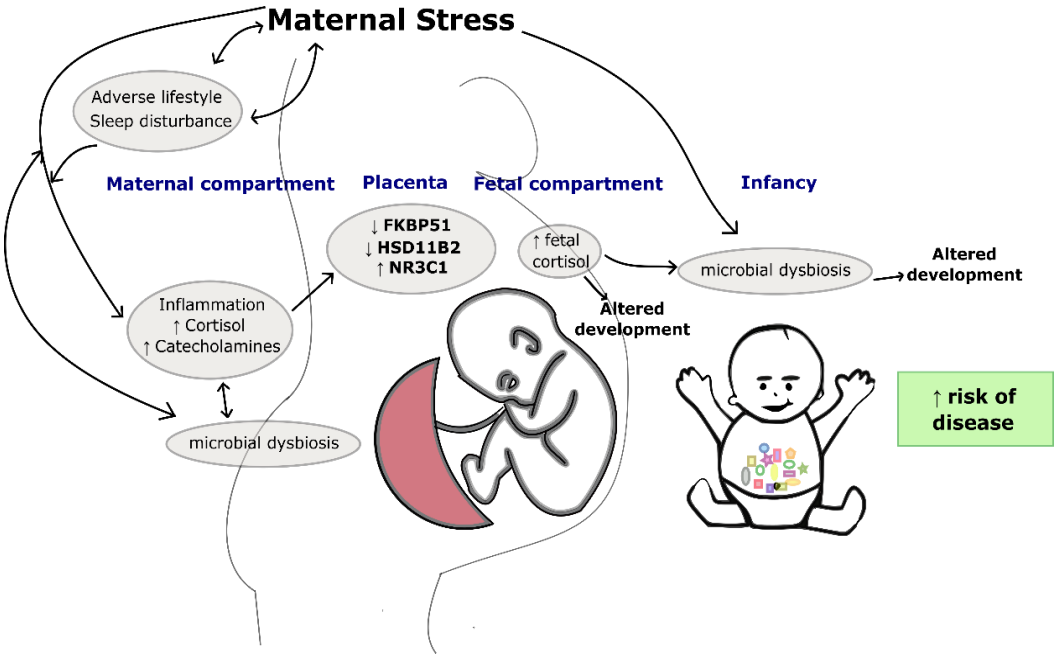

Figure 1: Summary figure of the potential biological underpinnings linking prenatal maternal distress to adverse outcomes, a complex relationship that involves a multitude of biological mediators. One potential mechanism includes stress-induced elevations in maternal cortisol, pro-inflammatory cytokines, catecholamine among other mediators which alter placental glucocorticoid signalling ultimately overexposing the fetus to cortisol. Cortisol overexposure alters fetal developmental processes during critical widows of fetal development that confers an increased risk to disease later in life. An alternative or contributory mechanism involves stressed induced alterations in the infant gut microbiome. These microbial alterations interfere with postnatal infant development, also conferring an increased risk of disease in later life.

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Abbreviations:

PNMD	Prenatal Maternal Distress
HSD11B2	11 beta hydroxysteroid dehydrogenase type 2 (gene)
HSD11B2	11 beta hydroxysteroid dehydrogenase type 2 (protein)
IL-	Interleukin-
PSS	Perceived Stress Scale
STAI	State Trait Anxiety Inventory
EPDS	Edinburgh Postnatal Depression Scale
NR3C1/GR	Glucocorticoid Receptor
SMArTI	Stressed Microbial Transfer to the Infant
FKBP51	FK506-bind protein 51
LBW	Low Birthweight
PTB	Preterm Birth
IUGR	Intrauterine Growth Restriction
HPA	Hypothalamic Pituitary Adrenal
WHO	World Health Organization
SGA	Small for Gestational Age
GL	Gestational Length
OR	Odds Ratio
RR	Risk Ratio
ASD	Autism Spectrum Disorders
ADHD	Attention deficit Hyperactivity Disorder
SAM	Sympatho-Adrenomedullary
CRH	Corticotrophin-releasing hormone
ACTH	Adrenocorticotrophin hormone
MDI	Mental Developmental Index
PDI	Psychomotor developmental Index
TNF-α	Tumor necrosis factor-alpha

E	Embryonic day
HAT	Histone Acetyl Transferase
HDAC	Histone Deacetylases
DNMT	DNA Methyltransferase
NNNS	NICU Network Neurobehavioral Scales
5-AZA	5-Aza-2'-Deoxycytidine
IP	Intraperitoneal
rRNA	Ribosomal RNA
OTU	Operational Taxonomic Units
GF	Germ Free
BMI	Body Mass Index
GDM	Gestational Diabetes Mellitus
CSTs	Community State Types
HMOs	Human Milk Oligosaccharides
VD	Vaginal Delivery
CS	Caesarean Section
BF	Breast-fed
FF	Formulae-fed
IAP	Intra-partum antibiotic prophylaxis
PD	Postnatal day
T1D	Type 1 diabetes
SIgA	Secretory IgA
GF	Germ Free
CUMH	Cork University Maternity Hospital
DMEM	Dulbecco's modified Eagle Medium Nutrient Mixture
FCS	Fetal Calf Serum
DMSO	Dimethyl Sulfoxide
HBSS	Hanks Balanced Salt Solution

MTT	Thiazolyl Blue Tetrazolium Bromide
PFA	Paraformaldehyde
PBS	Phosphate Buffer Solution
PBS-T	PBS Triton X
NICU	Neonatal Intensive Care Unit
PDQ	Pregnancy Distress Questionnaire
PSQI	Pittsburgh Sleep Quality Index
CTQ	Childhood Trauma Questionnaire
FFQ	Food Frequency Questionnaire
FKQ	Food Knowledge Questionnaire
Cort	Cortisol
HDI	HDAC Inhibitors

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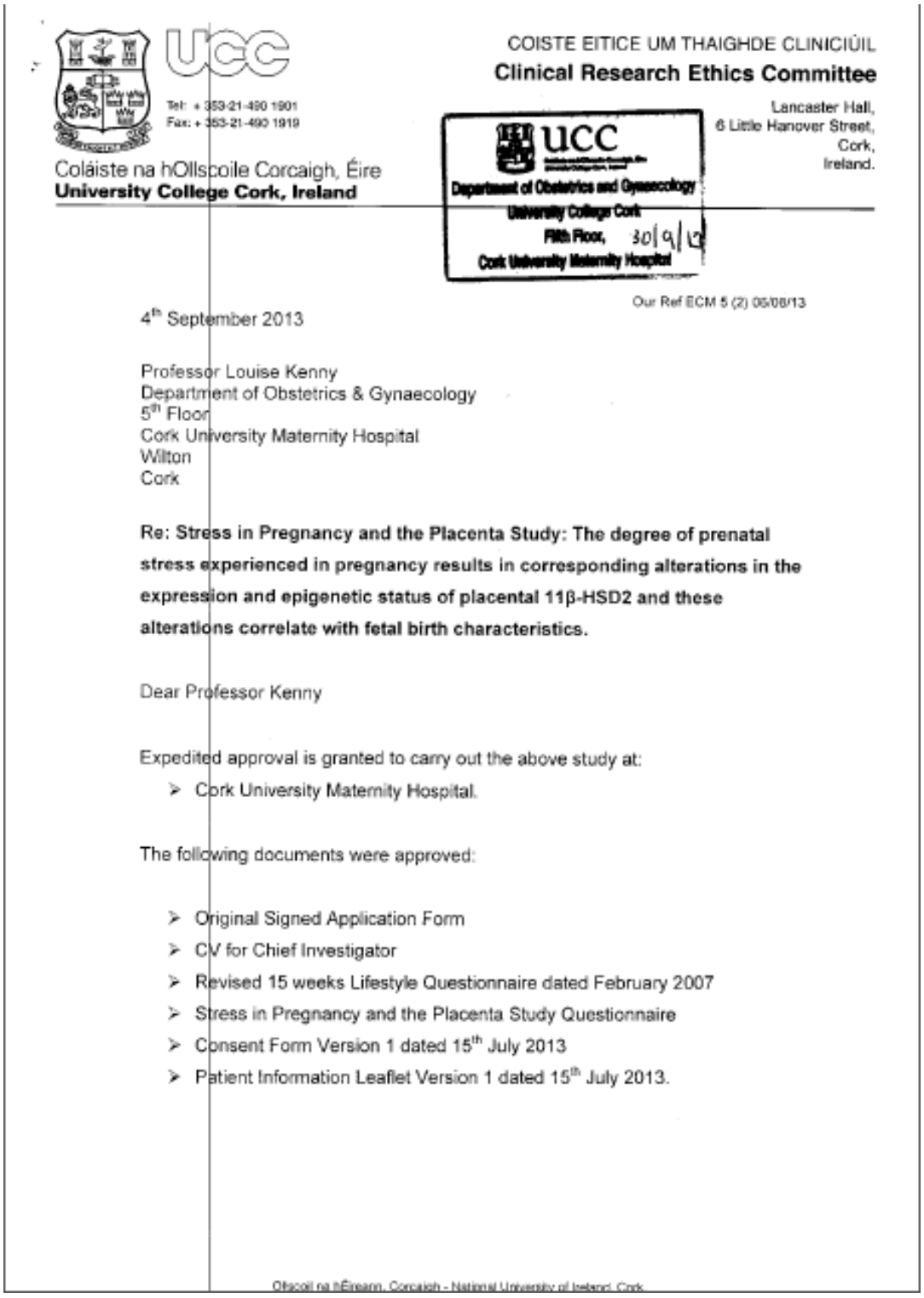
Appendix A:

Supplementary Information for Chapter 2:

Ethical approval letter:



UCC

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Coláiste na hOllscoile Corcaigh, Éire
University College Cork, Ireland

COISTE EITICE UM THAIGHDE CLINICIÚIL
Clinical Research Ethics Committee

Lancaster Hall,
6 Little Hanover Street,
Cork,
Ireland.

UCC
Department of Obstetrics and Gynaecology
University College Cork
Fifth Floor, 30/9/13
Cork University Maternity Hospital

Our Ref ECM 5 (2) 06/08/13

4th September 2013

Professor Louise Kenny
Department of Obstetrics & Gynaecology
5th Floor
Cork University Maternity Hospital
Wilton
Cork

Re: Stress in Pregnancy and the Placenta Study: The degree of prenatal stress experienced in pregnancy results in corresponding alterations in the expression and epigenetic status of placental 11β-HSD2 and these alterations correlate with fetal birth characteristics.

Dear Professor Kenny

Expedited approval is granted to carry out the above study at:

- Cork University Maternity Hospital.

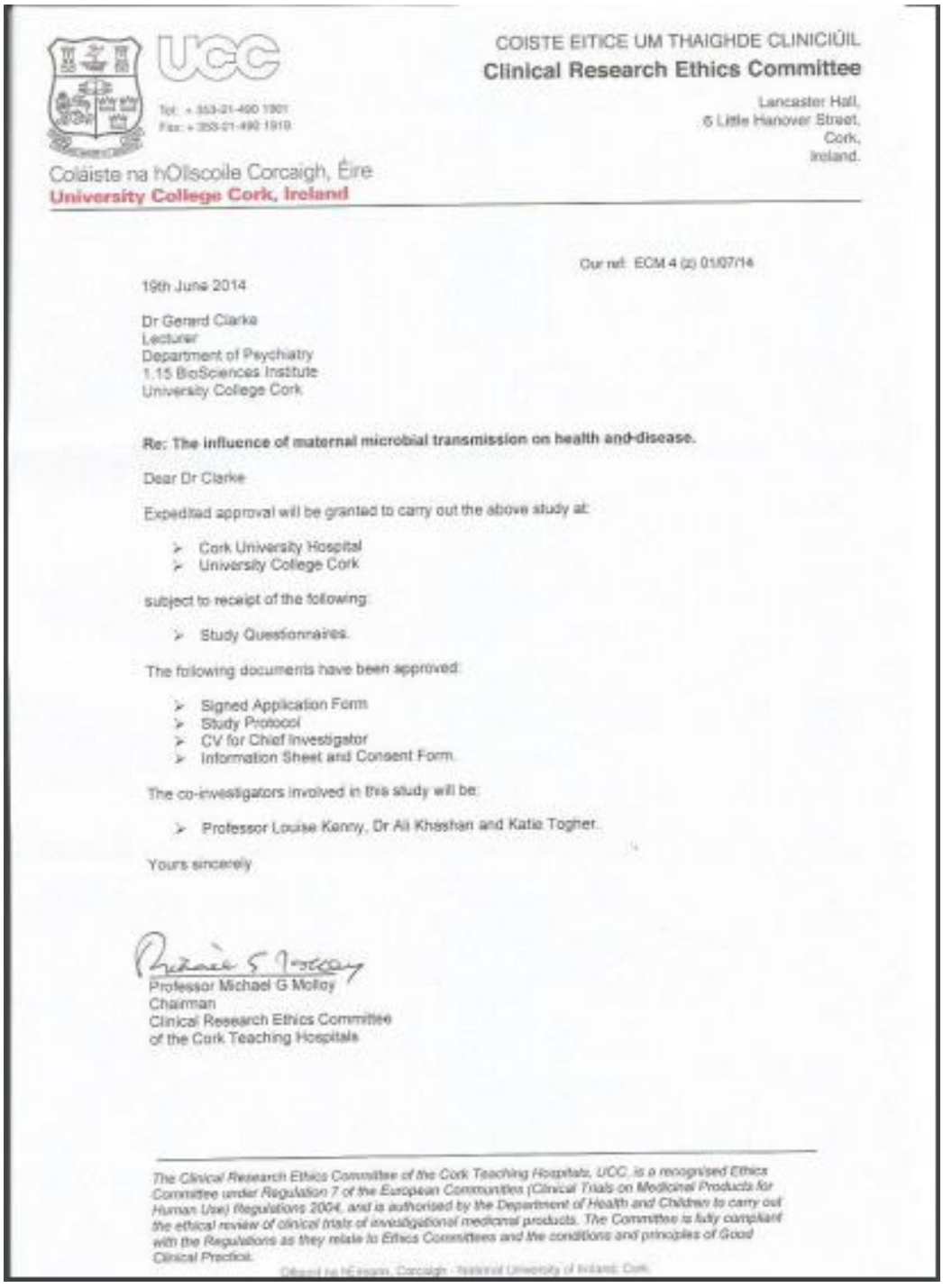
The following documents were approved:

- Original Signed Application Form
- CV for Chief Investigator
- Revised 15 weeks Lifestyle Questionnaire dated February 2007
- Stress in Pregnancy and the Placenta Study Questionnaire
- Consent Form Version 1 dated 15th July 2013
- Patient Information Leaflet Version 1 dated 15th July 2013.

Ollscoil na hÉireann, Corcaigh - National University of Ireland, Cork

Description: Ethical approval letter from the Clinical Research Ethics Committee of Cork Teaching Hospitals for work present in Chapter 4.

Ethical approval letter:



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Coláiste na hOllscoile Corcaigh, Éire
University College Cork, Ireland

COISTE EITICE UM THAIGHDE CLINICIÚIL
Clinical Research Ethics Committee

Lancaster Hall,
5 Little Hanover Street,
Cork,
Ireland.

Our ref: ECM 4 (p) 01/07/14

19th June 2014

Dr Gerard Clarke
Lecturer
Department of Psychiatry
1.15 BioSciences Institute
University College Cork

Re: The influence of maternal microbial transmission on health and disease.

Dear Dr Clarke

Expedited approval will be granted to carry out the above study at:

- Cork University Hospital
- University College Cork

subject to receipt of the following:

- Study Questionnaires.

The following documents have been approved:

- Signed Application Form
- Study Protocol
- CV for Chief Investigator
- Information Sheet and Consent Form.

The co-investigators involved in this study will be:

- Professor Louise Kenny, Dr Ali Khashan and Katie Togher.

Yours sincerely

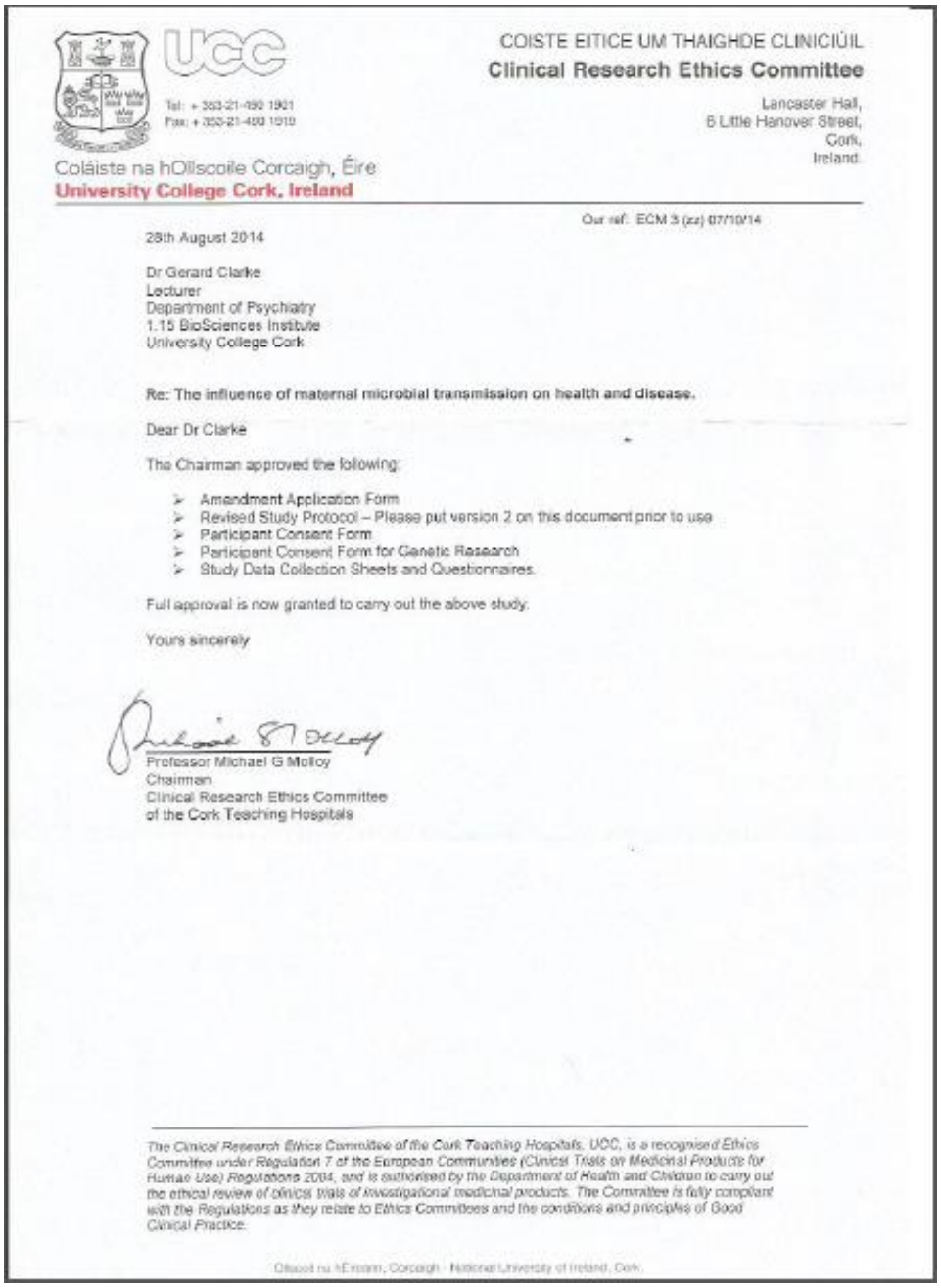
Professor Michael G Molloy
Chairman
Clinical Research Ethics Committee
of the Cork Teaching Hospitals

The Clinical Research Ethics Committee of the Cork Teaching Hospitals, UCC, is a recognised Ethics Committee under Regulation 7 of the European Communities (Clinical Trials on Medicinal Products for Human Use) Regulations 2004, and is authorised by the Department of Health and Children to carry out the ethical review of clinical trials of investigational medicinal products. The Committee is fully compliant with the Regulations as they relate to Ethics Committees and the conditions and principles of Good Clinical Practice.

Oibreoil na hEireann, Corcaigh - National University of Ireland, Cork

Description: Original ethical approval letter from the Clinical Research Ethics Committee of Cork Teaching Hospitals for work present in Chapter 5 & 6.

Ethical approval letter:



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Coláiste na hOllscoile Corcaigh, Éire
University College Cork, Ireland

COISTE EITICE UM THAIGHDE CLINICIÚIL
Clinical Research Ethics Committee

Lancaster Hall,
6 Little Hanover Street,
Cork,
Ireland.

Our ref: ECM 3 (zz) 07/10/14

28th August 2014

Dr Gerard Clarke
Lecturer
Department of Psychiatry
1.15 BioSciences Institute
University College Cork

Re: The influence of maternal microbial transmission on health and disease.

Dear Dr Clarke

The Chairman approved the following:

- Amendment Application Form
- Revised Study Protocol – Please put version 2 on this document prior to use
- Participant Consent Form
- Participant Consent Form for Genetic Research
- Study Data Collection Sheets and Questionnaires.

Full approval is now granted to carry out the above study.

Yours sincerely

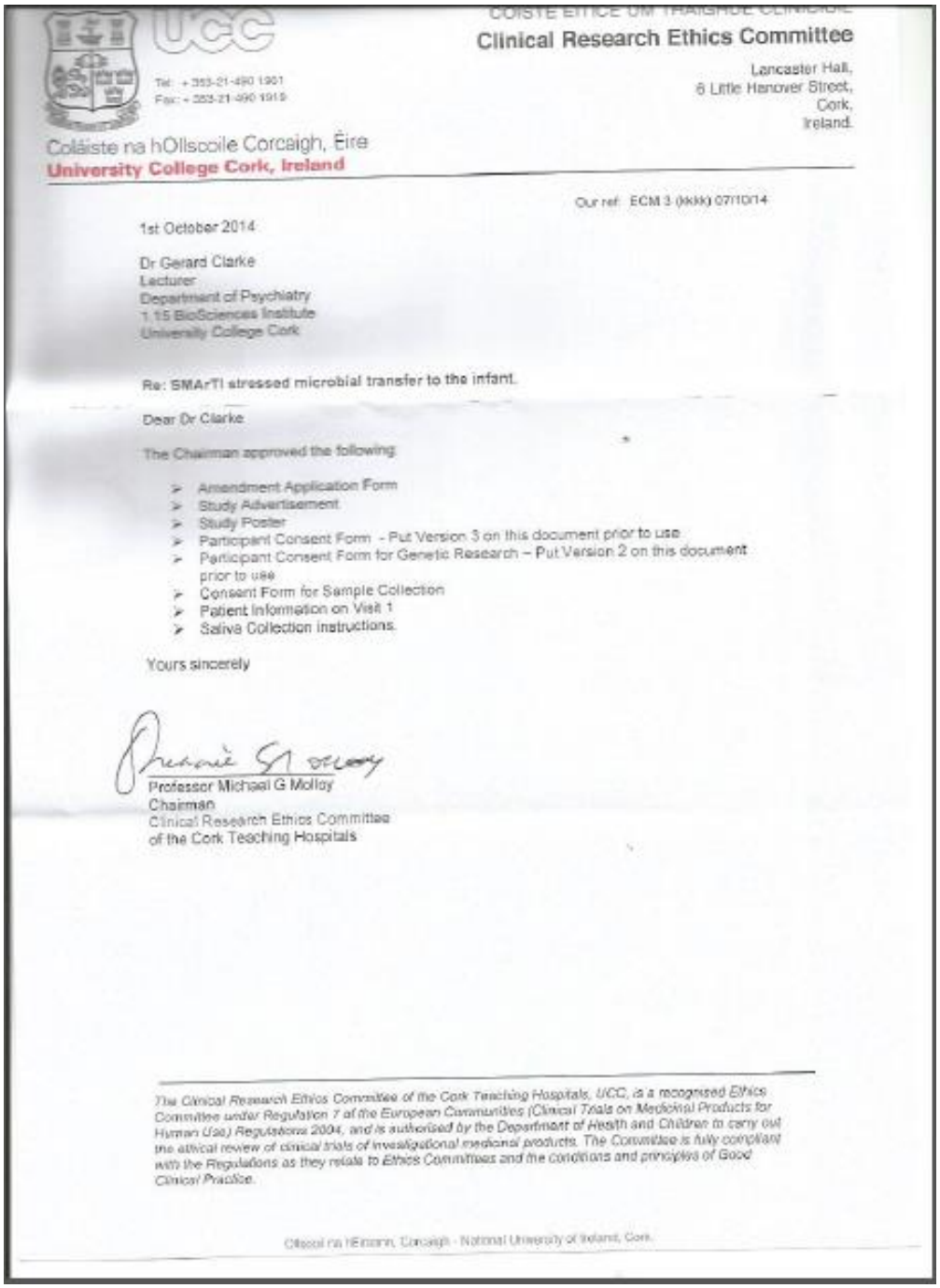
Professor Michael G Molloy
Chairman
Clinical Research Ethics Committee
of the Cork Teaching Hospitals

The Clinical Research Ethics Committee of the Cork Teaching Hospitals, UCC, is a recognised Ethics Committee under Regulation 7 of the European Communities (Clinical Trials on Medicinal Products for Human Use) Regulations 2004, and is authorised by the Department of Health and Children to carry out the ethical review of clinical trials of investigational medicinal products. The Committee is fully compliant with the Regulations as they relate to Ethics Committees and the conditions and principles of Good Clinical Practice.

Ollscoil na hÉireann, Corcaigh · National University of Ireland, Cork

Description: Ethical approval letter after the *first amendment* from the Clinical Research Ethics Committee of Cork Teaching Hospitals for work present in Chapter 5 & 6.

Ethical approval letter:



UCC

Clinical Research Ethics Committee

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Lancaster Hall,
6 Little Hanover Street,
Cork,
Ireland.

Coláiste na hOllscoile Corcaigh, Éire
University College Cork, Ireland

Our ref: ECM 3 (kkk) 07/10/14

1st October 2014

Dr Gerard Clarke
Lecturer
Department of Psychiatry
1.15 BioSciences Institute
University College Cork

Re: SMArTI stressed microbial transfer to the infant.

Dear Dr Clarke

The Chairman approved the following:

- Amendment Application Form
- Study Advertisement
- Study Poster
- Participant Consent Form - Put Version 3 on this document prior to use
- Participant Consent Form for Genetic Research – Put Version 2 on this document prior to use
- Consent Form for Sample Collection
- Patient Information on Visit 1
- Saliva Collection instructions.

Yours sincerely

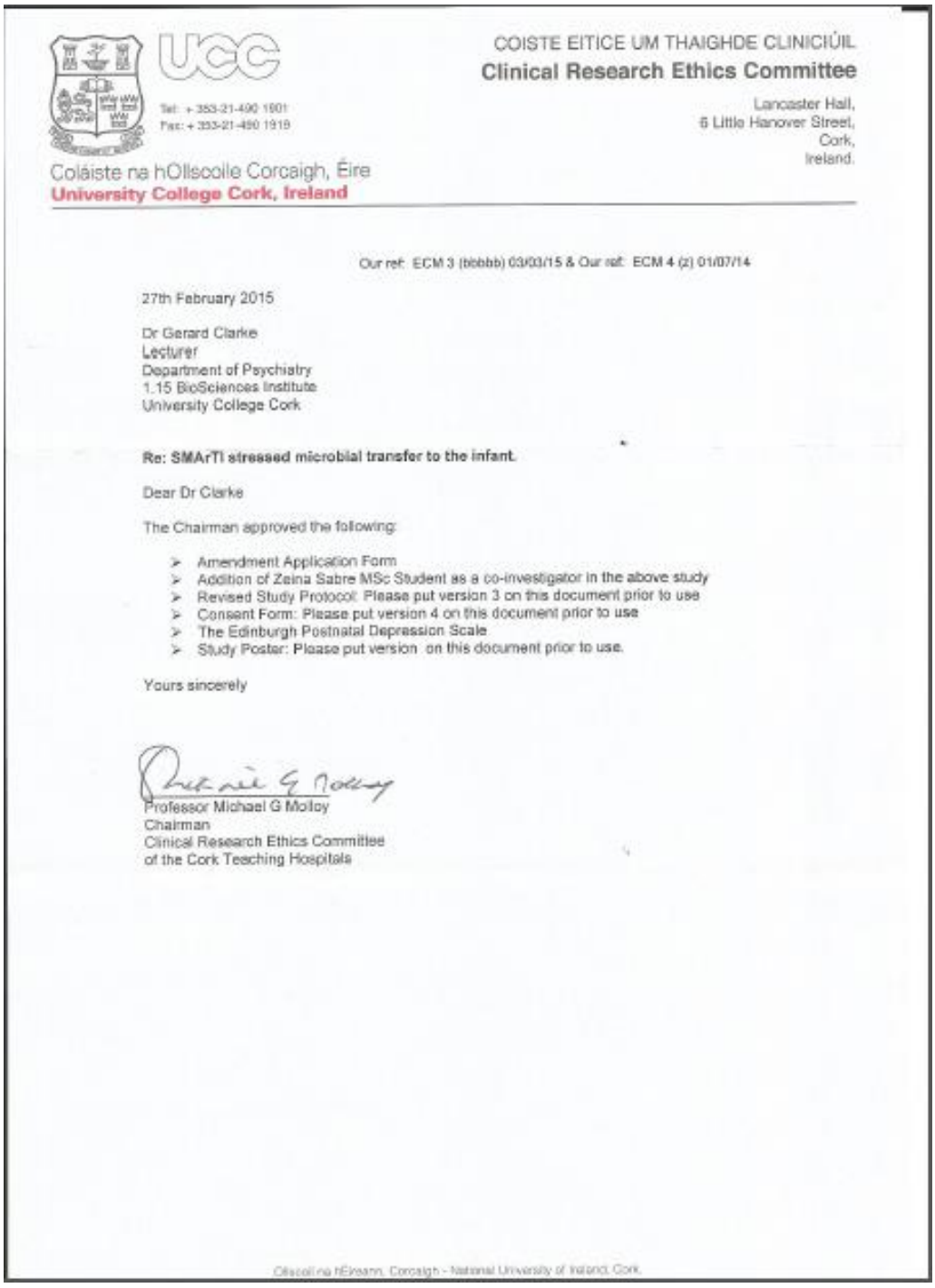
Professor Michael G Molloy
Chairman
Clinical Research Ethics Committee
of the Cork Teaching Hospitals

The Clinical Research Ethics Committee of the Cork Teaching Hospitals, UCC, is a recognised Ethics Committee under Regulation 7 of the European Communities (Clinical Trials on Medicinal Products for Human Use) Regulations 2004, and is authorised by the Department of Health and Children to carry out the ethical review of clinical trials of investigational medicinal products. The Committee is fully compliant with the Regulations as they relate to Ethics Committees and the conditions and principles of Good Clinical Practice.

Oifigeal na hEireann, Corcaigh - National University of Ireland, Cork.

Description: Ethical approval letter after the *second amendment* from the Clinical Research Ethics Committee of Cork Teaching Hospitals for work present in Chapter 5 & 6.

Ethical approval letter:



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Coláiste na hOllscoile Corcaigh, Éire
University College Cork, Ireland

COISTE EITICE UM THAIGHDE CLINICIÚIL
Clinical Research Ethics Committee

Lancaster Hall,
6 Little Hanover Street,
Cork,
Ireland.

Our ref: ECM 3 (bbbbb) 03/03/15 & Our ref: ECM 4 (z) 01/07/14

27th February 2015

Dr Gerard Clarke
Lecturer
Department of Psychiatry
1.15 BioSciences Institute
University College Cork

Re: SMArTI stressed microbial transfer to the infant.

Dear Dr Clarke

The Chairman approved the following:

- Amendment Application Form
- Addition of Zeina Sabre MSc Student as a co-investigator in the above study
- Revised Study Protocol: Please put version 3 on this document prior to use
- Consent Form: Please put version 4 on this document prior to use
- The Edinburgh Postnatal Depression Scale
- Study Poster: Please put version on this document prior to use.

Yours sincerely

Professor Michael G Molloy
Chairman
Clinical Research Ethics Committee
of the Cork Teaching Hospitals

Ollscoil na hÉireann, Corcaigh - National University of Ireland, Cork

Description: Ethical approval letter after the *third amendment* from the Clinical Research Ethics Committee of Cork Teaching Hospitals for work present in Chapter 5 & 6.

Detailed Protocol for the SMArTI Study:

Recruitment:

The IMPROvED consortium which is an FP-7-funded program lead by Professor Kenny, the Director of the Irish Centre for Fetal and Neonatal Translational Research (INFANT), UCC. This recently launched program is recruiting 5000 first time low risk pregnant women from six European centres at a cost of €6 million (http://www.fp7-improved.eu). It is expected that 1,500 women will be recruited in Cork University Maternity Hospital and the first baby is expected to be born in August 2014 and the final baby in August 2016. The added value of this cohort cannot be overestimated. In addition to a well-curated maternal biobank, pregnancies are extensively phenotyped with detailed maternal and paternal social, demographic and life-style data. The data are stored on a purpose built Internet-based database developed by MedSciNet. Therefore this is a unique and timely opportunity. We aim to recruit 150 women from the IMPROvED participants for the present study divided as follows: 50 women reporting low stress score (<the 25% percentile), 50 women reporting high stress score (>75% percentile) and 50 women with irritable bowel syndrome.

During Pregnancy:

Participating women in IMPROvED will complete the Perceived Stress Questionnaire (PSS), State Trait Anxiety Questionnaire and Edinburgh Postnatal Depression Scale (EPDS) at 3-4 time points during pregnancy to cover the three trimesters of pregnancy. We will recruit all women enrolled in IMPROvED until we have a quota of 50 women who rank as having high stress and 50 women who rank as having low stress as determined by these questionnaires. From the IMPROvED consortium we also aim to recruit 50 women who have been clinically diagnosed with IBS. We will ask these women to fill out a Gastrointestinal Symptom Severity form and the IBS Module Survey 3 times for the duration of the pregnancy. In addition to this, all women recruited to this study will be asked to complete a food frequency and food knowledge questionnaire in each trimester. This will allow us to assess how nutrition in pregnancy can influence short- and long-term outcomes in both the mother and offspring. Further, we will ask women to complete the Pittsburgh Sleep Quality Index in each trimester. Chronic Sleep loss in Pregnancy is associated with elevated stress in pregnancy and adverse pregnancy outcomes, further women who have sleep problems are more likely to identify as being stressed (Palagini et al., 2014), this will allow us to evaluate the association between sleep loss and adverse fetal outcomes. Finally, we will invite each participant to complete the Childhood Trauma Questionnaire, in an attempt to look at early life stress in these women. Participants will be given the option to fill out these questionnaires by hand on the day of their visit or online via Survey Monkey the day of or the day before their expected visit. If participants opt for the online version, we will acquire an email address from them and they will be sent the links to the surveys via email. Alternatively, if women opt for handwritten surveys and are unable to complete them on the day of their visit we will supply stamped envelopes for them to be

returned here to CUMH. We will also collect salivary cortisol at these time points for analysis of the cortisol awakening response (CAR), a physiological index of stress and a reliable marker of hypothalamic-pituitary-adrenal axis (HPA) activity. To get an accurate measure of HPA activity in relation to perceived stress women will be asked to donate their salivary sample within 24hours of completing the questionnaire. Each participant will be given a take home kit containing 4 salivettes (sarsedt) along with instructions on how to obtain the sample (attached). Women will be asked to take the sample on the morning of their IMPROvED visit and bring it with them to their appointment. Cortisol levels will be analysed using a commercially available ELISA assay from Enzo Life Sciences. On each visit participating women will be given the option to donate three stool and vaginal samples. Stool samples will be self-taken by the women via a home stool collection kit. Each kit comes with a detailed set of instructions (attached). Again participants will be asked to bring the sample on the day of their appointment. On the day of their appointment each participant will be asked to provide swabs from the oral cavity. Swabs will be collected by the study personnel at the time of appointment (See protocol 'Sampling of the Oral Cavity for SMarTI). DNA and RNA will be extracted from each sample using specialised DNA and RNA extraction kits and stored in the secure 'biobank' facility on the 5th floor of CUMH.

Upon Delivery:

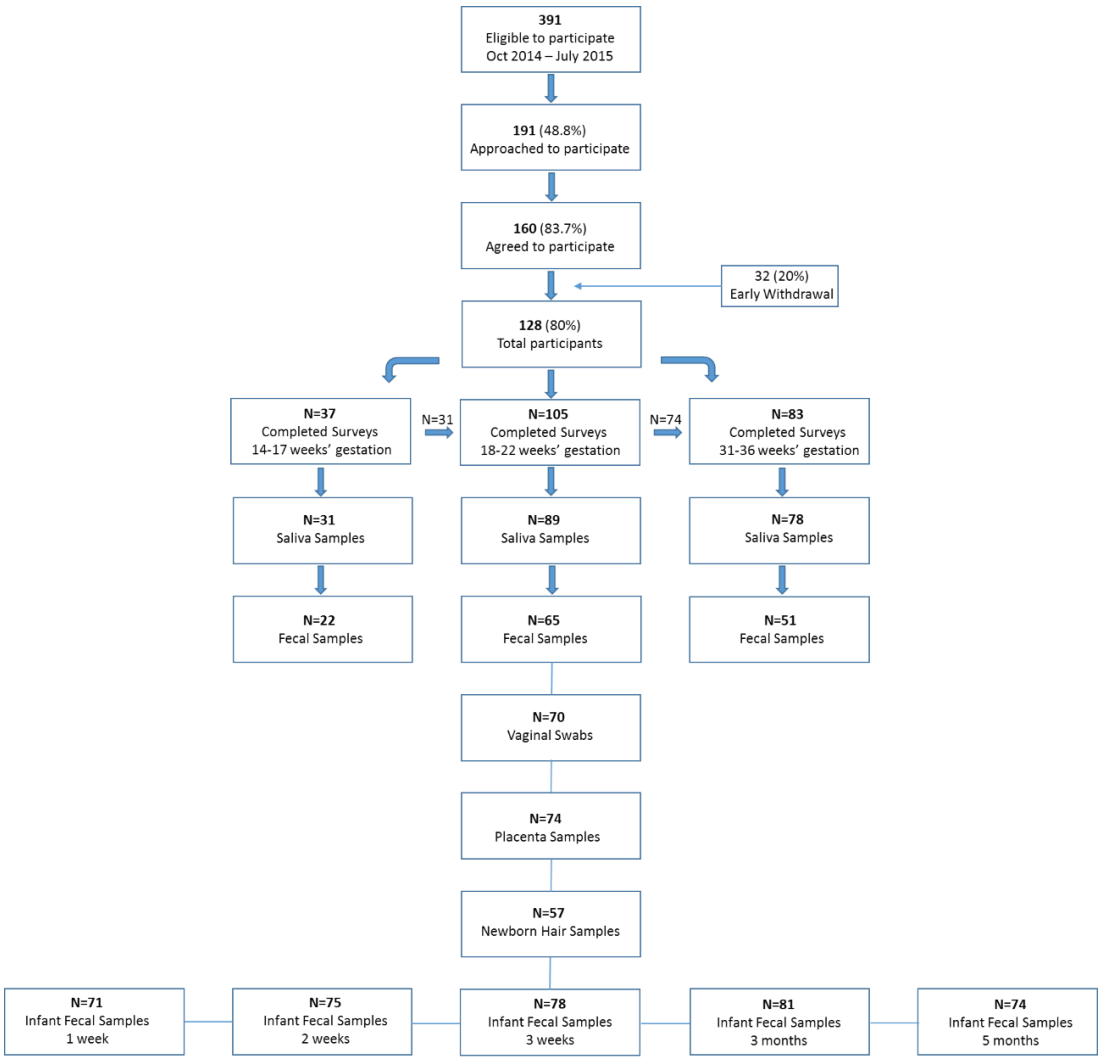
An additional vaginal swab and stool sample will be collected prior to delivery along with a maternal faecal sample. Similar to above, DNA will be extracted at stored in the biobank facility on the 5th floor of CUMH. After delivery we will collect a small sample of the placenta (1cm x 1cm x 1cm). The sample will be snap frozen in liquid nitrogen and stored the INFANT biobank. We potentially could be able use this sample for future research that at the moment cannot be foreseen. Further we will collect a hair sample from each infant at birth from the posterior vertex of the head. This sample will be used to assess cortisol levels in the infant. As cortisol present in the hair is believed to be a representation of long term HPA activity, we will use these samples to measure fetal cortisol exposure *in utero.* At birth we will take additional fetal characteristics such as gestational age, sex, weight, apgar score and head circumference. This data will be stored in a secure location of the 5th floor of CUMH. Only Chief- and co investigators on this study will have access to this information. We will use this date to determine an association (if any) between maternal stress/cortisol levels/IBS and birth outcome.

Follow up:

We will ask each participant to complete the EPDS postnatally to evaluate the relationship between postnatal depression and infant flora and behaviour. Each participant will be given the option to complete this survey by hand or online. Babies born by vaginal delivery will be followed up and stool samples will be collected at 1, 2 and 3 weeks, 3 and 5 months. Along with the stool sample, parents

will be asked to provide a urine sample from their infant at the 5 month collection. DNA and RNA will be extracted from each sample using specialised DNA and RNA extraction kits and stored in the secure 'biobank' facility on the 5th floor of CUMH. Once all samples are collected they will be shipped to an external sequencing company for determination of the metabogenomic composition of each individual's microbiota. This will allow us to examine the temporal changes in the gut and vaginal microbiome in relation to pregnancy only (low stress group) and in relation to exposure to high stress and IBS. In addition to very detailed data on the mothers and their partners and obstetric variables, data on breastfeeding and antibiotic use during pregnancy/delivery and in early life of the offspring will be available. Breastfeeding and antibiotics are known to alter the gut microbiota of the newborn therefore it is important to examine whether these factors play a role in any observed associations between stress and IBS and altered gut microbiota in the child. Finally we will use the revised version of the Infant Behavioural Questionnaire (IBQ-R) to assess infant temperament at 5 months of age. Parents will be asked to complete this questionnaire when their infant is 5 months of age (a time corresponding to our last infant stool collection). The IBQ-R will enable us detect if maternal stress and IBS affects infant temperament at 5 months of age. Further it will enable us to elucidate the role for the gut flora in this relationship.

Flowchart of SMArTI final numbers:



Description: Final cohort numbers from the SMArTI study.

SMArTI patient Information Leaflet:

ETHICS

This study had received ethical approval from the Cork University Hospitals Research Ethics Committee.

For more information contact

Katie Togher

Phone 089 416 1870

katie.togher@ucc.ie

Who We Are

About Us

The Alimentary Pharmabiotic Center

APC is a world leading research center that focuses on understanding the role of the gastrointestinal bacterial community (microbiota) in all aspects of health and disease.

http://www.ucc.ie/research/apc/content/

The Irish Center for Fetal and Neonatal Translational Research (INFANT)

INFANT is a world leading perinatal research center located in Cork University Maternity Hospital that specialized and dedicated to research to improve maternal and infant health.

http://www.infantcentre.ie/tf2w7gu3gw

SMArTI is a collaborative project of both these centers, combing expertise in gastrointestinal function and pregnancy with the ultimate goal to understand the influence of the gut microbiota in maternal and infant health

SMArTI

We need your help to carry out our research aimed at improving maternal and infant health

The aim of the SMArTI project is to determine the impact of Prenatal Stress and Irritable Bowel Syndrome on the development of the infant microbiome.

Current research into the human microbiome is beginning to make startling discoveries connecting the initial microbes that colonize your baby and their future health.

INTRODUCTION

You are invited to take part in a study focused on determining the effect of stress and Irritable Bowel Syndrome during pregnancy on the babies 'microbiome' in the first 5 months of life. Participation in this study is completely voluntary. Not taking part in this study will have no effect on your future care.

BACKGROUND

A large proportion of women experience some form of stress throughout their pregnancy. This experience of stress is completely normal; however large amounts of prolonged stress during pregnancy may alter infant development and increase the risk of disease in adulthood. Recently, the development of the infant gut 'microbiome' (the bacteria living in our bodies) has been put forward as a possible factor underlying the association between prenatal stress and infant development. SMArTI aims to test this hypothesis by determining the effect that prenatal stress and Irritable Bowel Syndrome (IBS) has on the development of the infants gut microbiome. Understanding these mechanisms has the potential to facilitate the development of microbiome-based therapeutic interventions aimed at reducing disease risk later in life.

ELIGIBILITY

To participate in this study you must be enrolled in the IMPROvED study (http://www.fp7improved.eu/)

WHAT AM I BEING ASKED TO DO?

Each visit corresponds to the exact time and date of your IMPROvED visit. **SMArTI requires no extra appointments.**

Visit 1: On your first visit we will meet you at your IMPROvED appointment to describe the study to you. If you agree to participate we will have you sign the consent forms to enroll you in the study. You will complete a short interview and be given the sample collection kits. All samples will be collected by you at home and brought back at your next IMPROvED visit. This visit will take approximately 30 minutes.

Visit 2: You will complete your surveys at home as close as possible to this visit. You will bring your samples to this visit. We will collect your samples from you and give you a kit for your next visit. This visit will take approximately 5 minutes.

Visit 3: You will complete your surveys at home as close as possible to this visit. You will bring your samples to this visit. We will collect your samples from you and mark your chart. This visit will take approximately 5 minutes.

Visit 4: You will complete your surveys at home as close as possible to this visit. You will bring your samples to this visit. We will collect your samples from you. This visit will take approximately 5 minutes

Postnatal: We will ask you to collect nappy samples from your baby at various time points in the first 5 months of life. These samples are collected by you, at home using a kit that will be provided to you by us. Once you have taken these samples we will have them collected from you at your home as soon as possible.

PARTICIPATION

Participation in this study is completely voluntary. You can choose to withdraw from this study at any point. Choosing to withdraw will have no effect on your future health care.

RISKS AND BENEFITS

This study will not directly benefit you in your pregnancy but will help future women who suffer from stress/anxiety/depression and gut dysfunction throughout their pregnancy. There are no anticipated risks to you or your baby associated with this study.

CONFIDENTIALLITY

A study number will be given to each participant in this study. This study number will ensure that no material can personally identify you.

RESULTS

The results of this study will be published in medical journals. Unfortunately you will not have access to your individual results

Description: The information leaflet given to patients and placed in antenatal clinics during recruitment of the SMArTI study.

SMArTI poster:

Description: The poster given to patients and placed in antenatal clinics during recruitment of the SMArTI study.

SMArTI consent form:

Participants Consent Form

(version 5)

SECTION 1: Overview

Patients name: _____
(please print)

MRN: _____

What are you being asked to do?
You are being asked to participate in a research study. At INFANT we study normal pregnancies and complications of pregnancy and their impact on mother and baby. We do this to try to develop improved methods of diagnosis, treatment and prevention. In order to decide whether or not you want to be a part of this research study, you should understand enough about its risks and benefits to make an informed judgment. This process is known as informed consent. This consent form gives detailed information about the research study, which will be discussed with you. Once you understand the study, you will be asked to sign this form if you wish to participate.

SECTION 2: What is involved and are there any risks?

1. What is this study about?
Children whose mothers suffer from stress and/or Irritable Bowel Syndrome (IBS) during pregnancy are thought to have an increased risk for disease later in life. Exactly why this occurs is unclear but one possible mechanism may be through alterations in the gut microbiota. The term 'microbiota' refers to the microbe population (bacteria etc.) that resides in our gut. The first microbes that enter our gut are thought to do so at birth, where they are passed to us from our mothers as we make our journey through the birth canal. The establishment of a healthy microbiota is essential for normal health, as alterations in the gut microbiome has been linked to diseases such as anxiety. In this study we aim to determine if stress and IBS during pregnancy alters the normal gut microbiota. We then aim to determine if this altered microbiota population is transmitted to the infant at birth. Understanding these mechanisms has the potential to facilitate therapeutic interventions to prevent disease later in life.

2. What do you require me to do?
We will ask you to complete a number of questionnaires 3 times during your pregnancy. These questionnaire will take approx. 45 minutes to complete and will assess your levels of stress, anxiety and depression, as well as your IBS symptom severity (if applicable), sleep patterns, childhood experiences and diet. All the data will be stored in a secure location here at CUMH and will be anonymous. You will also be asked to give salivary samples, stool samples (optional) and oral swabs at these times. Before delivery we would ask for an additional vaginal swap. After delivery we would like your permission to take a small biopsy (1cmx1cm) of your placenta, along with a hair sample from your newborn. In addition to this we would ask for stool samples from your infant at 1, 2 and 3 weeks, 3 and 5 months and for you to complete a postnatal depression test. We would also ask for a urine sample from your infant at 5 months. All samples will be stored in a secure "BioBank" facility in the APC and INFANT research Centers.

3. What are the potential risk and benefits?
There are no risks to you or your baby by taking part in this study.

4. What happens if I don't want to participate?
f you do not wish to participate in research in our group at this time, we completely understand. Please be assured that this will not impact in any way on the medical care you will or are currently receiving. Any support received from you is a generous gift without strings attached, and will always be regarded as such by us.

SECTION 3: Agreement to consent

A) The research project and the treatment procedures associated with it have been fully explained to me. All experimental procedures have been identified and no guarantee has been given about the possible results. I have had the opportunity to ask questions concerning any and all aspects of the project and any procedures involved. I am aware that participation is voluntary and that I may withdraw my consent at any time. I am aware that my decision not to participate or to withdraw will not restrict my access to health care services normally available to me. Confidentiality of records concerning my involvement in this project will be maintained in an appropriate manner. When required by law, the records of this research may be reviewed by government agencies and sponsors of the research.

B) I understand that the sponsors and investigators have such insurance as is required by law in the event of injury resulting from this research.

C) I, the undersigned, hereby consent to participate as a subject in the above described project conducted at the Cork Teaching Hospitals. I have received a copy of this consent form for my records. I understand that if I have any questions concerning this research, I can contact the doctor(s) listed above. If I have further queries concerning my rights in connection with the research, I can contact the Clinical Research Ethics Committee of the Cork Teaching Hospitals, Lancaster Hall, 6 Little Hanover Street, Cork.

D) After reading the entire consent form, if you have no further questions about giving consent, please sign where indicated.

Doctor: _____.

Signature of patient: _____.

Witness: _____.

Time: _____.

(Circle) **AM** **PM**

References:
1. Human and Tissue and Biological Samples for Use in Research. Interim Operational and Ethical Guidelines Issued by the Medical Research Council. Published for Consultation November 1999 (email: www.mrc.ac.uk/tissue_gde.pdf)
2. EU Directive 95/46/EC ei@dg15.cec.be

SMArTI

'Stressed MicrobiAl Transfer to Infants'

Participants Consent Form for genetic research

Version2

SECTION 4: Consent Form for genetic research

Patients name: _____

(please print)

MRN: _____

What are you being asked to do?

We are asking you to give us permission to for long term storage of the samples we have taken from you. These samples are very valuable to us and we potentially could be able use them for future research that at the moment cannot be foreseen. We therefore ask that you give consent to allow us to store this sample in a secure "BioBank" facility in the INFANT ad APC research Centers and use it in our future research if we deem this appropriate. All our current and future studies will always be carried out with full ethical approval from the Clinical Research Ethics Committee Of The Cork Teaching Hospitals and comply with National and International rules and regulations.

	Please Initial Boxes
1. I have read the attached information leaflet on the above project dated ..... and have been supplied with a copy. The information has been fully explained to me.	
2. I agree to give a sample of DNA for the above research. I agree that my sample and the information gathered from me can be stored in computer or manual format and looked after by the INFANT directorate.	
3. I give permission for my medical records to be reviewed and information to be taken from them to be analyzed in confidence by Dr Khashan and Dr Clarke or a representative authorized them.	
4. I understand that all medical information pertaining to me, including my samples, will be protected by the principles of confidentiality and both National and EU Data Protection Legislation.	
5. I agree to the storage of my samples at INFANT in Cork University Maternity Hospital for .....years for use later in genetic research.	

Signature of patient: _____. **Date:** _____.

SECTION 5: Consent Form for sample collection (version 2):

Patients name: _____

(please print)

MRN: _____

I agree to..... (please tick the box where appropriate):

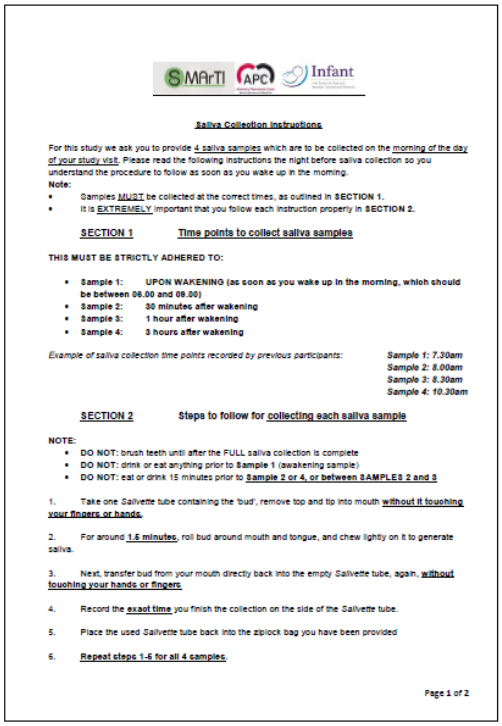
1. Stress/Depression/Anxiety Questionnaire	
2. Pregnancy Distress Questionnaire	
3. IBS Severity Scale	
4. Food Frequency and knowledge Questionnaire	
5. Childhood Trauma Questionnaire	
6. Pittsburgh Sleep Quality Index	
7. Salivary Sample – During pregnancy	
8. Stool Sample – During pregnancy (optional)	
9. Oral Swab – During pregnancy	
10. Vaginal Swab - At Birth	
11. Infant hair sample - At Birth	
12. Placenta Sample – At Birth	
13. Infant stool samples at 1 wk, 2wk, 3wk, 3months and 5months	
14. Infant urine at 5 months	
15. Infant Behaviour Questionnaire at 5 months	
I agree to be contacted for research in the future	

Witness: _____. **Date:** _____.

Signature of patient: _____. **Date:** _____.

Biological Sample Collection:

Saliva collection:



Saliva Collection Instructions

For this study we ask you to provide 4 saliva samples which are to be collected on the morning of the day of your study visit. Please read the following instructions the night before saliva collection so you understand the procedure to follow as soon as you wake up in the morning.

Note:

- Samples MUST be collected at the correct times, as outlined in **SECTION 1.**
- It is EXTREMELY important that you follow each instruction properly in **SECTION 2.**

SECTION 1 **Time points to collect saliva samples**

THIS MUST BE STRICTLY ADHERED TO:

- **Sample 1: UPON WAKENING (as soon as you wake up in the morning, which should be between 06.00 and 09.00)**
- **Sample 2: 30 minutes after wakening**
- **Sample 3: 1 hour after wakening**
- **Sample 4: 3 hours after wakening**

Example of saliva collection time points recorded by previous participants:

Sample 1: 7.30am
Sample 2: 8.00am
Sample 3: 8.30am
Sample 4: 10.30am

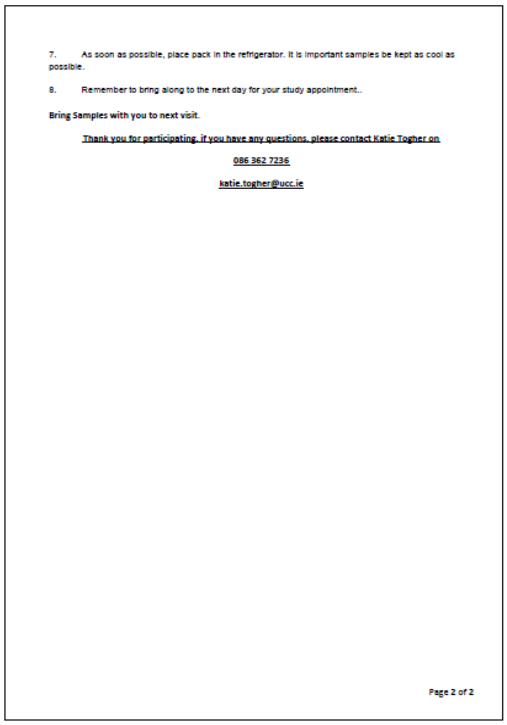
SECTION 2 **Steps to follow for collecting each saliva sample**

NOTE:

- **DO NOT:** brush teeth until after the FULL saliva collection is complete
- **DO NOT:** drink or eat anything prior to **Sample 1** (awakening sample)
- **DO NOT:** eat or drink 15 minutes prior to **Sample 2 or 4, or between SAMPLES 2 and 3**

1. Take one *Salivette* tube containing the 'bud', remove top and tip into mouth **without it touching your fingers or hands.**
2. For around **1.5 minutes**, roll bud around mouth and tongue, and chew lightly on it to generate saliva.
3. Next, transfer bud from your mouth directly back into the empty *Salivette* tube, again, **without touching your hands or fingers.**
4. Record the **exact time** you finish the collection on the side of the *Salivette* tube.
5. Place the used *Salivette* tube back into the ziplock bag you have been provided
6. **Repeat steps 1-5 for all 4 samples.**

Page 1 of 2



7. As soon as possible, place pack in the refrigerator. It is important samples be kept as cool as possible.
8. Remember to bring along to the next day for your study appointment..

Bring Samples with you to next visit.

Thank you for participating, if you have any questions, please contact Katie Togher on

086 362 7236

katie.togher@ucc.ie

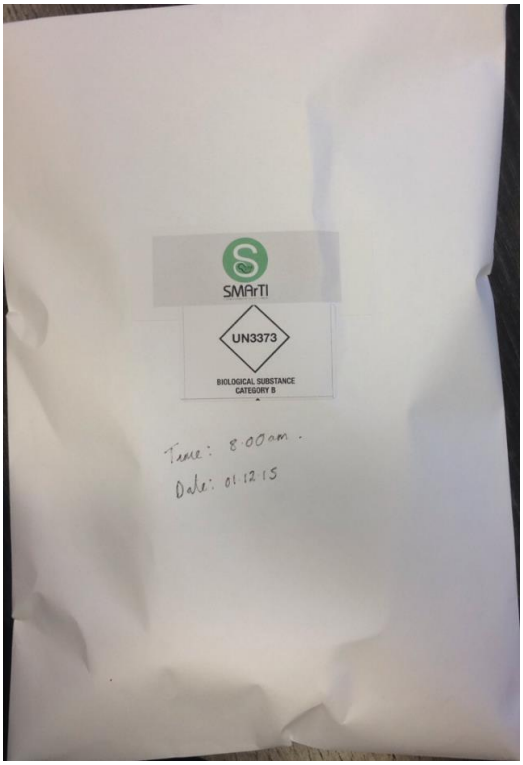
Page 2 of 2

Contents:

(a) Detailed Instructions
(b) Salivettes (x4)
(c) Zip-lock bag (x1)

Description: Instructions and home collection kit given to SMArTI participants for collection of saliva samples at home.

Maternal fecal sample collection kit:

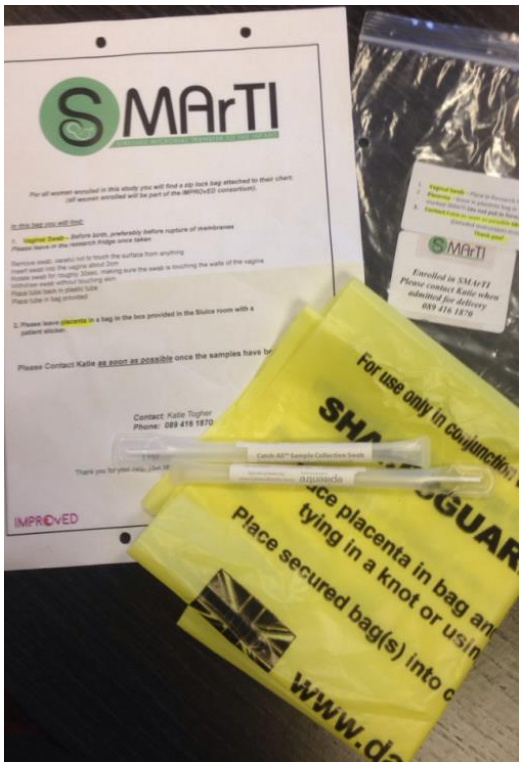


Contents:

(d) Detailed Instructions
(e) Latex gloves
(f) Plastic container contain AnaeroGen Sachet
(g) Zip-lock bag (x2)
(h) Envelope

Description: Kit given to SMArTI participants for home collection of fecal samples at home.

Vaginal swab and Placenta Collection:



Contents:

(i) Detailed Instructions
(j) Yellow Placenta Bag
(k) Epicentre Sterile Swabs (x2)
(l) Zip-lock bag
(m) Short Instructions

Description: This kit was inserted into participants chart so staff on the delivery ward knew they were enrolled in the SMArTI study and could collect the samples.

Infant stool sample Collection:

We would first like to sincerely thank you for your ongoing participation in our study SMArTI. So far for this study you have completed a number of questionnaires and provided various samples, all of which are invaluable to us. In the final part of this study we hope to collect a number of stool samples from your infant. These stool samples will enable us to characterise the bacteria within the gut and watch them grow over the first few months of life. The establishment of these bacteria are crucial for infant health and with your help we hope to identify what during pregnancy plays a role in determining how the bacteria develop.

Below is a brief guide on how to collect faecal samples from your infant:

1. We will provide you with tubes (brown cap) to collect the faecal samples.

2. On the day the sample is required we will ring/text you
   - 1 week old
   - 2 weeks old
   - 3 weeks old
   - 3 months old
   - 5 months old

3. At any point during that day when a dirty nappy is produced, use the brown capped tube to collect a faecal sample. Simply scrape some faeces from the nappy with the scoop provided on the tube. Close tube and repeat for second tube.

4. Place samples in the envelope provided and label with time and date. Place envelope in the zip lock bag.

5. Please keep the sample as cool as possible once collected, ideally in the fridge.

6. Once you have the sample ring or text Katie **(086 362 7236)** to let her know the sample has been collected.

7. We will arrange for our courier to collect the sample from you as soon as possible.

Thank you for your participation ☺

Description: Instructions given to participants for collection of infant fecal samples.

Questionnaires:

Perceived Stress Scale:

These questions ask about your feelings and thoughts during THE LAST MONTH.

In each case, you will be asked to indicate how often you felt or thought a certain way. Although some of the questions are similar, there are differences between them and you should treat each one as a separate question. The best approach is to answer each question fairly quickly. Don't try to count up the number of times you felt a particular way, but rather ***circle the answer that you think best fits for you***.

	Never	**Almost Never**	**Some-times**	**Fairly Often**	**Very Often**
In the last month, how often have you been upset because of something that happened unexpectedly?	0	1	2	3	4
In the last month, how often have you felt that you were unable to control the important things in your life?	4	3	2	1	0
In the last month, how often have you felt nervous and stressed?	0	1	2	3	4
In the last month, how often have you felt confident about your ability to handle your personal problems?	4	3	2	1	0
In the last month, how often have you felt that things were going your way?	4	3	2	1	0
In the last month, how often have you found that you could not cope with all the things you had to do?	0	1	2	3	4
In the last month, how often have you been able to control irritations in your life?	4	3	2	1	0
In the last month, how often have you felt that you were on top of things?	4	3	2	1	0
In the last month, how often have you been angered because of things that happened that were outside of your control?	0	1	2	3	4
In the last month, how often have you felt difficulties were piling up so high that you could not overcome them?	0	1	2	3	4

State Trait Anxiety Inventory

Please read each of the following statements and circle the most appropriate number to the right of the statement to indicate **how you feel right now**, at this moment. Do not spend too much time on any one statement but ***circle*** the answer which seems to describe your present feelings best.

	Not at all	**Somewhat**	**Moderately**	**Very Much**
I feel calm	3	2	1	0
I feel tense	0	1	2	3
I feel upset	0	1	2	3
I feel relaxed	3	2	1	0
I feel content	3	2	1	0
I feel worried	0	1	2	3

Edinburgh Postnatal Depression Scale:

These questions ask about your mood.

Please ***circle*** the answer that best describes to how you have felt in **THE PAST WEEK.**

(1) I have been able to laugh and see the funny side of things:

- As much as I always could (0)
- Not quite so much now (1)
- Definitely not so much now (2)
- Not at all (3)

(2) I have looked forward with enjoyment to things:

- As much as I ever did (0)
- Rather less than I used to (1)
- Definitely less than I used to (2)
- Hardly at all (3)

(3) I have blamed myself unnecessarily when things went wrong:

- No not at all (0)
- Hardly ever (1)
- Yes, sometimes (2)
- Yes, very often (3)

(4) I have felt anxious or worried for no very good reason:

- Yes, quite a lot (3)
- Yes, sometimes (2)
- No, not much (1)
- No, not at all (0)

(5) I have felt scared or panicky for no very good reason:

- Yes, quite a lot (3)
- Yes, sometimes (2)
- No, not much (1)
- No, not at all (0)

(6) Things have been getting on top of me:

- Yes, most of the time I haven't been able to cope at all (3)
- Yes, sometimes I haven't been coping as well as usual (2)
- No, Most of the time I have coped quite well (1)
- No, I have been coping as well as ever (0)

(7) I have been so unhappy that I have had difficulty sleeping:

- Yes, most of the time (3)
- Yes, sometimes (2)
- Not very often (1)
- No, not at all (0)

(8) I have felt sad or miserable:

- Yes, most of the time (3)
- Yes, sometimes (2)
- Not very often (1)
- No, not at all (0)

(9) I have been so unhappy that I have been crying:

- Yes, most of the time (3)
- Yes, sometimes (2)
- Not very often (1)
- No, not at all (0)

(10) The thought of harming myself has occurred to me:

- Yes, quite often (3)
- Sometimes (2)
- Hardly ever (1)
- Never (0)

Pregnancy Distress Questionnaire

(1) I find weight gain during pregnancy troubling

- Not at all worried (0)
- Slightly worried (1)
- Moderately worried (2)
- Quite worried (3)
- Extremely worried (4)

(2) Physical symptoms of pregnancy, such as nausea, vomiting, swollen feet or backache, irritate me

- Not at all (0)
- Slightly (1)
- Moderately (2)
- Quite (3)
- Extremely (4)

(3) I am worried about handling the infant when I first come home from the hospital

- Not at all worried (0)
- Slightly worried (1)
- Moderately worried (2)
- Quite worried (3)
- Extremely worried (4)

(4) Emotional ups and downs during pregnancy annoy me

- Not at all (0)
- Slightly (1)
- Moderately (2)
- Quite (3)
- Extremely (4)

(5) I am troubled that my relationships with other people important to me are changing due to my pregnancy

- Not at all worried (0)
- Slightly worried (1)
- Moderately worried (2)
- Quite worried (3)
- Extremely worried (4)

(6) I am worried about eating healthy foods and a balanced diet for the infant

- Not at all worried (0)
- Slightly worried (1)
- Moderately worried (2)
- Quite worried (3)
- Extremely worried (4)

(7) Overall, the changes in my body shape and size during pregnancy bothers me

- Not at all (0)
- Slightly (1)
- Moderately (2)
- Quite (3)
- Extremely (4)

(8) I am concerned that having a new infant will alter my relationship with the infants' father

- Not at all worried (0)
- Slightly worried (1)
- Moderately worried (2)
- Quite worried (3)
- Extremely worried (4)

(9) I worry about having an unhealthy infant

- Not at all worried (0)
- Slightly worried (1)
- Moderately worried (2)
- Quite worried (3)
- Extremely worried (4)

(10) I am anxious about labour and childbirth

- Not at all worried (0)
- Slightly worried (1)
- Moderately worried (2)
- Quite worried (3)
- Extremely worried (4)

(11) The possibility or premature childbirth frightens me

- Not at all worried (0)
- Slightly worried (1)
- Moderately worried (2)
- Quite worried (3)
- Extremely worried (4)

(12) I am worried that i might not become emotionally attached to the infant

- Not at all worried (0)
- Slightly worried (1)
- Moderately worried (2)
- Quite worried (3)
- Extremely worried (4)

Pittsburgh Sleep Quality Index

The following questions relate to your usual sleep habits during the past month only. Your answers should indicate the most accurate reply for the majority of days and nights in the past month. Please answer all questions.

During the past month,

(1) When have you usually gone to bed?

(2) How long (in minutes) has it taken you to fall asleep each night?

(3) When have you usually gotten up in the morning?

(4) How many hours of actual sleep do you get at night? (This may be different than the number of hours you spend in bed)

(5) During the past month, how often have you had trouble sleeping because you…

(a) Cannot get to sleep within 30 minutes
- Not during the past month (0)
- Less than once a week (1)
- Once or twice a week (2)
- Three or more times a week (3)

(b) Wake up in the middle of the night or early morning
- Not during the past month (0)
- Less than once a week (1)
- Once or twice a week (2)
- Three or more times a week (3)

(c) Have to get up to use the bathroom
- Not during the past month (0)
- Less than once a week (1)
- Once or twice a week (2)
- Three or more times a week (3)

(d) Cannot breathe comfortably
- Not during the past month (0)
- Less than once a week (1)
- Once or twice a week (2)
- Three or more times a week (3)

(e) Cough or snore loudly
- Not during the past month (0)
- Less than once a week (1)
- Once or twice a week (2)
- Three or more times a week (3)

(f) Feel too cold
- Not during the past month (0)
- Less than once a week (1)
- Once or twice a week (2)
- Three or more times a week (3)

(g) Feel too hot

- Not during the past month (0)
- Less than once a week (1)
- Once or twice a week (2)
- Three or more times a week (3)

(h) Have bad dreams

- Not during the past month (0)
- Less than once a week (1)
- Once or twice a week (2)
- Three or more times a week (3)

(i) Have pain

- Not during the past month (0)
- Less than once a week (1)
- Once or twice a week (2)
- Three or more times a week (3)

(j) Other reason(s), please describe, including how often you

- Not during the past month (0)
- Less than once a week (1)
- Once or twice a week (2)
- Three or more times a week (3)

(6) During the past month, how often have you taken medicine (prescribed or "over the counter") to help you sleep?

- Not during the past month (0)
- Less than once a week (1)
- Once or twice a week (2)
- Three or more times a week (3)

(7) During the past month, how often have you had trouble staying awake while driving, eating meals, or engaging in social activity?

- Not during the past month (0)
- Less than once a week (1)
- Once or twice a week (2)
- Three or more times a week (3)

(8) During the past month, how much of a problem has it been for you to keep up enthusiasm to get things done?

- Not during the past month (0)
- Less than once a week (1)
- Once or twice a week (2)
- Three or more times a week (3)

(9) During the past month, how would you rate your sleep quality overall?

- Very good (0)
- Fairly good (1)
- Fairly bad (2)
- Very bad (3)
-

Scoring PSQI

- Component 1: #9 Score
  C1_____

- Component 2: #2 Score (<15min=0; 16-30 min=1; 31-60 min=2, >60 min=3) + #5a Score (if sum is equal 0=0; 1-2=1; 3-4=2; 5-6=3)
  C2_____
- Component 3 #4 Score (>7=0; 6-7=1; 5-6=2; <5=3)
  C3_____

- Component 4 (total # of hours asleep)/(total # of hours in bed) x 100 (>85%=0, 75%-84%=1, 65%-74%=2, <65%=3)
  C4_____

- Component 5 Sum of Scores #5b to #5j (0=0; 1-9=1; 10-18=2; 19-27=3)
  C5_____

- Component 6 #6 Score
  C6_____

- Component 7 #7 Score + #8 Score (0=0; 1-2=1; 3-4=2; 5-6=3)
  C7_____

Add the seven component scores together _____ **Global PSQI Score** _____

Childhood Trauma Questionnaire

This questionnaire studies different aspects of your childhood.

Please circle one answer per question which most accurately represents your experience

Please answer every question as accurate as possible.

When I was growing up……..	**Never true**	**Rarely true**	**Some-times true**	**Often true**	**Very Often true**
1. I didn't have enough to eat	0	1	2	3	4
2. I knew that there was someone to take care of me and protect me	4	3	2	1	0
3. People in my family called me things like stupid, lazy or ugly**.**	0	1	2	3	4
4. My parents were too drunk or high to take care of the family	0	1	2	3	4
5. There was somebody in my family who helped me feel like I was important or special	4	3	2	1	0
6. I had to wear dirty clothes	0	1	2	3	4
7. I felt loved	4	3	2	1	0
8. I thought that my parents wished that I had never been born	0	1	2	3	4
9. I got hit so hard by someone in my family that I had to see a doctor or go to the hospital	0	1	2	3	4
10. There was nothing I wanted to change about my family	4	3	2	1	0
11. People in my family hit me so hard that it left me with bruises or marks	0	1	2	3	4
12. I was punished with a belt, a cord or some other hard object	0	1	2	3	4
13. People in my family looked out for each other	4	3	2	1	0
14. People in my family said hurtful or insulting things to me	0	1	2	3	4

15. I believe that I was physically abused	0	1	2	3	4
16. I had the perfect childhood	4	3	2	1	0
17. I got hit or beaten so badly that it was noticed by someone like a teacher, neighbour or doctor	0	1	2	3	4
18. I felt that someone in my family hated me	0	1	2	3	4
19. People in my family felt close to each other	4	3	2	1	0
20. Someone tried to touch me in a sexual way, or tried to make me touch them	0	1	2	3	4
21. Someone threatened to hurt me or tell lies about me unless I did something sexual with them	0	1	2	3	4
22. I had the best family in the world	4	3	2	1	0
23. Someone tried to make me do sexual things or watch sexual things	0	1	2	3	4
24. Someone molested me	0	1	2	3	4
25. I believe that I was emotionally abused	0	1	2	3	4
26. There was someone to take me to the doctor if I needed it	4	3	2	1	0
27. I believe that I was sexually abused	0	1	2	3	4
28. My Family was a source of strength and support	4	3	2	1	0

CTQ subscales

- Physical neglect: Q 1, 2, 4, 6 & 26
- Emotional abuse: Q 3, 8, 14, 18 & 25
- Emotional neglect: Q 5, 7, 13, 19 & 28
- Physical abuse: Q 9, 11, 12, 15 & 17
- Sexual abuse: Q 20, 21, 23, 24 & 27
- Minimalizing/denial scale: Q 10, 16 & 22

IBS severity Questionnaire:

In the past month, how often did you have discomfort or pain anywhere in your abdomen?

- Never (0; skip remaining questions)
- Less than one day a month (1)
- One day a month (2)
- Two to three days a month (3)
- One day a week (4)
- More than one day a week (5)
- Every day (6)

Have you had this discomfort or pain for 6 months or longer?

- No (0)
- Yes (1)

How often did this discomfort or pain get better or stop after you had a bowel movement?

- Never or rarely (0)
- Sometimes (1)
- Often (2)
- Most of the time (3)
- Always (4)

When this discomfort or pain started, did you have more frequent bowel movements?

- Never or rarely (0)
- Sometimes (1)
- Often (2)
- Most of the time (3)
- Always (4)

When this discomfort or pain started, were your stools (bowel movements) looser?

- Never or rarely (0)
- Sometimes (1)
- Often (2)
- Most of the time (3)
- Always (4)

When this discomfort or pain started, how often did you have harder stools?

- Never or rarely (0)
- Sometimes (1)
- Often (2)
- Most of the time (3)
- Always (4)

In the past month, how often did you have hard or lumpy stools?

- Never or rarely (0)
- Sometimes (1)
- Often (2)
- Most of the time (3)
- Always (4)

In the past month, how often did you have loose, mushy or watery stools?

- Never or rarely (0)
- Sometimes (1)

- Often (2)
- Most of the time (3)
- Always (4)

Do you have abdominal pain at this time?
- No (0)
- Yes (1)

If yes, how severe in your abdominal pain?
- No pain (0)
- Mild (1)
- Moderately (2)
- Severe (3)
- Very severe (4)

Do you currently have abdominal fullness, bloating or swelling?
- No (0)
- Yes (1)

If yes, how severe is your abdominal fullness, bloating or swelling?
- None (0)
- Mild (1)
- Moderately (2)
- Severe (3)
- Very severe (4)

How satisfied are you with your bowel habit?
- Very unhappy (0)
- Unhappy (1)
- Quite happy (2)
- Happy (3)
- Very happy (4)

How much do your Gastrointestinal Symptoms affect or interfere with your life in general?
- Not at all (0)
- Not much (1)
- Quite a lot (2)

How much do you currently feel the urge to have a bowel movement?
- Not at all (0)
- Not much (1)
- Quite a lot (2)

Food Frequency Questionnaire

The questionnaire is organised into 9 different food categories and you will be asked to record your average frequency of consumption of each food item over the last year.

YOUR DIET OVER THE LAST YEAR

For each food there is an amount shown, either what we think is a "medium serving" or a common household unit such as a slice or teaspoon. Please put a tick in the box to indicate how often, on average, you have eaten the specified amount of each food, to the nearest whole number during the past year i.e. from when you receive this questionnaire to the same month the previous year. Please estimate your average food use as best you can. Please answer every question, do not leave ANY lines blank.

Please read the questions and instructions carefully and complete each section to the best of your ability.

MEAT, FISH & POULTRY (Medium serving – the size of a deck of cards)	**Never or less than once per month**	**1-3 per month**	**Once a week**	**2-4 per week**	**5-6 per week**	**Once a day**	**2-3 per day**	**4-5 per day**	**6+ per day**
Beef: roast									
Beef: steak									
Beef: mince									
Beef: stew									
Beef burger (1 burger)									
Pork: roast									
Pork: chops									
Pork: slices/ escalope									
Lamb: roast									
Lamb: chops									
Lamb: stew									
Chicken portion or other poultry e.g. turkey: Roast									
Breaded chicken, chicken nuggets, chicken burger									
Bacon									
Ham									
Corned beef, Spam, Luncheon									

meats									
Sausages, Frankfurters (1 sausage)									
Savoury pies (e.g. meat pie, pork pie, steak & kidney pie, sausage rolls)									
Liver, heart, kidney									
Liver paté									
Fish fried in batter, as in fish and chips									
Fish fried in breadcrumbs									
Oven baked/grilled fish (in breadcrumbs or batter)									
Fish fingers/fish cakes									
Other white fish, fresh or frozen (e.g. cod, haddock, plaice, sole, halibut, coli)									
Oily fish, fresh or canned (e.g. mackerel, kippers, tuna, salmon, sardines, herring)									
Shellfish (e.g. crab, prawns, mussels)									

BREAD AND SAVOURY BISCUITS (One slice or one biscuit)	**Never or less than once per month**	**1-3 per month**	**Once a week**	**2-4 per week**	**5-6 per week**	**Once a day**	**2-3 per day**	**4-5 per day**	**6+ per day**

White bread and rolls (including ciabatta and panini bread)									
Brown bread and rolls									
Wholemeal bread and rolls									
Cream crackers, cheese biscuits									
Crisp bread, e.g. Ryvita									
Pancakes, muffins, oatcakes									

CEREALS (One medium sized bowl)	**Never or less than once per month**	**1-3 per month**	**Once a week**	**2-4 per week**	**5-6 per week**	**Once a day**	**2-3 per day**	**4-5 per day**	**6+ per day**
Porridge, Readybrek									
All Bran, Weetabix, Shredded Wheat									
Branflakes, Bran Buds									
Cornflakes, Rice Krispies									
Muesli (e.g. Country Store, Alpen, sugar coated									
Sugar Coated Cereals (e.g. Frosties, Crunchy Nut Cornflakes, Crunchy Sugar Coated Muesli)									

POTATOES, RICE AND PASTA	**Never or less than**	**1-3 per month**	**Once a week**	**2-4 per week**	**5-6 per week**	**Once a day**	**2-3 per day**	**4-5 per day**	**6+ per day**

(Medium serving – about a cupful)	**once per month**								
Boiled, instant or jacket potatoes									
Mashed potatoes									
Chips									
Roast potatoes									
Potato salad									
White rice									
Brown rice									
White/yellow or green pastas (e.g. spaghetti, macaroni, noodles)									
Wholemeal pasta									
Lasagne (meat based)									
Lasagne (vegetarian)									
Moussaka									
Pizza									
Macaroni Cheese									

DAIRY PRODUCTS AND FATS	**Never or less than once per month**	**1-3 per month**	**Once a week**	**2-4 per week**	**5-6 per week**	**Once a day**	**2-3 per day**	**4-5 per day**	**6+ per day**
Cream (Tablespoon)									
Full-fat yoghurt or Greek-style yoghurt (125g carton)									
Low-fat yoghurt, fromage frais (125g carton)									
Dairy desserts (125g carton)									
Cheddar cheese (medium serving)									

Brie, Edam type cheese (medium serving)									
Low-fat cheddar cheese (medium serving)									
Cottage cheese, cream cheese, low-fat soft cheese (medium serving)									
Eggs as boiled, fried, scrambled, poached (one)									
Quiche (medium serving)									
Light salad cream or light mayonnaise (tablespoon)									
Salad cream, mayonnaise (Tablespoon)									
French dressing (tablespoon)									
Other salad dressing (Tablespoon)									

THE FOLLOWING ON BREAD OR VEGETABLES	**Never or less than once per month**	**1-3 per month**	**Once a week**	**2-4 per week**	**5-6 per week**	**Once a day**	**2-3 per day**	**4-5 per day**	**6+ per day**
Butter (teaspoon)									
Lite Butter e.g. Dawn Lite, Connacht Gold (teaspoon)									
Sunflower margarine e.g. Flora (teaspoon)									
Low-fat margarine (e.g.									

Low-low)									
Cholesterol Lowering Spreads e.g. Flora Pro Active, Dairy Gold Heart (teaspoon)									
Cream & Vegetable Oil spread e.g. Golden Pasture, Kerrymaid, Dairy Gold – teaspoon									
Olive oil spread e.g. Golden Olive (teaspoon)									

FRUIT (1 Fruit or medium serving)	**Never or less than once per month**	**1-3 per month**	**Once a week**	**2-4 per week**	**5-6 per week**	**Once a day**	**2-3 per day**	**4-5 per day**	**6+ per day**
Apples									
Pears									
Oranges, Satsumas, Mandarins									
Grapefruit									
Bananas									
Grapes									
Melon									
Peaches, Plums, Apricot									
Strawberries, Raspberries, Kiwi fruit									
Tinned fruit									
Dried fruit e.g. raisins									
Frozen fruit									

VEGETABLES Fresh, frozen or tinned (Medium serving – 2 tablespoons)	**Never or less than once per month**	**1-3 per month**	**Once a week**	**2-4 per week**	**5-6 per week**	**Once a day**	**2-3 per day**	**4-5 per day**	**6+ per day**

Carrots									
Spinach									
Broccoli, Spring Greens, Kale									
Brussel Sprouts									
Cabbage									
Peas									
Green Beans, Broad Beans, Runner Beans									
Marrow, Courgettes									
Cauliflower									
Parsnips, Turnips									
Leeks									
Onions									
Garlic									
Mushrooms									
Sweet Peppers									
Beansprouts									
Green salad, Lettuce									
Cucumber, Celery									
Watercress									
Tomatoes									
Sweetcorn									
Beetroot									
Coleslaw									
Avocado									
Baked Beans									
Dried lentils, beans, peas									
Tofu, Soya Meat, TVP, Vegeburger									

SWEETS AND SNACKS (Medium serving)	**Never or less than once per month**	**1-3 per month**	**Once a week**	**2-4 per week**	**5-6 per week**	**Once a day**	**2-3 per day**	**4-5 per day**	**6+ per day**
Chocolate coated sweet biscuits e.g. digestive (one)									
Plain sweet biscuits e.g. Marietta,									

digestives, rich tea (one)									
Cakes e.g. fruit, sponge									
Scones, flapjacks									
Buns, pastries e.g. croissants, doughnuts									
Fruit pies, tarts, crumbles									
Sponge puddings									
Milk puddings e.g. rice, custard, trifle									
Ice cream, choc ices, Frozen desserts									
Chocolates, singles or squares									
Sweets, toffees, mints									
Sugar added to tea coffee, cereal (teaspoon)									
Sugar substitute e.g. canderel added to tea coffee, cereal (teaspoon)									
Crisps or other packet snacks									
Peanuts or other nuts									

SOUPS, SAUCES AND SPREADS	**Never or less than once per month**	**1-3 per month**	**Once a week**	**2-4 per week**	**5-6 per week**	**Once a day**	**2-3 per day**	**4-5 per day**	**6+ per day**
Vegetable soups: Homemade/Fresh (1 bowl)									
Vegetable soups: Tinned/packet (1 bowl)									

Meat or cream soups: Homemade/Fresh (1 bowl)									
Meat or cream soups: Tinned/packet (1 bowl)									
Sauces e.g. white sauce, cheese sauce, gravy (Tablespoon)									
Tomato based sauces e.g. pasta sauces									
Curry-type sauces									
Pickles, chutney (Tablespoon)									
Marmite, Bovril (Tablespoon)									
Jam, marmalade, honey, syrup (teaspoon)									
Peanut butter (teaspoon)									

DRINKS	**Never or less than once per month**	**1-3 per month**	**Once a week**	**2-4 per week**	**5-6 per week**	**Once a day**	**2-3 per day**	**4-5 per day**	**6+ per day**
Tea (cup)									
Coffee instant (cup)									
Coffee, decaffeinated (cup)									
Coffee whitener e.g. coffee-mate (teaspoon)									
Cocoa, Hot Chocolate (cup)									
Horlicks, Ovaltine (cup)									
Wine (glass)									
Beer, Larger or Cider (half pint)									
Alcopops e.g. Bacardi Breezer (bottle)									
Port, Sherry, Vermouth, Liqueurs (glass)									

Spirits e.g. Gin, Whiskey (single measure)									
Low calorie or diet soft drink, fizzy (glass)									
Fizzy soft drinks e.g. Coca Cola (glass)									
Pure fruit drinks e.g. orange juice (small glass)									
Fruit squash (small glass)									

Food Knowledge Questionnaire

Here we are interested in finding out about your knowledge of food.

(1) Advice from the Health Experts
The first few questions are about what advice you think experts are giving us

(a) Do you think health experts recommend that people should be eating more, the same amount, or less of these foods? (*Fill in one box per food)*

	More	Same	Less	Not sure
Vegetables				
Sugary foods				
Meat				
Starchy foods				
Fatty foods				
High fibre foods				
Fruit				
Salty foods				

(b) How many servings of fruit and vegetables a day do you think experts are advising people to eat? (One serving could be, for example, an apple or a handful of chopped carrots)

(c) Which fat do experts say is most important for people to cut down on? *(Only fill one box)*

- Monounsaturated fat
- Poly unsaturated fat
- Saturated fat
- Not sure

(d) What version of dairy foods do experts say people should eat? *(Only fill one box)*

- Full fat
- Lower fat
- Mixture of full fat and lower fat
- Neither, dairy foods should be cut out
- Not sure

(2) Food Groups and Nutritional Content of Foods

This section is concerned with food groups and the nutritional content of foods.

(a) Do you think these foods are *high or low in added sugar*? (*Fill in one box per food)*

	High	Low	Not sure
Bananas			
Unflavoured yogurt			
Ice-cream			
Orange juice			
Tomato ketchup			
Tinned fruit in natural juice			

(b) Do you think these foods are *high or low in fat*? (*Fill in one box per food)*

	High	Low	Not sure
Pasta (without sauce)			
Low fat spread			
Baked beans			
Lunch/sandwich meat (e.g. corned beef)			
Honey			
Meat pastry pie			
Nuts			
Bread			
Cottage cheese			
Polyunsaturated margarine			

(c) Do you think experts put these in the starchy foods group? (*Fill in one box per food)*

	High	Low	Not sure
Cheese			
Pasta			
Butter			
Nuts			
Rice			
Porridge			

(d) Do you think these foods are high or low in salt? (*Fill in one box per food)*

	High	Low	Not sure
Sausages			
Pasta			
Kippers			
Red meat			

Frozen vegetables			
Cheese			
Tinned soup			

(e) Do you think these foods are *high or low in protein*? (*Fill in one box per food)*

	High	Low	Not sure
Chicken			
Cheese			
Fruit			
Baked beans			
Butter			
Cream			

(f) Do you think these foods are *high or low in fibre/roughage*? (*Fill in one box per food)*

	High	Low	Not sure
Cornflakes			
Bananas			
Eggs			
Red meat			
Broccoli			
Nuts			
Fish			
Baked potatoes with skins			
Chicken			
Baked beans			

(g) Do you think these fatty foods are *high or low in saturated fat*? (*Fill in one box per food)*

	High	Low	Not sure
Mackerel			
Whole milk			
Olive oil			
Red meat			
Broccoli			
Sunflower margarine			
Chocolate			

(h) Some foods contain a lot of fat but no cholesterol

- Agree
- Disagree
- Not sure

(i) Do you think experts call these a healthy alternative to red meat? (*Fill in one box per food)*

	Yes	No	Not sure
Liver pate			

Lunch/sandwich meat (e.g. corned beef)			
Baked beans			
Nuts			
Low fat cheese			
Quiche			

(j) A glass of unsweetened fruit juice counts as a helping of fruit

- Agree
- Disagree
- Not sure

(k) Saturated fats are mainly found in: *(only fill one box)*

- Vegetable oils
- Dairy products
- Both
- Not sure

(l) Brown sugar is a healthy alternative to white sugar

- Agree
- Disagree
- Not sure

(m) There is more protein in a glass of whole milk than in a glass of skimmed milk

- Agree
- Disagree
- Not sure

(n) Polyunsaturated margarine contains less fat than butter

- Agree
- Disagree
- Not sure

(o) Which of these breads contain the most vitamins and minerals? *(Only fill one box)*

- White
- Brown
- Wholegrain
- Not sure

(p) Which do you think is higher in calories: butter or regular margarine? *(Only fill one box)*

- Butter
- Regular margarine
- Both the same
- Not sure

(q) A type of oil which contains mostly monounsaturated fat is: *(only fill one box)*

- Coconut oil
- Sunflower oil
- Olive oil
- Palm oil
- Not sure

(r) There is more calcium in a glass of whole milk than a glass of skimmed milk

- Agree
- Disagree
- Not sure

(s) Which one of the following has the most calories for the same weight? *(Only fill one box)*

- Sugar
- Starchy foods
- Fibre/roughage
- Fat
- Not sure

(t) Harder fats contain more: *(only fill one box)*

- Monounsaturated
- Polyunsaturated
- Saturates
- Not sure

(u) Polyunsaturated fats are mainly found in: *(only fill one box)*

- Vegetable oils
- Dairy products
- Both
- Not sure

(3) Food Choice

The next few items are about choosing foods.
Please answer what is being asked and not whether you like or dislike the food!
For example, suppose you were asked……
'If a person wanted to cut down on fat, which cheese would be best to eat?'
(a) Cheddar cheese
(b) Camembert
(c) Cream cheese
(d) Cottage cheese
If you didn't like cottage cheese, but knew it was the right answer, you would still fill in the box for cottage cheese.

(a) What is the best choice for a low fat, high fibre snack? *(Only fill one box)*

- Diet strawberry yoghurt
- Raisins
- Muesli bars
- Wholemeal crackers and cheddar cheese

(b) What is the best choice for a low fat, high fibre light meal? *(Only fill one box)*

- Grilled chicken
- Cheese with wholemeal toast
- Beans on wholemeal toast
- Quiche

(c) Which kind of sandwich is healthier? *(Only fill one box)*

- Two thick slices of bread with a thin slice of cheddar cheese filling
- Two thin slices of bread with a thick slice of cheddar cheese filling

(d) Many people eat spaghetti Bolognese (pasta with tomato and meat sauce). Which option is healthier? *(Only fill one box)*

- A large amount of pasta with a little sauce on top
- A small amount of pasta with a lot of sauce on top

(e) If a person wanted to reduce the amount of fat in their diet, which would be the best choice? *(Only fill one box)*

- Steak, grilled
- Sausages, grilled
- Turkey, grilled
- Pork chop, grilled

(f) If a person wanted to reduce the amount of fat in their diet, but didn't want to give up chips, which one would be the best choice? *(Only fill one box)*

- Thick cut chips
- Thin cut chips
- Crinkle cut chips

(g) If a person felt like something sweet, but was trying to cut down on sugar, which would be the best choice? *(Only fill one box)*

- Honey on toast
- A cereal snack bar
- Plain digestive biscuit
- Banana with plain yoghurt

(h) Which of these would be the healthiest pudding? *(Only fill one box)*

- Baked apple
- Strawberry yoghurt

- Wholemeal crackers with cheddar cheese
- Carrot cake with cream cheese topping

(i) Which cheese would be the best choice as a lower fat option? *(Only fill one box)*

- Plain cream cheese
- Edam
- Cheddar
- Stilton

(j) If a person wanted to reduce the amount of salt in their diet, which would be the best choice? *(Only fill one box)*

- Ready-made frozen shepherd's pie
- Gammon with pineapple
- Mushroom omelette
- Stir fry vegetables with soy sauce

(k) Which one of these would be the right portion size for a serving of cheese? *(Only fill one box)*

- 1 match-box size portion
- 2 match-box portion
- Palm of the hand

(l) Which one of these would be the right portion size for a serving of peanut butter? *(Only fill one box)*

(a) 1 teaspoon (5ml)
(b) 2 teaspoons (10ml)
(c) 3 teaspoons (15ml)

(4) **Diet and Disease**

This section is about the relationship between diet and health problems or diseases.

(a) Are you aware of any major health problems or diseases that are related to a low intake of fruit and vegetables?

- Yes
- No
- Not sure

(b) If yes, what diseases or health problems do you think are related to a *low intake of fruit and vegetables?*

_____
_____

(c) Are you aware of any major health problems or diseases that are related to a *low intake of fibre?*

- Yes
- No
- Not sure

(d) If yes, what diseases or health problems do you think are related to *a low intake of fibre*?

(e) Are you aware of any major health problems or diseases that are related to *how much sugar* people eat?

- Yes
- No
- Not sure

(f) If yes, what diseases or health problems do you think are related to sugar?

_____

(g) Are you aware of any major health problems or diseases that are related to *how much salt or sodium* people eat?

- Yes
- No
- Not sure

(h) If yes, what diseases or health problems do you think are related to salt?

(i) Are you aware of any major health problems or diseases that are related to the *amount of fat* people eat?

- Yes
- No
- Not sure

(j) If yes, what diseases or health problems do you think are related to fat?

_____

(k) Do you think these help to reduce the chances of getting certain kinds of cancer? *(Answer each one)*

	Yes	No	Not sure
Eating more fibre			
Eating less sugar			
Eating less fruit			
Eating less salt			
Eating more fruit & vegetables			
Eating less preservatives/additives			

(l) Do you think these help prevent heart disease? (*Answer each one*)

	Yes	No	Not sure
Eating more fibre			
Eating less saturated food			
Eating less salt			
Eating more fruit & vegetables			
Eating less preservatives/additives			

(m) Which one of these is more likely to raise people's blood cholesterol level? *(Only fill one box)*

- Antioxidants
- Polyunsaturated fats
- Saturated fats
- Cholesterol in the diet
- Not sure

(n) Have you heard of antioxidant vitamins?

- Yes
- No

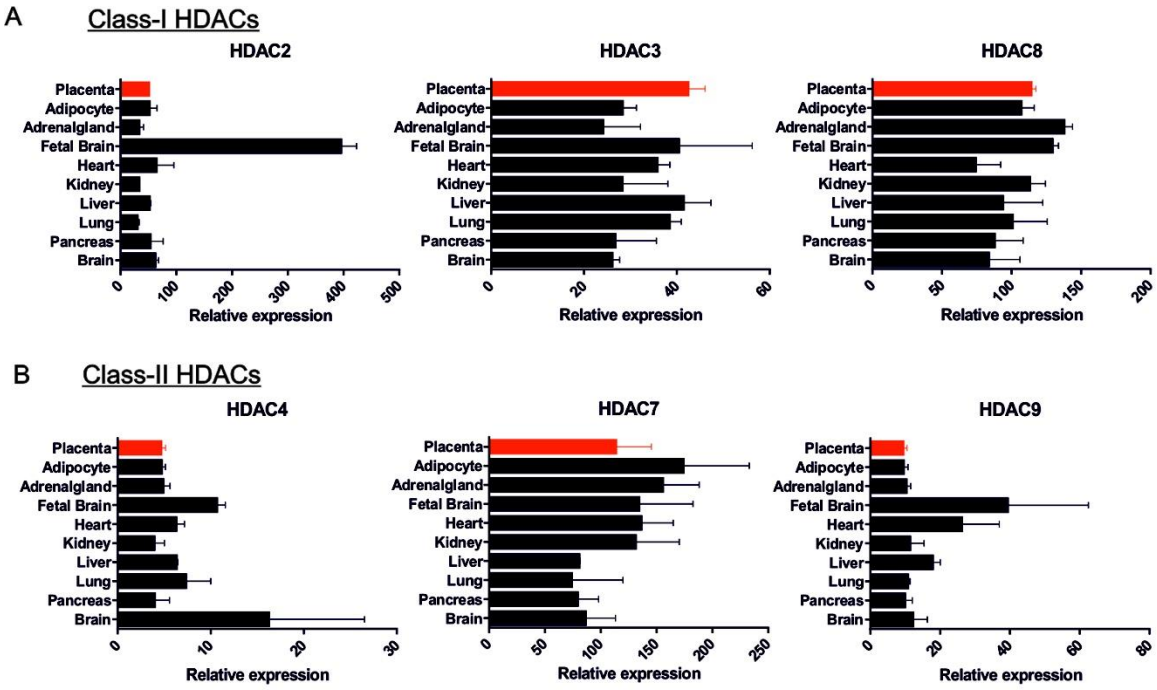
(o) If YES to question J46, do you think these vitamins are antioxidant vitamins? (*Answer each one*)

	Yes	No	Not sure
Vitamin A			
B complex vitamins			
Vitamin C			
Vitamin D			
Vitamin E			
Vitamin K			

Appendix B:

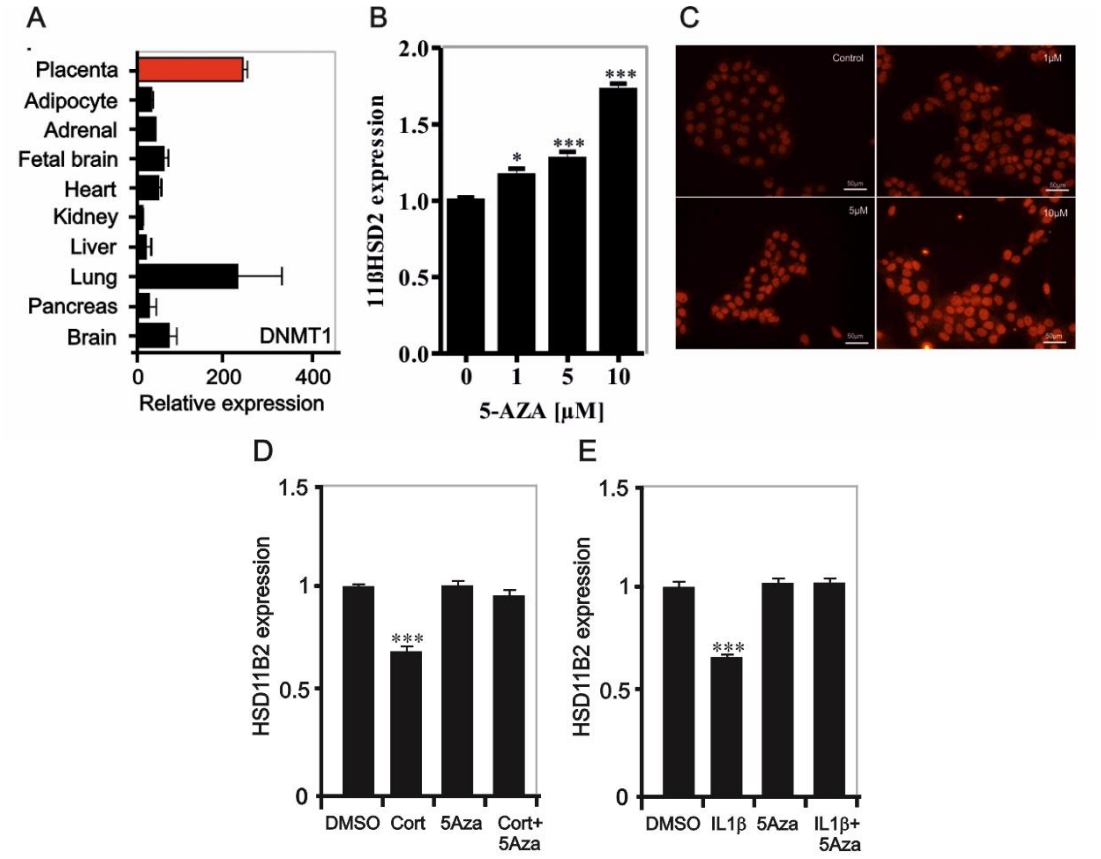
Supplementary Information for Chapter 3:

Figure 1:



Supplementary Figure 1: Expression levels of different classes of HDACs: (A,B) Expression data from the BioGPS database showing the relative expression of (A) Class-I HDACs, HDAC 2, 3 and 8 and (B) Class-IIa HDACs, HDAC 4, 7 and 9 in the placenta (red) relative to multiple human tissues and fetal brain.

Figure 2:



Description: DNMT Inhibitor on HSD11B2 regulation in the placenta (A) Expression data from the BioGPS database showing the relative expression of a DNMT1in the placenta (red) relative to multiple human tissues and fetal brain. (B) Graphical representation of HSD11B2 expression in JEG-3 cells treated with 0-10mM of DNMT1 inhibitor 5-aza-2'-deoxycytidine (5-AZA) for 24h. (D) Representative photomicrographs of JEG-3 cells immunocytochemically stained for HSD11B2 after treatment with (0-10µM) 5-AZA for 24h. (D, E) Graphical representation and of HSD11B2 expression in JEG-3 cells treated with 2mM Cort (D) or (E) 10ng/ml IL1b in the presence or absence of 10mM 5-AZAfor 24h. Data are expressed as mean ± SEM (*** $p < 0.001$ compared to control; one-way ANOVA with *post-hoc* Tukey's test; N = 3). Scale bar = 50µm.

Appendix C:

Supplementary Information for Chapter 4:

Table 1: Prenatal stress, anxiety and depression and other neonatal outcomes

Perceived Stress Scale		
	OR (CI ; *p*-value)	aOR (CI ; *p*-value)
Birth centiles	0.429 (0.115 – 1.606 ; 0.209)	0.395 (0.104 – 1.501 ; 0.173)
Neonatal Resuscitation	0.404 (0.109 – 1.501 ; 0.176)	0.460 (0.122 – 1.743 ; 0.253)
NICU Admission	1.405 (0.225 – 8.774 ; 0.716)	1.572 (0.240 – 10.312 ; 0.637)
Stat Trait Anxiety Inventory		
	OR (CI ; *p*-value)	aOR (CI ; *p*-value)
Birth centiles	1.279 (0.407 – 4.019 ; 0.673)	1.272 (0.396 – 4.080 ; 0.686)
Neonatal Resuscitation	1.142 (0.368 – 3.544 ; 0.818)	1.206 (0.375 – 3.873 ; 0.753)
NICU Admission	4.448 (0.708 – 27.954 ; 0.111)	1.109 (0.930 – 1.323 ; 0.250)
Edinburgh Postnatal Depression Scale		
	OR (CI ; *p*-value)	aOR (CI ; *p*-value)
Birth centiles	0.650 (0.172 – 2.456 ; 0.525)	0.602 (0.152 – 2.382 ; 0.469)
Neonatal Resuscitation	0.618 (0.164 – 2.324 ; 0.476)	0.654 (0.165 – 2.593 ; 0.546)
NICU Admission	2.071 (0.329 – 13.032 ; 0.438)	1.857 (0.277 – 12.460 ; 0.524)
Cumulative		
	OR (CI ; p-value)	aOR (CI ; p-value)
Birth centiles	0.363 (0.045 – 2.945 ; 0.342)	0.334 (0.039 – 2.855 ; 0.316)
Neonatal Resuscitation	0.764 (0.158 – 3.689 ; 0.738)	0.795 (0.158 – 4.003 ; 0.781)
NICU Admission	4.444 (0.685 – 28.828 ; 0.118)	1.007 (0.878 – 1.155 ; 0.919)

Table 1: Relationship between scoring in the PSS, STAI, and/or EPDS with infants in the lower 10th birth centile, neonatal resuscitations and NICU Admissions. Binary logistic regression shows no significant difference between groups P>0.05. Adjusted for maternal age, BMI and social class.

Figure 1: Participants groupings based on Questionnaire Scorings

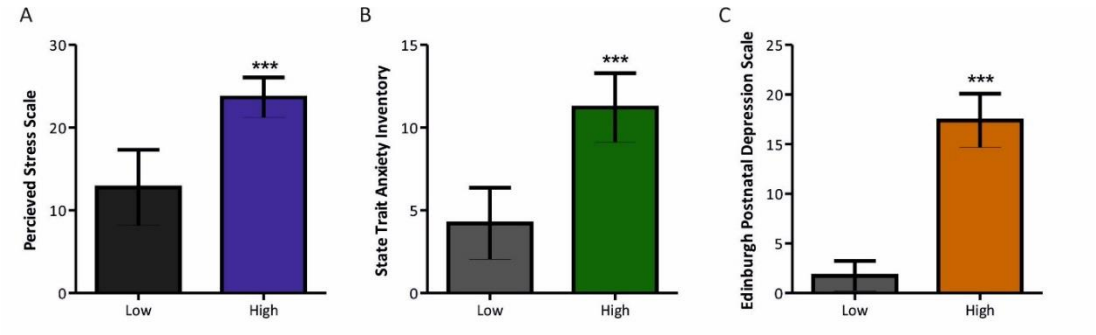


Figure 1: Graphical representation of Perceived Stress Scale (A), State Trait Anxiety Inventory (B) and Edinburgh Postnatal Depression Scale (C) scores between women ranked in the low and high categories. Unpaired student t-test show a significant difference between groups $P < 0.001$ ***, N=121.

Table 2: Global sample means of measured variables

Measure	*Population*	*Low*	*High*
	Mean ± SD (N)	Mean ± SD (N)	Mean ± SD (N)
Perceived Stress Scale	15.88 ± 6.5 (121)	12.75 ± 4.5 (81)	23.65 ± 2.4 (40)
State Trait Anxiety Inventory	6.17 ± 3.8 (121)	4.20 ± 2.1 (87)	11.21 ± 2.0 (34)
Edinburgh Postnatal Depression Scale	9.07 ± 5.6 (121)	6.52 ± 3.7 (90)	16.48 ± 2.9 (31)

Table 2: Global sample means of measured variables expressed as mean ± SD. High stress (PSS ≥ 20), low stress (PSS ≤ 19). High anxiety (STAI ≥ 9), low anxiety (STAI ≤ 8). High probability of depression (EPDS ≥ 13), low probability of depression (EPDS ≤ 12).

Figure 2: Intercorrelations of questionnaire scores

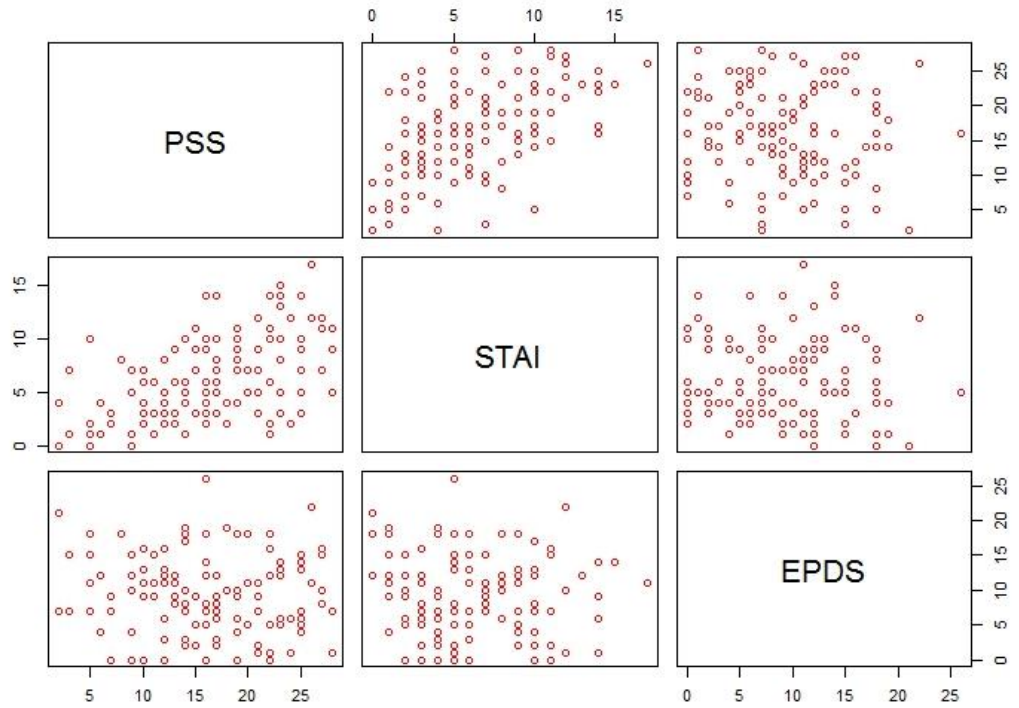


Figure 2: Correlation matrices showing relationship between PSS, STAI and EPDS scores. Pearson correlation shows each questionnaire is significantly correlated. PSS and STAI (r = 0.521; P < 0.001 ***), PSS and EPDS (r = 0.724; P < 0.001 ***) and STAI and EPDS (r = 0.678; P > 0.001 ***). RStudio Graphics.

Table 3: Prenatal distress with number of newborn adversities

Perceived Stress Scale		
	OR (CI ; *p*-value)	aOR (CI ; *p*-value)
1 adverse outcome	0.669 (-1.245 – 0.442; 0.351)	0.648 (-1.302 - 0.435; 0.328)
2 adverse outcomes	0.341 (-3.276 – 1.122; 0.337)	0.335 (-3.310 – 1.124; 0.334)
3 adverse outcomes	0.852 (-2.607 – 2.287; 0.898)	0.878 (-2.628 – 2.468; 0.474)
Stat Trait Anxiety Inventory		
	OR (CI ; *p*-value)	aOR (CI ; *p*-value)
1 adverse outcome	0.914 (-0.974 – 0.794; 0.842)	0.860 (-1.076 – 0.773; 0.748)
2 adverse outcomes	1.325 (-1.492 – 2.055; 0.756)	1.440 (-1.445 – 2.174; 0.693)
3 adverse outcomes	1.325 (-2.174 – 2.736; 0.822)	0.809 (-2.769 – 2.346; 0.871)
Edinburgh Postnatal Depression Scale		
	OR (CI ; *p*-value)	aOR (CI ; *p*-value)
1 adverse outcome	0.914 (-0.974 – 0.794; 0.842)	0.914 (-1.019 – 0.839; 0.849)
2 adverse outcomes	--	--
3 adverse outcomes	1.325 (-2.174 – 2.736; 0.822)	1.090 (-2.517 – 2.690; 0.948)
Cumulative		
	OR (CI ; p-value)	aOR (CI ; p-value)
1 adverse outcome	1.025 (-1.056 – 1.105; 0.965)	0.986 (-1.148 – 1.120; 0.981)
2 adverse outcomes	--	--
3 adverse outcomes	2.818 (-1.449 – 3.521; 0.414)	1.610 (-2.143 – 3.094; 0.722)

Table 3: Odds ratios assessing the relationship between prenatal distress and number of newborn adversities. Newborns were grouped into number of adverse birth outcomes based on six parameters (a) Admission to the NICU (b) Newborn Resuscitation received (c) Delivered before 37 weeks gestation (d) 5 minute Apgar score ≤ 7 (e) Birth Temperature < 36.5°C and (f) Birth Centile ≤ 10 or ≥ 90. Ordinal logistic regression analysis revealed no significant effect (P > 0.05 *). Adjusted for maternal age, BMI and social class.

Table 4: Linear Regression analysis of placental HSD11B2 mRNA expression with maternal distress, demographics and neonatal outcomes

HSD11B2	*R2*	*β-coefficient*	*95% CI*	*p-value*
Birthweight	0.019	0.137	-0.783 – 1.547	0.505
Placental weight	0.037	0.119	-0.144 – 0.382	0.360
PWR	0.030	0.173	0.00 – 0.00	0.408
Birthweight centiles	0.012	0.109	-0.068 – 0.116	0.597
1 min Apgar	0.012	0.109	-0.002 – 0.003	0.597
5 min Apgar	0.011	0.103	-0.001 – 0.002	0.617
Birth Temperature	0.003	0.054	-0.001 – 0.001	0.794
Head Circumference	0.023	0.153	-0.003 – 0.006	0.476
Gravidity	0.000	-0.009	-0.009 – 0.009	0.964
Maternal Age	0.004	-0.064	-0.647 – 0.472	0.472
Maternal BMI	0.062	-0.248	-0.001 – 0.000	0.212
Social Class	0.009	-0.095	-0.954 – 0.594	0.636
PSS	*0.137*	*-0.370*	*-0.051 – 0.001*	*0.058*
STAI	0.070	-0.265	-0.025 – 0.005	0.181
EPDS	0.068	-0.260	-0.038 – 0.008	0.190

Table 4: Placental HSD11B2 expression and continuous variables. Linear regression analysis. P > 0.05

Table 5: Linear Regression analysis of placental NR3C1 mRNA expression with maternal distress, demographics and neonatal outcomes

NR3C1	*R2*	*β-coefficient*	*95% CI*	*p-value*
Birthweight	0.083	-0.288	-1961 – 325.796	0.153
Placental weight	***0.159***	***-0.398***	***-790.258 - -2.667***	***0.049***
PWR	0.086	-0.293	-0.867 – 0.146	0.155
Birthweight centiles	***0.170***	***-0.412***	***-180 - -6.380***	***0.036***
1 min Apgar	0.004	-0.064	-2.870 – 2.111	0.756
5 min Apgar	0.00	0.010	-1.714 – 1.794	0.962
Birth Temperature	0.038	-0.195	-1.267 – 0.455	0.340
Head Circumference	0.042	-0.206	-10.938 – 3.888	0.355
Gravidity	0.003	-0.054	-9.854 – 7.583	0.791
Maternal Age	000	0.015	-547.008 – 588.035	0.941
Maternal BMI	0.023	0.151	-0.292 – 0.635	0.453
Social Class	0.005	0.074	-644.397 – 955.528	0.715
PSS	0.075	0.273	-8.437 – 45.959	0.168
STAI	0.003	0.052	-13.496 – 17.405	0.797
EPDS	0.007	0.084	-18.893 – 28.563	0.678

Table 5: Placental NR3C1 expression and continuous variables. Linear regression analysis. Placental weight and birthweight centiles associated with NR3C1 levels. $P < 0.05$ *

Table 6: Linear Regression analysis of placental FKBP51 mRNA expression with maternal distress, demographics and neonatal outcomes

FKBP51	*R2*	*β-coefficient*	*95% CI*	*p-value*
Birthweight	0.033	-0.183	-2955.102 – 1147.112	0.372
Placental weight	0.109	-0.330	-1010.482 – 106.235	0.107
PWR	0.020	-0.140	-0.959 – 0.487	0.506
Birthweight centiles	*0.150*	*-0.387*	*-308.452 – 0.616*	*0.051*
1 min Apgar	0.011	0.107	-3.244 – 5.473	0.603
5 min Apgar	0.002	0.05	-2.714 – 3.444	0.809
Birth Temperature	0.015	-0.122	-1.985 – 1.086	0.551
Head Circumference	0.049	-0.222	-14.815 – 4.733	0.296
Gravidity	*0.012*	*-0.122*	*-19.559 – 11.174*	*0.579*
Maternal Age	*0.137*	*-0.370*	*-47.048 – 1.302*	*0.063*
Maternal BMI	***0.185***	***0.430***	***3.069 – 42.498***	***0.025***
Social Class	0.023	0.153	-862.086 – 1893.458	0.448
PSS	0.038	0.194	-25.554 – 72.702	0.332
STAI	0.008	0.088	-21.420 – 33.170	0.661
EPDS	0.045	0.212	-1.231 – 3.962	0.289

Table 6: Placental FKBP5 expression and continuous variables. Linear regression analysis. Maternal BMI positively associated with FKBP5 levels. $P < 0.05$ *.

Figure 3: Placental Gene expression, gender and obstetric outcomes

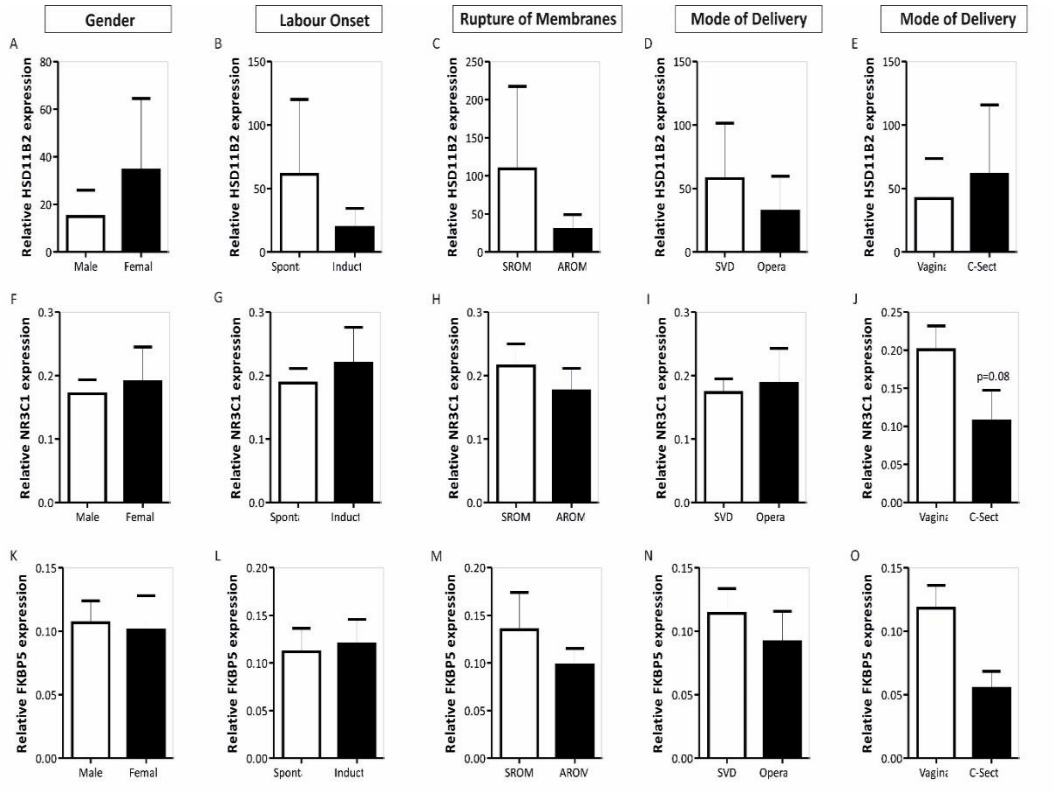


Figure 3: Expression of placental HSD11B2, NR3C1 and FKBP5 in relation newborn sex and obstetric outcomes. No significant differences between groups. Unpaired Student's t-test. $P < 0.005$. Spontaneous (Spont), Induction (Induct), Spontaneous rupture of membranes (SROM), Artificial Rupture of membranes (AROM), Spontaneous vaginal delivery (SVD), Operative delivery (Opera) Vaginal delivery (Vagin) and C-Section delivery (C-Sect).

Appendix D:

Supplementary Information for Chapter 6:

Table 1: Descriptive statistics of continuous variables

Measure	Mean ± SD (N)	Range
Age	30.67 ± 4.4 (105)	19 - 41
BMI 15wks gestation (kg/m^2)	25.43 ± 3.8 (105)	18.1 - 39.0
BMI 20wks gestation (kg/m^2)	26.34 ± 3.6 (105)	18.9 – 39.7
BMI 32wks gestation (kg/m^2)	28.87 ± 3.9 (90)	20.0 – 41.7
Gestational Age at Delivery	39.68 ± 1.3 (105)	34 - 42
1 minute Apgar score	8.57 ± 1.1 (105)	3 - 10
5 minute Apgar score	9.52 ± 0.6 (105)	6 - 10
Birthweight (g)	3548.76 ± 477.5 (105)	2060 - 4980
Birthweight Centiles	49.17 ± 27.1 (105)	1 – 100
Maximum temperature in labour (®C)	37.06 ± 0.48 (86)	36.0 – 38.6

Supplementary table 1: Descriptive statistics of maternal age, body mass index (BMI) and obstetric and neonatal outcomes of study cohort. Data shown are cohort Mean ± Standard deviation (SD) and range.

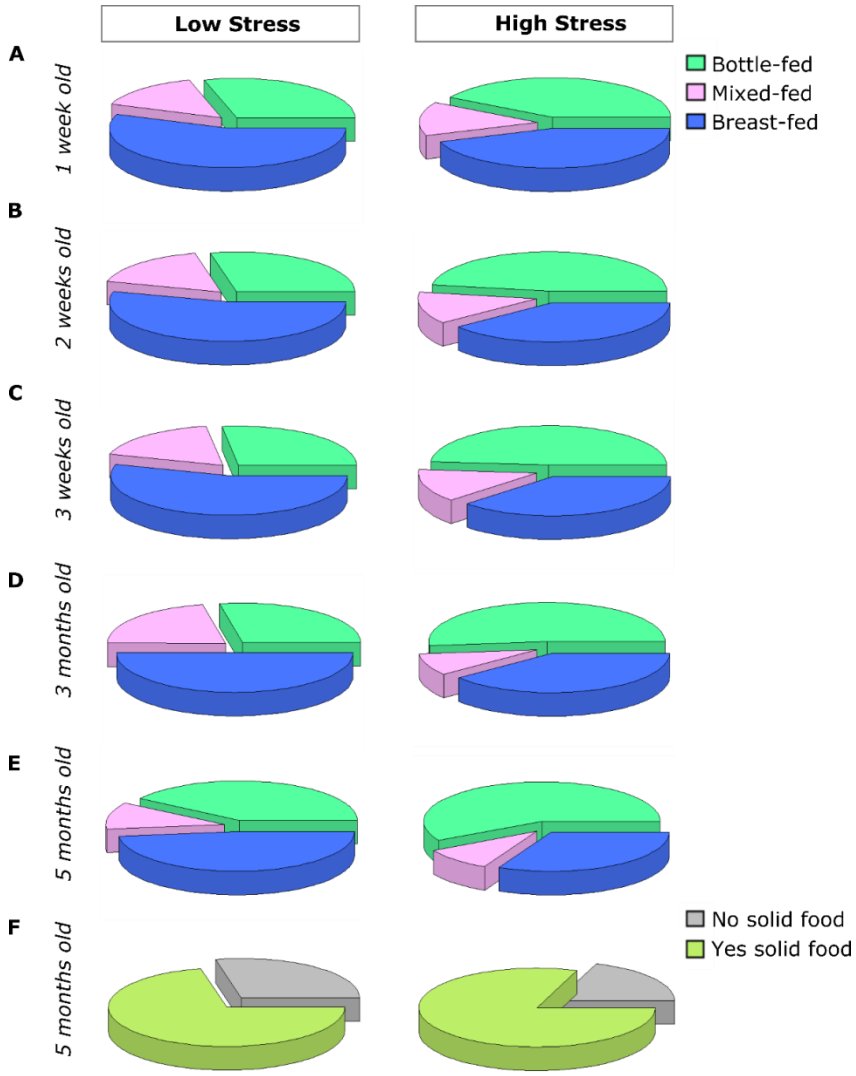
Table 2: Descriptive statistics of categorical variables

Measure	Frequency (N)
Country of birth	
Europe	92.8 (103)
South America	0.9(1)
Africa	0.9 (1)
*Missing	5.4 (6)
Marital status	
Married	56.8 (63)
Defacto	27.0 (30)
Single	10.8 (12)
*Missing	5.4 (6)
Tertiary Education	
Graduated	67.6 (75)
None	25.2 (28)
Still attending	1.8 (2)
*Missing	5.4 (6)
Employment	
Employed	86.2 (96)
Unemployed	8,1 (9)
*Missing	5.4 (6)
Income	
< €21K	8.1 (9)

€21K – €42K	13.5 (15)
€43K – €63K	20.7 (23)
> €64K	49.5 (55)
*Missing	8.1 (9)
Hx IBD	
No	91.9 (103)
Yes	2.7 (3)
*Missing	5.4 (6)
Hx Coeliac disease	
No	92.8 (103)
Yes	1.8 (2)
*Missing	5.4 (6)
Hx IBS	
No	85.6 (95)
Yes	9.0 (10)
*Missing	5.4 (6)
Hx Depression	
No	83.8 (93)
Yes	10.8 (12)
*Missing	5.4 (6)
Sex	
Male	46.8 (52)
Female	47.7 (53)
*Missing	5.4 (6)
Onset of delivery	
Spontaneous	51.4 (57)
Induction	24.3 (27)
Pre-labour caesarean	9.9 (11)
PROM	9.0 (10)
*Missing	5.4 (6)
Mode of delivery	
Unassisted vaginal	39.6 (44)
Operative vaginal	32.4 (36)
Caesarean in labour	12.6 (14)
Pre-labour caesarean	9.9 (11)
*Missing	5.4 (6)

Supplementary table 2: Table shows descriptive statistics of maternal demographics, pregnancy and neonatal outcomes for entire study cohort. Data shown are cohort frequencies. Abbreviations: History (Hx), Irritable Bowel Disease (IBD), Irritable Bowel Syndrome (IBS).

Figure 1: Mode of feeding in infants by second trimester maternla stress



Supplementary Figure 1: Pie Charts showing the percentage of infant feeding practises grouped by maternal stress (Perceived Stress Scale, low < 13, high > 14) in the second trimester. (A) 1 week old low stress versus high stress; 29.4% v 41.6% bottle-fed, 55.8% v 44.4% breast-fed, 14.7% v 13.8% mixed-fed. (B) 2 weeks old low stress versus high stress; 28.5% v 47.3% bottle-fed, 54.2% v 39.4% breast-fed, 17.1% v 13.1% mixed-fed. (C) 3 weeks old low stress versus high stress; 27.2% v 48.6% bottle-fed, 54.5% v 37.8% breast-fed, 18.1% v 13.5% mixed-fed. (D) 3 months old low stress versus high stress; 28.1% v 51.5% bottle-fed, 50% v 39.3% breast-fed, 21.8% v 9.0% mixed-fed. (E) 5 months old low stress versus high stress; 40.7% v 58.0% bottle-fed, 48.1% v 32.2% breast-fed, 11.1% v 9.6% mixed-fed. (F) Eating solids by 5 months old low stress versus high stress; 28.5% v 20% no, 71.4% v 80% high. P > 0.05 Chi Square Test.

Table 3: Maternal gut in the 2nd trimester by 2nd trimester stress

OTU	Relative Abundance (%)		p-value
Maternal gut (2nd trimester)	**Low PSS**	**High PSS**	
Chao1 Index	**405.0**	**356.5**	***t*(44) = 2.01, *p*=0.049**
Simpson Index	0.96	0.96	*t*(44) = -0.26, *p*=0.792
Shannon Index	5.7	5.7	*t*(44) = 0.23, *p*=0.815
Phylogenetic diversity	20.4	18.9	*t*(44) = 1.42, *p*=0.160
Observed Species	315.6	288.9	*t*(44) = 1.59, *p*=0.118
Actinobacteria	4.5	4.4	W=276, p=0.800
Bacteroidetes	21.8	21.4	W=231, p=0.478
Firmicutes	69.8	69.7	W=280, p=0.735
Proteobacteria	1.7	1.8	W=242, p=0.639
Verrucomicrobia	1.3	2.1	W=215.5, p=0.290
Bifidobacteriaceae	2.8	2.5	W=288, p=0.608
Coriobacteriaceae	1.5	1.3	W=271.5, p=0.877
Bacteroidaceae	11.3	12.7	W=191.5, p=0.113
Porphyromonadaceae	3.8	3.0	W=248.5, p=0.741
Prevotellaceae	2.6	2.2	W=314.5, p=0.270
Rikenellaceae	3.3	3.2	W=263, p=0.991
Christensenellaceae	2.0	1.3	W=318.5, p=0.235
Clostridiaceae1	1.5	1.2	W=298.5, p=0.454
Lachnospiraceae	34.2	35.3	W=233, p=0.506
Peptostreptococcaceae	3.0	3.3	W=265, p=0.991
Ruminococcaceae	**21.8**	**18.7**	**W=357, p=0.041**
Erysipelotrichaceae	2.0	2.2	W=278, p=0.766
Acidaminococcaceae	0.8	1.3	W=228, p=0.422
Veillonellaceae	2.4	3.9	W=244, p=0.668
Oxalobacteraceae	**0.0**	**0.0**	**W=345, p=0.044**
Campylobacteraceae	**0.0**	**0.0**	**W=220, p=0.050**
Verrucomicrobiaceae	1.3	2.0	W=214.5, p=0.280
Bifidobacterium	2.8	2.5	W=288, p=0.608
Bacteroides	11.3	12.7	W=191.5, p=0.113
Barnesiella	1.2	1.1	W=249, p=0.749
Prevotella	2.3	1.8	W=270, p=0.902
Alistipes	3.1	3.2	W=255, p=0.853
Christensenellaceae uncultured	2.0	1.2	W=323.5, p=0.194
Clostridium sensu stricto1	1.5	1.2	W=298.5, p=0.454

Anaerostipes	1.3	2.0	W=231, p=0.474
Eubacterium	**0.0**	**0.0**	**W=220, p=0.050**
Blautia	4.4	4.8	W=203, p=0.183
Coprococcus	1.0	1.1	W=254.5, p=0.843
Lachnospiraceaen Icertae Sedis	10.2	11.9	W=216.5, p=0.301
Pseudobutyrivibrio	3.0	2.9	W=297.5, p=0.468
Roseburia	1.6	1.2	W=299, p=0.448
Shuttleworthia	**0.0**	**0.0**	**W=353.5, p=0.044**
Lachnospiraceae uncultured	11.3	10.0	W=295, p=0.506
Peptostreptococcaceae Incertae Sedis	0.9	1.0	W=261.5, p=0.964
Peptostreptococcaceae uncultured	2.0	2.3	W=273.5, p=0.843
Faecalibacterium	6.1	4.8	W=312, p=0.296
Ruminococcaceae Incertae Sedis	1.7	2.0	W=276, p=0.800
Oscillibacter	**0.0**	**0.0**	**W=353.5, p=0.047**
Ruminococcus	1.9	1.4	W=281.5, p=0.708
Subdoligranulum	3.7	2.7	W=312, p=0.296
Ruminococcaceae uncultured	7.9	7.3	W=279, p=0.752
Dialister	2.2	2.8	W=270, p=0.903
Campylobacter	**0.0**	**0.0**	**W=220, p=0.050**
Akkermansia	1.3	2.0	W=214.5, p=0.280

Supplementary table 3: Relative abundance of bacterial OTUs in the *2nd trimester* maternal gut by *maternal stress* in the *2nd trimester*. Listed are all OTUs with an abundance of greater than 1% and significant OTUs with an abundance of less than 1%. Low stress (PSS < 13; N=22), high stress (PSS > 4; N=25). Unpaired students t-test and Mann-Whitney-Wilcoxon test. Abbreviations: Operational taxonomic unit (OTU), Perceived Stress Scale (PSS).

Table 4: Maternal gut in the 2nd trimester by 2nd trimester anxiety

OTU	**Relative Abundance (%)**		**p-value**
Maternal gut (2nd trimester)	**Low STAI**	**High STAI**	
Chao1 Index	382.5	372.6	$t(44) = 0.37$, $p=0.712$
Simpson Index	0.9	0.9	$t(44) = -1.00$, $p=0.319$
Shannon Index	5.6	5.8	$t(44) = -1.03$, $p=0.307$
Phylogenetic diversity	19.7	19.3	$t(44) = 0.25$, $p=0.802$
Observed Species	299.5	300.1	$t(44) = -0.03$, $p=0.974$
Actinobacteria	4.3	4.6	W=250, p=0.766
Bacteroidetes	23.2	20.2	W=274, p=0.836
Firmicutes	68.3	71.2	W=232, p=0.492
Proteobacteria	1.5	2.0	W=211, p=0.251

Verrucomicrobia	2.1	1.4	W=313.5, p=0.280
Bifidobacteriaceae	2.6	2.7	W=260, p=0.939
Coriobacteriaceae	1.3	1.3	W=242, p=0.636
Bacteroidaceae	12.2	12.1	W=220.5, p=0.344
Porphyromonadaceae	3.5	3.3	W=212.5, p=0.262
Prevotellaceae	**3.4**	**1.2**	**W=341, p=0.091**
Rikenellaceae	3.4	3.1	W=246, p=0.719
Bacteroidales247	**0.4**	**0.1**	**W=348, p=0.030**
Christensenellaceae	1.8	1.5	W=260.5, p=0.947
Clostridiaceae1	1.6	1.2	W=272.5, p=0.860
Lachnospiraceae	33.5	35.9	W=216, p=0.299
Peptostreptococcaceae	3.0	3.4	W=264, p=1
Ruminococcaceae	20.4	19.8	W=272, p=0.870
Erysipelotrichaceae	1.7	2.6	W=214.5, p=0.281
Acidaminococcaceae	0.8	1.3	W=222, p=0.348
Veillonellaceae	3.1	3.4	W=296, p=0.488
Verrucomicrobiaceae	2.0	1.4	W=310.5, p=0.311
Bifidobacterium	2.6	2.7	W=260, p=0.939
Slackia	**0.0**	**0.0**	**W=183, p=0.029**
Bacteroides	12.2	12.1	W=220.5, p=0.244
Barnesiella	1.1	1.3	W=196.5, p=0.140
Prevotella	3.0	1.1	W=317, p=0.242
Alistipes	3.2	3.1	W=235, p=0.534
Rikenellaceae RC9 gut group	**0.1**	**0.0**	**W=331.5, p=0.025**
Christensenellaceae uncultured	1.7	1.4	W=261.5, p=0.964
Clostridium sensu stricto1	1.6	1.2	W=272.5, p=0.860
Anaerostipes	1.2	1.9	W=225.5, p=0.403
Blautia	4.7	4.4	W=243, p=0.652
Anaerotruncus	**0.1**	**0.3**	**W=118.5, p=0.001**
Hydrogenoanaero bacterium	**0.0**	**0.0**	**W=201.5, p=0.029**
Erysipelotrichaceae Incertae Sedis	**0.4**	**1.5**	**W=164.5, p=0.029**
Coprococcus	1.0	1.1	W=245, p=0.684
Lachnospiraceaen Icertae Sedis	10.2	11.7	W=213.5, p=0.271
Pseudobutyrivibrio	3.0	3.0	W=270, p=0.903
Roseburia	1.3	1.5	W=221.5, p=0.355
Lachnospiraceae uncultured	10.6	10.7	W=255, p=0.853
Peptostreptococcaceae Incertae Sedis	0.8	1.1	W=227, p=0.422

Peptostreptococcaceae uncultured	2.2	2.3	W=278, p=0.766
Faecalibacterium	5.4	05.4	W=256, p=0.869
Ruminococcaceae Incertae Sedis	2.0	1.7	W=268.5, p=0.929
Ruminococcus	1.6	1.6	W=247.5, p=0.725
Subdoligranulum	3.3	3.0	W=277, p=0.783
Ruminococcaceae uncultured	7.6	7.6	W=261, p=0.956
Dialister	2.2	3.0	W=295, p=0.501
Akkermansia	2.0	1.4	W=310.5, p=0.311

Supplementary table 4: Relative abundance of bacterial OTUs in the *2nd trimester* maternal gut by *maternal anxiety* in the *2nd trimester*. Listed are all OTUs with an abundance of greater than 1% and significant OTUs with an abundance of less than 1%. Low anxiety (STAI < 4; N=24), high anxiety (STAI > 5; N=22). Unpaired students t-test and Mann-Whitney-Wilcoxon test. Abbreviations: Operational taxonomic unit (OTU), State Trait Anxiety Inventory (STAI).

Table 5: Maternal gut in the 2nd trimester by 2nd trimester depressive symptoms

OTU	**Relative Abundance (%)**		**p-value**
Maternal gut (2nd trimester)	**Low EPDS**	**High EPDS**	
Chao1 Index	**392.1**	**326.2**	***t*(44) = 2.11, *p*=0.040**
Simpson Index	0.9	0.9	*t*(44) = 0.84, *p*=0.400
Shannon Index	5.8	5.5	*t*(44) = 1.41, *p*=0.163
Phylogenetic diversity	**20.2**	**16.9**	***t*(44) = 2.33, *p*=0.024**
Observed Species	**310.3**	**262.2**	***t*(44) = 2.13, *p*=0.038**
Euryarchaeota	**0.1**	**0.0**	**W=240, p=0.040**
Actinobacteria	4.4	4.7	W=174, p=0.883
Bacteroidetes	21.2	23.8	W=140, p=0.297
Firmicutes	70.2	67.7	W=210, p=0.438
Cyanobacteria	**0.2**	**0.0**	**W=266.5, p=0.020**
Proteobacteria	1.6	2.3	W=127, p=0.164
Tenericutes	**0.0**	**0.0**	**W=244, p=0.081**
Verrucomicrobia	1.9	1.2	W=206, p=0.496
Methanobacteriaceae	**0.1**	**0.0**	**W=240, p=0.040**
Bifidobacteriaceae	2.7	2.5	W=186, p=0.885
Coriobacteriaceae	1.4	1.1	W=193.5, p=0.729
Bacteroidaceae	**11.4**	**14.9**	**W=112, p=0.072**
Porphyromonadaceae	3.3	3.8	W=119, p=0.1071
Prevotellaceae	2.6	1.5	W=172, p=0.841
Rikenellaceae	3.2	3.4	W=173, p=0.864
Christensenellaceae	1.8	1.0	W=235.5, p=0.143

Clostridiaceae1	1.5	0.8	W=221.5, p=0.274
Lachnospiraceae	**33.5**	**38.6**	**W=108, p=0.056**
Peptostreptococcaceae	3.5	2.0	W=215, p=0.363
Ruminococcaceae	**20.9**	**17.2**	**W=250, p=0.063**
Erysipelotrichaceae	2.4	1.3	W=234, p=0.154
Acidaminococcaceae	1.0	1.2	W=179, p=0.989
Veillonellaceae	3.0	4.2	W=148, p=0.401
Oxalobacteraceae	**0.0**	**0.0**	**W=250.5, p=0.034**
Verrucomicrobiaceae	1.9	2.7	W=203, p=0.548
Methanobrevibacter	**0.1**	**0.0**	**W=240, p=0.040**
Bifidobacterium	1.2	2.5	W=186, p=0.885
Bacteroides	**11.4**	**14.9**	**W=112, p=0.072**
Barnesiella	1.1	1.3	W=133.5, p=0.220
Prevotella	2.3	1.3	W=161.5, p=0.628
Alistipes	3.1	3.4	W=167, p=0.743
Christensenella	**0.1**	**0.0**	**W=262, p=0.029**
Christensenellaceae uncultured	1.7	0.9	W=231.5, p=0.174
Clostridium sensu stricto1	1.5	0.8	W=221.5, p=0.274
Anaerostipes	1.3	2.6	W=119.5, p=0.110
Blautia	4.5	4.8	W=148, p=0.401
Coprococcus	1.1	0.8	W=196.5, p=0.670
Howardella	**0.0**	**0.0**	**W=240, p=0.040**
Lachnospiraceaen Icertae Sedis	10.5	12.6	W=136, p=0.246
Pseudobutyrivibrio	2.7	4.1	W=155, p=0.514
Roseburia	1.4	1.3	W=171, p=0.820
Lachnospiraceae uncultured	10.6	10.8	W=170, p=0.803
Peptostreptococcaceae Incertae Sedis	1.0	0.8	W=183, p=0.946
Peptostreptococcaceae uncultured	2.5	1.1	W=229, p=0.196
Faecalibacterium	**4.7**	**7.7**	**W=98, p=0.029**
Ruminococcaceae Incertae Sedis	2.1	1.0	W=228, p=0.205
Ruminococcus	1.8	0.8	W=237.5, p=0.129
Subdoligranulum	3.4	2.5	W=219.5, p=0.299
Ruminococcaceae uncultured	**8.4**	**4.8**	**W=265, p=0.022**
Dialister	2.4	3.1	W=178, p=0.968
Oxalobacter	**0.0**	**0.0**	**W=246, p=0.043**
Akkermansia	1.9	1.2	W=203, p=0.548

Supplementary table 5: Relative abundance of bacterial OTUs in the *2nd trimester* maternal gut by *maternal depressive symptoms* in the *2nd trimester*. Listed are all OTUs with an abundance of greater than 1% and significant OTUs with an abundance of less than 1%. Low depressive symptoms (EPDS < 8; N=36), high depressive symptoms (EPDS > 9; N=10). Unpaired students t-test and Mann-Whitney-Wilcoxon test. Abbreviations: Operational taxonomic unit (OTU), Edinburgh Postnatal Depression Scale (EPDS).

Table 6: Maternal gut in the 2nd trimester by 2nd trimester cortisol

OTU	**Relative Abundance (%)**		**p-value**
Maternal gut (2nd trimester)	**Low CAR**	**High CAR**	
Chao1 Index	357.3	390.2	*t*(36) = -1.19, *p*=0.2391
Simpson Index	0.9	0.9	*t*(36) = -0.82, *p*=0.414
Shannon Index	5.6	5.8	*t*(36) = -1.61, *p*=0.115
Phylogenetic diversity	18.5	20.3	*t*(36) = -1.41, *p*=0.165
Observed Species	283.15	311.8	*t*(36) = -1.44, *p*=0.156
Actinobacteria	3.4	3.9	W=194, p=0.693
Bacteroidetes	21.9	22.1	W=169, p=0.761
Firmicutes	70.6	70.3	W=193, p=0.717
Proteobacteria	1.7	1.9	W=170, p=0.783
Verrucomicrobia	1.7	1.0	W=189.5, p=0.792
Bifidobacteriaceae	1.9	2.3	W=205, p=0.478
Coriobacteriaceae	1.0	1.4	W=177, p=0.907
Bacteroidaceae	14.3	11.2	W=205, p=0.478
Porphyromonadaceae	3.0	2.9	W=180, p=1
Prevotellaceae	1.3	3.9	W=164, p=0.649
Rikenellaceae	**2.7**	**3.6**	**W=121, p=0.087**
Christensenellaceae	1.2	1.8	W=138.5, p=0.230
Clostridiaceae1	1.5	1.2	W=203, p=0.510
Lachnospiraceae	35.2	36.0	W=171, p=0.806
Peptostreptococcaceae	3.4	2.4	W=197, p=0.633
Ruminococcaceae	20.5	20.4	W=185, p=0.896
Erysipelotrichaceae	1.8	2.3	W=199.5, p=0.578
Acidaminococcaceae	1.2	1.0	W=214, p=0.314
Veillonellaceae	3.7	2.9	W=197, p=0.629
Verrucomicrobiaceae	1.7	1.0	W=187.5, p=0.837
Bifidobacterium	1.9	2.2	W=205, p=0.478
Bacteroides	14.3	11.2	W=,205 p=0.478
Barnesiella	1.4	0.9	W=196.5, p=0.639
Odoribacter	**0.0**	**0.0**	**W=109, p=0.039**

Prevotella	1.0	3.5	W=156.5, p=0.493
Alistipes	2.6	3.4	W=124, p=0.105
Christensenellaceae uncultured	1.1	1.8	W=136, p=0.206
Clostridium sensu stricto1	1.5	1.2	W=203, p=0.510
Anaerostipes	1.3	1.6	W=159.5, p=0.558
Blautia	4.3	5.2	W=146, p=0.327
Coprococcus	1.1	1.4	W=161.5, p=0.598
Lachnospiraceaen Icertae Sedis	11.7	10.4	W=215.5, p=0.306
Pseudobutyrivibrio	3.4	3.6	W=171, p=0.803
Roseburia	1.4	1.1	W=203, p=0.510
Lachnospiraceae uncultured	10.5	11.1	W=158, p=0.534
Peptostreptococcaceae uncultured	2.4	1.6	W=177, p=0.941
Faecalibacterium	5.9	6.2	W=162.5, p=0.619
Ruminococcaceae Incertae Sedis	2.2	1.3	W=175.5, p=0.906
Ruminococcus	1.9	2.1	W=149.5, p=0.380
Subdoligranulum	3.6	2.6	W=228, p=0.166
Ruminococcaceae uncultured	6.4	7.6	W=147, p=0.346
Phascolarctobacterium	1.1	0.9	W=212, p=0.344
Dialister	2.8	2.2	W=220, p=0.246
Akkermansia	1.7	1.0	W=187.5, p=0.837

Supplementary table 6: Relative abundance of bacterial OTUs in the *2nd trimester* maternal gut by *maternal cortisol* in the *2nd trimester*. Listed are all OTUs with an abundance of greater than 1% and significant OTUs with an abundance of less than 1%. Low cortisol (CAR < 545131; N=20), high cortisol (CAR > 545132; N=18). Unpaired students t-test and Mann-Whitney-Wilcoxon test. Abbreviations: Operational taxonomic unit (OTU), Cortisol Awakening Response (CAR).

Table 7: Maternal gut in the 3rd trimester by 2nd trimester stress

OTU	**Relative Abundance (%)**		**p-value**
Maternal gut (3rd trimester)	**Low PSS**	**High PSS**	
Chao1 Index	390.7	362.0	$t(29) = 0.98$, $p=0.330$
Simpson Idnex	0.9	0.9	$t(29) = -0.60$, $p=0.546$
Shannon Index	5.8	5.7	$t(29) = 0.35$, $p=0.728$
Phylogenetic diversity	19.9	18.8	$t(29) = 0.74$, $p=0.459$
Observed Species	306.1	287.4	$t(29) = 0.85$, $p=0.401$
Actinobacteria	6	6	W=105, p=0.571
Bacteroidetes	17	19	W=107, p=0.626
Firmicutes	72	69	W=131, p=0.682

Proteobacteria	2	2	W=116, p=0.891
Verrucomicrobia	2	3	W=117, p=0.921
Bifidobacteriaceae	3	4	W=117, p=0.922
Nocardiaceae	2	1	W=126, p=0.827
Coriobacteriaceae	2	2	W=87, p=0.202
Bacteroidaceae	11	12	W=102, p=0.494
Porphyromonadaceae	3	3	W=117, p=0.922
Prevotellaceae	0	2	W=93.5, p=0.301
Rikenellaceae	3	2	W=129, p=0.740
Christensenellaceae	3	2	W=145.5, p=0.323
Clostridiaceae1	2	1	W=152.5, p=0.205
Lachnospiraceae	34	36	W=108, p=0.653
Peptostreptococcaceae	3	2	W=141, p=0.417
Ruminococcaceae	22	20	W=137, p=0.514
Erysipelotrichaceae	2	2	W=115, p=0.860
Acidaminococcaceae	2	1	W=125.5, p=0.838
Veillonellaceae	3	4	W=119, p=0.984
Alcaligenaceae	1	1	W=116.5, p=0.905
Verrucomicrobiaceae	2	3	W=116, p=0.889
Bifidobacterium	3	4	W=118, p=0.953
Rhodococcus	2	1	W=126, p=0.827
Bacteroides	11	12	W=102, p=0.494
Barnesiella	1	1	W=136, p=0.539
Porphyromonadaceae uncultured	1	1	W=95.5, p=0.342
Prevotella	**0**	**3**	**W=77, p=0.082**
Alistipes	1	2	W=128, p=0.770
Christensenellaceae uncultured	3	2	W=144, p=0.352
Clostridium sensu stricto1	2	1	W=153, p=0.198
Anaerostipes	1	1	W=104, p=0.545
Blautia	5	5	W=102, p=0.489
Coprococcus	1	1	W=122.5, p=0.937
Lachnospiraceae Incertae Sedis	10	10	W=114, p=0.827
Pseudobutyrivibrio	3	4	W=95, p=0.889
Roseburia	2	2	W=116, p=0.889
Lachnospiraceae uncultured	10	11	W=112, p=0.658
Peptostreptococcaceae Incertae Sedis	1	1	W=133.5, p=0.607
Peptostreptococcaceae uncultured	1	1	W=126, p=0.823

Faecalibacterium	6	7	W=117, p=0.921
Ruminococcaceae Incertae Sedis	2	2	W=125, p=0.858
Ruminococcus	2	1	W=128, p=0.766
Subdoligranulum	4	4	W=130, p=0.711
Ruminococcaceae uncultured	8	7	W=128, p=0.770
Phascolarctobacterium	2	1	W=126.5, p=0.806
Dialister	2	2	W=138, p=0.486
Megamonas	1	2	W=94, p=0.250
Akkermansia	2	3	W=116, p=0.889
Turicibacter	**0.4**	**0.0**	**W=178.5, p=0.021**

Supplementary table 7: Relative abundance of bacterial OTUs in the *3rd trimester* maternal gut by *maternal stress* in the *2nd trimester*. Listed are all OTUs with an abundance of greater than 1% and significant OTUs with an abundance of less than 1%. High stress (PSS < 13; N=16), low stress (PSS > 14; N=15). Unpaired students t-test and Mann-Whitney-Wilcoxon test. Abbreviations: Operational taxonomic unit (OTU), Perceived Stress Scale (PSS)

Table 8: Maternal gut in the 3rd trimester by 2nd trimester anxiety

OTU	**Relative Abundance (%)**		**p-value**
Maternal gut (3rd trimester)	**Low STAI**	**High STAI**	
Chao1 Index	385.0	365.5	$t(29) = 0.65$, $p=0.514$
Simpson Index	0.9	0.9	$t(29) = 1.15$, $p=0.256$
Shannon Index	5.8	5.7	$t(29) = 1.00$, $p=0.323$
Phylogenetic diversity	19.8	18.8	$t(29) = 0.65$, $p=0.516$
Observed Species	300.8	291.9	$t(29) = 0.39$, $p=0.693$
Actinobacteria	7	6	W=124, p=0.797
Bacteroidetes	18	19	W=106, p=0.678
Firmicutes	70	72	W=109, p=0.767
Proteobacteria	2	2	W=129, p=0.650
Verrucomicrobia	3	1	W=136, p=0.458
Bifidobacteriaceae	4	2	W=153, p=0.157
Nocardiaceae	1	2	W=114, p=0.920
Coriobacteriaceae	2	1	W=140, p=0.373
Bacteroidaceae	11	12	W=97, p=0.440
Porphyromonadaceae	3	3	W=107, p=0.707
Prevotellaceae	2	1	W=148.5, p=0.212
Rikenellaceae	2	3	W=95, p=0.394
Christensenellaceae	2	2	W=106.5, p=0.688
Clostridiaceae1	2	1	W=140, p=0.367

Lachnospiraceae	33	37	W=93, p=0.352
Peptostreptococcaceae	3	2	W=139, p=0.389
Ruminococcaceae	20	22	W=95.5, p=0.400
Erysipelotrichaceae	2	2	W=95, p=0.394
Acidaminococcaceae	1	1	W=116.5, p=0.1
Veillonellaceae	4	3	W=135.5, p=0.471
Alcaligenaceae	1	1	W=114, p=0.920
Verrucomicrobiaceae	3	1	W=137, p=0.434
Bifidobacterium	4	2	W=153, p=0.157
Rhodococcus	1	2	W=114, p=0.920
Atopobium	**0.0**	**0.0**	**W=155, p=0.050**
Bacteroides	11	12	W=97, p=0.440
Barnesiella	1	1	W=100, p=0.508
Porphyromonadaceae uncultured	1	1	W=100.5, p=0.521
Paraprevotella	**0.1**	**0.0**	**W=162, p=0.047**
Alistipes	2	3	W=94, p=0.373
Bacteroidales uncultured	**0.2**	**0.0**	**W=159.5, p=0.049**
Christensenellaceae uncultured	2	2	W=109.5, p=0.779
Clostridium sensu stricto1	2	1	W=139.5, p=0.378
Anaerostipes	1	2	W=95, p=0.394
Blautia	4	5	W=95, p=0.389
Coprococcus	1	1	W=122.5, p=0.841
Lachnospiraceae Incertae Sedis	**9**	**11**	**W=74, p=0.088**
Pseudobutyrivibrio	3	4	W=111, p=0.828
Roseburia	2	2	W=122, p=0.857
Lachnospiraceae uncultured	10	11	W=110, p=0.797
Peptostreptococcaceae Incertae Sedis	1	1	W=132, p=0.561
Peptostreptococcaceae uncultured	2	1	W=96, p=0.400
Faecalibacterium	6	7	W=124, p=0.794
Ruminococcaceae Incertae Sedis	2	2	W=133.5, p=0.521
Ruminococcus	1	2	W=111, p=0.825
Subdoligranulum	4	3	W=139, p=0.394
Ruminococcaceae uncultured	6	8	W=99, p=0.489
Phascolarctobacterium	1	1	W=117.5, p=1
Dialister	2	1	W=155, p=0.131
Megamonas	1	2	W=114, p=0.909
Akkermansia	3	1	W=137, p=0.434

Escherichia Shigella	**0.0**	**0.2**	**W=61.5, p=0.024**

Supplementary table 8: Relative abundance of bacterial OTUs in the *3rd trimester* maternal gut by *maternal anxiety* in the *2nd trimester*. Listed are all OTUs with an abundance of greater than 1% and significant OTUs with an abundance of less than 1%. High anxiety (STAI < 4; N=18), low anxiety (STAI > 5; N=13). Unpaired students t-test and Mann-Whitney-Wilcoxon test. Abbreviations: Operational taxonomic unit (OTU), State Trait Anxiety Inventory (STAI).

Table 9: Maternal gut in the 3rd trimester by 2nd trimester depressive symptoms

OTU	**Relative Abundance (%)**		**p-value**
Maternal gut (3rd trimester)	**Low EPDS**	**High EPDS**	
Chao1 Index	377.4	374.9	$t(29) = 0.07$, $p=0.939$
Simpson Index	0.9	0.9	$t(29) = -0.47$, $p=0.636$
Shannon Index	5.8	5.7	$t(29) = 0.40$, $p=0.684$
Phylogenetic diversity	19.6	18.8	$t(29) = 0.51$, $p=0.612$
Observed Species	299.1	291.2	$t(29) = 0.31$, $p=0.757$
Actinobacteria	6	6	W=70, p=0.338
Bacteroidetes	19	15	W=103, p=0.642
Firmicutes	69	75	W=67, p=0.274
Proteobacteria	2	2	W=78, p=0.594
Verrucomicrobia	3	2	W=109, p=0.456
Bifidobacteriaceae	4	3	W=86, p=0.807
Nocardiaceae	1	2	W=86, p=0.803
Coriobacteriaceae	2	1	W=95, p=0.912
Bacteroidaceae	11	11	W=91, p=0.982
Porphyromonadaceae	3	2	W=102, p=0.674
Prevotellaceae	2	0	W=110.5, p=0.414
Rikenellaceae	3	2	W=102, p=0.674
Christensenellaceae	2	1	W=92.5, p=1
Clostridiaceae1	2	1	W=123.5, p=0.161
Lachnospiraceae	**33**	**41**	**W=54, p=0.090**
Peptostreptococcaceae	3	2	W=123, p=0.168
Ruminococcaceae	21	22	W=77.5, p=0.527
Erysipelotrichaceae	2	2	W=91, p=0.982
Acidaminococcaceae	1	2	W=85, p=0.762
Veillonellaceae	4	4	W=90.5, p=0.964
Alcaligenaceae	1	1	W=70, p=0.331
Verrucomicrobiaceae	3	2	W=109, p=0.456

Bifidobacterium	4	3	W=86, p=0.807
Rhodococcus	1	2	W=86, p=0.803
Bacteroides	11	11	W=91, p=0.982
Barnesiella	1	1	W=76, p=0.483
Porphyromonadaceae uncultured	1	1	W=110.5, p=0.416
Alistipes	2	2	W=102, p=0.674
Christensenellaceae uncultured	2	1	W=92.5, p=1
Clostridium sensu stricto1	2	1	W=123, p=0.168
Anaerostipes	1	2	W=84, p=0.740
Blautia	**4**	**6**	**W=46, p=0.039**
Coprococcus	1	2	W=80.5, p=0.619
Lachnospiraceae Incertae Sedis	10	10	W=83, p=0.701
Pseudobutyrivibrio	3	5	W=62, p=0.186
Roseburia	2	2	W=73, p=0.403
Lachnospiraceae uncultured	10	12	W=67, p=0.274
Peptostreptococcaceae Incertae Sedis	1	1	W=108.5, p=0.470
Peptostreptococcaceae uncultured	**2**	**1**	**W=135, p=0.054**
Faecalibacterium	6	7	W=89, p=0.910
Ruminococcaceae Incertae Sedis	2	2	W=84, p=0.734
Ruminococcus	2	2	W=112, p=0.378
Subdoligranulum	4	4	W=82, p=0.674
Ruminococcaceae uncultured	7	7	W=94, p=0.947
Phascolarctobacterium	1	2	W=86, p=0.798
Dialister	2	1	W=126, p=0.128
Megamonas	1	2	W=70.5, p=0.279
Akkermansia	3	2	W=109, p=0.456
Parasutterella	**0.3**	**0.8**	**W=124, p=0.012**

Supplementary table 9: Relative abundance of bacterial OTUs in the *3rd trimester* maternal gut by *maternal depressive symptoms* in the *2nd trimester*. Listed are all OTUs with an abundance of greater than 1% and significant OTUs with an abundance of less than 1%. High depressive (EPDS < 8; N=23), low depressive (EPDS > 9; N=8). Unpaired students t-test and Mann-Whitney-Wilcoxon test. Abbreviations: Operational taxonomic unit (OTU), Edinburgh Postnatal Depression Scale (EPDS).

Table 10: Maternal gut in the 3rd trimester by 2nd trimester cortisol

OTU	**Relative Abundance (%)**		**p-value**
Maternal gut (3rd trimester)	**Low CAR**	**High CAR**	
Chao1 Index	364.7	410.6	$t(26) = -1.53$, $p=0.136$

Simpson Index	0.9	0.9	$t(26) = 0.62$, $p=0.536$
Shannon Index	5.7	5.8	$t(26) = -0.55$, $p=0.583$
Phylogenetic diversity	**18.6**	**21.4**	**$t(26) = -2.05$, $p=0.049$**
Observed Species	**284.6**	**327.3**	**$t(26) = -1.95$, $p=0.061$**
Actinobacteria	5.6	7.7	W=108, p=0.650
Euryarchaeota	**0.0**	**0.1**	**W=64, p=0.034**
Bacteroidetes	19.0	17.8	W=103, p=0.820
Firmicutes	71.2	67.9	W=119, p=0.338
Proteobacteria	2.2	1.5	W=131, p=0.130
Verrucomicrobia	1.5	4.0	W=91, p=0.782
Bifidobacteriaceae	3.0	3.8	W=117, p=0.387
Nocardiaceae	1.1	1.9	W=88.5, p=0.695
Coriobacteriaceae	1.4	1.9	W=90, p=0.751
Bacteroidaceae	12.4	10.5	W=113, p=0.495
Porphyromonadaceae	3.1	2.8	W=112, p=0.525
Prevotellaceae	1.4	0.9	W=83, p=0.517
Rikenellaceae	1.9	2.8	W=78, p=0.387
Christensenellaceae	1.4	3.4	W=65.5, p=0.146
Clostridiaceae1	0.8	2.5	W=77, p=0.356
Lachnospiraceae	**37.2**	**29.4**	**W=134, p=0.097**
Peptostreptococcaceae	**1.5**	**3.7**	**W=59, p=0.079**
Ruminococcaceae	21.3	20.5	W=115, p=0.433
Erysipelotrichaceae	**1.3**	**2.3**	**W=52, p=0.036**
Acidaminococcaceae	1.5	1.6	W=78.5, p=0.386
Veillonellaceae	4.5	1.9	W=118, p=0.356
Alcaligenaceae	**1.1**	**0.6**	**W=149, p=0.018**
Verrucomicrobiaceae	1.4	3.9	W91=, p=0.782
Methanobrevibacter	**0.0**	**0.1**	**W=64, p=0.034**
Bifidobacterium	3.0	3.8	W=117, p=0.387
Rhodococcus	1.1	1.9	W=88.5, p=0.695
Bacteroides	12.4	10.5	W=113, p=0.495
Barnesiella	1.0	0.9	W=112.5, p=0.504
Porphyromonadaceae uncultured	0.5	0.7	W=84.5, p=0.564
Alistipes	1.9	2.8	W=78, p=0.387
Bacteroidete uncultured	**0.0**	**0.3**	**W=144.5, p=0.031**
Christensenellaceae uncultured	1.3	3.4	W=64, p=0.128
Clostridium sensu stricto1	0.8	2.5	W=58, p=0.381

Anaerostipes	1.3	1.3	W=85, p=0.586
Blautia	4.9	3.8	W=129, p=0.153
Coprococcus	1.6	1.1	W=116.5, p=0.394
Howardella	**0.0**	**0.0**	**W=61, p=0.029**
Lachnospiraceae Incertae Sedis	9.8	9.2	W=107, p=0.678
Pseudobutyrivibrio	4.6	2.5	W=131, p=0.130
Roseburia	1.8	1.3	W=125.5, p=0.204
Lachnospiraceae uncultured	11.4	8.7	W=127, p=0.184
Peptostreptococcaceae Incertae Sedis	0.8	1.7	W=73.5, p=0.278
Peptostreptococcaceae uncultured	**0.7**	**2.0**	**W=47.5, p=0.022**
Faecalibacterium	7.6	5.3	W=133, p=0.107
Ruminococcaceae Incertae Sedis	1.7	1.8	W=85, p=0.580
Ruminococcus	1.8	1.1	W=100, p=0.926
Subdoligranulum	3.5	3.5	W=103, p=0.820
Ruminococcaceae uncultured	6.1	8.2	W=81, p=0.467
Asteroleplasma	**0.0**	**0.1**	**W=61, p=0.029**
Turicibacter	**0.1**	**0.6**	**W=39, p=0.007**
Phascolarctobacterium	1.5	1.6	W=77.5, p=0.360
Dialister	1.1	1.6	W=92, p=0.816
Megamonas	2.8	0.0	W=124, p=0.173
Enterobacter	**0.0**	**0.1**	**W=65.5, p=0.042**
Tenericutes Mollicutes uncultured	**0.0**	**0.0**	**W=53, p=0.032**
Akkermansia	1.4	3.9	W=91, p=0.782

Supplementary table 10: Relative abundance of bacterial OTUs in the *3rd trimester* maternal gut by *maternal cortisol* in the *2nd trimester*. Listed are all OTUs with an abundance of greater than 1% and significant OTUs with an abundance of less than 1%. Low cortisol (CAR < 545131; N=15), high cortisol (CAR > 545132; N=13). Unpaired students t-test and Mann-Whitney-Wilcoxon test. Abbreviations: Operational taxonomic unit (OTU), Cortisol Awakening Response (CAR).

Table 11: Maternal gut in the 3rd trimester by 3rd trimester stress

OTU	**Relative Abundance (%)**		**p-value**
Maternal gut (3rd trimester)	**Low PSS**	**High PSS**	
Chao1 Index	355.7	370.3	$t(31) = -0.62$, $p=0.536$
Simpson Index	0.9	0.9	$t(31) = -0.04$, $p=0.967$
Shannon Index	5.7	5.7	$t(31) = -0.25$, $p=0.799$
Phylogenetic diversity	18.6	19.0	$t(31) = -0.37$, $p=0.711$
Observed Species	283.1	295.0	$t(31) = -0.61$, $p=0.544$

Actinobacteria	7	5	W=128, p=0.816
Bacteroidetes	18	22	W=106, p=0.306
Firmicutes	71	67	W=165, p=0.290
Proteobacteria	2	2	W=157, p=0.436
Verrucomicrobia	2	3	W=133.5, p=0.971
Bifidobacteriaceae	4	3	W=145, p=0.734
Nocardiaceae	1	1	W=116.5, p=0.514
Coriobacteriaceae	2	2	W=128, p=0.814
Bacteroidaceae	11	14	W=100, p=0.215
Porphyromonadaceae	4	4	W=104, p=0.274
Prevotellaceae	1	2	W=146, p=0.702
Rikenellaceae	**2**	**3**	**W=83, p=0.061**
Christensenellaceae	2	2	W=119, p=0.575
Clostridiaceae1	1	1	W=142, p=0.814
Lachnospiraceae	37	36	W=145, p=0.734
Peptostreptococcaceae	3	2	W=169, p=0.225
Ruminococcaceae	19	19	W=132.5, p=0.942
Erysipelotrichaceae	2	1	W=160, p=0.380
Acidaminococcaceae	1	2	W=93.5=, p=0.126
Veillonellaceae	**4**	**2**	**W=181, p=0.099**
Alcaligenaceae	1	1	W=164, p=0.302
Verrucomicrobiaceae	2	3	W=133.5, p=0.971
Bifidobacterium	4	3	W=145, p=0.734
Rhodococcus	1	1	W=116.5, p=0.514
Bacteroides	11	14	W=100, p=0.215
Barnesiella	1	1	W=106.5, p=0.311
Porphyromonadaceae uncultured	2	1	W=107, p=0.319
Alistipes	**2**	**3**	**W=81.5, p=0.055**
Christensenellaceae uncultured	2	2	W=119, p=0.575
Clostridium sensu stricto1	1	1	W=142.5, p=0.800
Anaerostipes	2	2	W=118, p=0.555
Blautia	5	5	W=159, p=0.395
Coprococcus	1	1	W=136.5, p=0.971
Lachnospiraceae Incertae Sedis	11	10	W=138, p=0.928
Pseudobutyrivibrio	3	4	W=111, p=0.400
Roseburia	2	2	W=134, p=0.985
Lachnospiraceae uncultured	11	11	W=142, p=0.816

Peptostreptococcaceae Incertae Sedis	1	1	W=159, p=0.395
Peptostreptococcaceae uncultured	1	1	W=157.5, p=0.110
Faecalibacterium	6	6	W=141, p=0.842
Ruminococcaceae Incertae Sedis	2	2	W=166, p=0.274
Ruminococcus	2	1	W=157.5, p=0.426
Subdoligranulum	3	3	W=137, p=0.957
Ruminococcaceae uncultured	6	6	W=113, p=0.442
Gelria	**0.0**	**0.0**	**W=79, p=0.016**
Phascolarctobacterium	1	2	W=94.5, p=0.135
Dialister	**3**	**1**	**W=189, p=0.051**
Megamonas	**1**	**1**	**W=172.5, p=0.031**
Escherichia Shigella	**0.0**	**0.0**	**W=72, p=0.021**
Akkermansia	2	3	W=133.5, p=0.974

Supplementary table 11: Relative abundance of bacterial OTUs in the *3rd trimester* maternal gut by *maternal stress* in the *3rd trimester*. Listed are all OTUs with an abundance of greater than 1% and significant OTUs with an abundance of less than 1%. Low stress (PSS < 11; N=18), high stress (PSS > 12; N=14). Unpaired students t-test and Mann-Whitney-Wilcoxon test. Abbreviations: Operational taxonomic unit (OTU), Perceived Stress Scale (PSS).

Table 12: Maternal gut in the 3rd trimester by 3rd trimester anxiety

OTU	**Relative Abundance (%)**		**p-value**
Maternal gut (3rd trimester)	**Low STAI**	**High STAI**	
Chao1 Index	365.1	362.5	$t(31) = 0.08$, $p=0.936$
Simpson Index	0.9	0.9	$t(31) = -0.11$, $p=0.906$
Shannon Index	5.7	5.7	$t(31) = 0.27$, $p=0.782$
Phylogenetic diversity	19.2	18.4	$t(31) = 0.49$, $p=0.621$
Observed Species	292.3	286.0	$t(31) = 0.23$, $p=0.812$
Actinobacteria	7	6	W=125, p=0.709
Bacteroidetes	17	22	W=106, p=0.292
Firmicutes	69	68	W=140, p=0.901
Proteobacteria	2	1	W=173, p=0.188
Verrucomicrobia	4	2	W=155, p=0.504
Bifidobacteriaceae	5	3	W=157, p=0.465
Nocardiaceae	0	1	W=120, p=0.576
Coriobacteriaceae	2	2	W=123.5, p=0.665
Bacteroidaceae	11	13	W=104, p=0.260
Porphyromonadaceae	3	5	W=111, p=0.382

Prevotellaceae	1	2	W=165, p=0.302
Rikenellaceae	2	3	W=110, p=0.363
Christensenellaceae	2	1	W=137, p=0.985
Clostridiaceae1	2	1	W=171.5, p=0.207
Lachnospiraceae	34	37	W=104, p=0.260
Peptostreptococcaceae	3	2	W=182, p=0.101
Ruminococcaceae	19	20	W=126.5, p=0.745
Erysipelotrichaceae	2	2	W=128, p=0.789
Acidaminococcaceae	1	1	W=122.5, p=0.628
Veillonellaceae	4	2	W=165, p=0.304
Alcaligenaceae	1	1	W=156.5, p=0.471
Verrucomicrobiaceae	3	2	W=155, p=0.504
Bifidobacterium	5	3	W=157, p=0.465
Rhodococcus	0	1	W=120, p=0.576
Bacteroides	11	13	W=104, p=0.260
Barnesiella	1	1	W=116, p=0.482
Porphyromonadaceae uncultured	1	2	W=120, p=0.576
Alistipes	2	3	W=110, p=0.358
Bacteroidales uncultured	**0.3**	**0.0**	**W=188, p=0.026**
Christensenellaceae uncultured	2	1	W=136, p=1
Clostridium sensu stricto1	2	1	W=171, p=0.213
Anaerostipes	1	2	W=111, p=0.382
Blautia	5	5	W=128, p=0.814
Coprococcus	1	1	W=140.5, p=0.885
Lachnospiraceae Incertae Sedis	10	11	W=93, p=0.125
Pseudobutyrivibrio	3	4	W=100, p=0.203
Roseburia	2	2	W=141, p=0.871
Lachnospiraceae uncultured	10	11	W=117, p=0.510
Peptostreptococcaceae Incertae Sedis	1	1	W=159, p=0.417
Peptostreptococcaceae uncultured	**2**	**1**	**W=190, p=0.053**
Faecalibacterium	6	6	W=128, p=0.787
Ruminococcaceae Incertae Sedis	2	2	W=161, p=0.382
Ruminococcus	2	1	W=152.2, p=0.564
Subdoligranulum	3	3	W=149, p=0.656
Ruminococcaceae uncultured	6	7	W=123, p=0.656
Phascolarctobacterium	1	1	W=123.5, p=0.655
Dialister	**3**	**1**	**W=189, p=0.057**

Megamonas	1	1	W=118.5, p=0.477
Akkermansia	3	2	W=155, p=0.504

Supplementary table 12: Relative abundance of bacterial OTUs in the *3rd trimester* maternal gut by *maternal anxiety* in the *3rd trimester*. Listed are all OTUs with an abundance of greater than 1% and significant OTUs with an abundance of less than 1%. Low anxiety (STAI < 4; N=16), high anxiety (STAI > 5; N=17). Unpaired students t-test and Mann-Whitney-Wilcoxon test. Abbreviations: Operational taxonomic unit (OTU), State Trait Anxiety Inventory (STAI).

Table 13: Maternal gut in the 3rd trimester by 3rd trimester depressive symptoms

OTU	**Relative Abundance (%)**		**p-value**
Maternal gut (3rd trimester)	**Low EPDS**	**High EPDS**	
Chao1 Index	363.9	364.1	*t*(31) = -0.00, *p*=0.995
Simpson Index	0.9	0.9	*t*(31) = 0.55, *p*=0.586
Shannon Index	5.6	5.6	*t*(31) = 0.41, *p*=0.681
Phylogenetic diversity	18.1	18.1	*t*(31) = 0.57, *p*=0.568
Observed Species	287.5	287.5	*t*(31) = 0.10, *p*=0.920
Actinobacteria	8	6	W=93, p=0.789
Bacteroidetes	18	25	W=62, p=0.116
Firmicutes	71	64	W=138, p=0.116
Proteobacteria	2	2	W=97.5, p=0.933
Verrucomicrobia	2	4	W=96.5, p=0.899
Bifidobacteriaceae	4	4	W=93, p=0.789
Nocardiaceae	**1**	**1**	**W=59.5, p=0.092**
Coriobacteriaceae	**2**	**1**	**W=141, p=0.088**
Bacteroidaceae	11	15	W=63, p=0.127
Porphyromonadaceae	**4**	**5**	**W=57, p=0.073**
Prevotellaceae	2	0	W=125.5, p=0.291
Rikenellaceae	**2**	**4**	**W=56, p=0.066**
Christensenellaceae	2	2	W=75, p=0.303
Clostridiaceae1	1	1	W=126, p=0.284
Lachnospiraceae	37	32	W=121, p=0.396
Peptostreptococcaceae	2	2	W=119.5, p=0.424
Ruminococcaceae	20	19	W=102.5, p=0.933
Erysipelotrichaceae	2	2	W=103, p=0.918
Acidaminococcaceae	1	2	W=72.5, p=0.241
Veillonellaceae	4	3	W=115, p=0.542
Alcaligenaceae	1	1	W=120.5, p=0.400

Verrucomicrobiaceae	2	4	W=96.5, p=0.899
Bifidobacterium	4	4	W=93, p=0.789
Rhodococcus	**1**	**1**	**W=59.5, p=0.092**
Bacteroides	11	15	W=63, p=0.127
Barnesiella	**1**	**2**	**W=44, p=0.019**
Coprobacter	**0.0**	**0.0**	**W=48.5, p=0.024**
Porphyromonadaceae uncultured	1	1	W=72, p=0.247
Alistipes	**2**	**4**	**W=55, p=0.061**
Christensenellaceae uncultured	2	2	W=74.5, p=0.293
Clostridium sensu stricto1	1	1	W=125.2, p=0.293
Anaerostipes	2	1	W=120, p=0.420
Blautia	**6**	**3**	**W=142, p=0.081**
Coprococcus	1	1	W=107, p=0.784
Lachnospiraceae Incertae Sedis	11	11	W=89, p=0.659
Pseudobutyrivibrio	3	4	W=94, p=0.820
Roseburia	2	2	W=102, p=0.949
Lachnospiraceae uncultured	11	9	W=117, p=0.495
Peptostreptococcaceae Incertae Sedis	1	1	W=95.5, p=0.866
Peptostreptococcaceae uncultured	1	1	W=134.5, p=0.153
Faecalibacterium	6	6	W=106, p=0.817
Ruminococcaceae Incertae Sedis	2	1	W=119, p=0.444
Ruminococcus	**2**	**1**	**W=143.5, p=0.070**
Subdoligranulum	3	3	W=90, p=0.695
Ruminococcaceae uncultured	6	7	W=90, p=0.695
Phascolarctobacterium	1	2	W=70.5, p=0.208
Dialister	**2**	**1**	**W=140, p=0.095**
Megamonas	1	2	W=127, p=0.196
Akkermansia	2	4	W=96.5, p=0.899

Supplementary table 13: Relative abundance of bacterial OTUs in the *3rd trimester* maternal gut by *maternal depressive symptoms* in the *3rd trimester*. Listed are all OTUs with an abundance of greater than 1% and significant OTUs with an abundance of less than 1%. Low depressive symptoms (EPDS < 8; N=25), high depressive symptoms (EPDS > 9; N=8). Unpaired students t-test and Mann-Whitney-Wilcoxon test. Abbreviations: Operational taxonomic unit (OTU), Edinburgh Postnatal Depression Scale (EPDS).

Table 14: Maternal gut in the 3rd trimester by 3rd trimester cortisol

OTU	**Relative Abundance (%)**		**p-value**
Maternal gut (3rd trimester)	**Low CAR**	**High CAR**	

Chao1 Index	365.3	384.4	$t(31) = -0.63$, $p=0.527$
Simpson Index	0.9	0.9	$t(31) = -0.49$, $p=0.622$
Shannon Index	5.7	5.7	$t(31) = -0.07$, $p=0.944$
Phylogenetic diversity	19.0	19.8	$t(31) = -0.52$, $p=0.603$
Observed Species	290.5	306.5	$t(31) = -0.69$, $p=0.493$
Actinobacteria	5.2	8.1	W=78, p=0.155
Bacteroidetes	21.2	16.0	W=133, p=0.499
Firmicutes	68.6	70.5	W=120, p=0.552
Proteobacteria	1.8	1.9	W=96.5, p=0.480
Verrucomicrobia	2.4	2.8	W=126.5, p=0.666
Bifidobacteriaceae	2.7	4.3	W=91, p=0.363
Nocardiaceae	1.1	1.8	W=77.5, p=0.146
Coriobacteriaceae	1.3	1.8	W=103, p=0.652
Bacteroidaceae	12.2	11.2	W=115, p=1
Porphyromonadaceae	4.4	2.4	W=147, p=0.221
Prevotellaceae	1.6	0.1	W=103.5, p=0.664
Rikenellaceae	2.3	2.0	W=122, p=0.802
Christensenellaceae	2.0	2.2	W=114, p=0.984
Clostridiaceae1	0.9	2.0	W=137.5, p=0.388
Lachnospiraceae	35.3	33.8	W=118, p=0.923
Peptostreptococcaceae	1.7	3.4	W=91, p=0.363
Ruminococcaceae	20.6	19.9	W=127, p=0.652
Erysipelotrichaceae	1.5	2.4	W=88, p=0.304
Acidaminococcaceae	1.4	1.6	W=103, p=0.642
Veillonellaceae	3.2	3.1	W=114.5, p=1
Alcaligenaceae	1.0	0.8	W=119, p=0.890
Verrucomicrobiaceae	2.3	2.8	W125.5=, p=0.695
Bifidobacterium	2.7	4.3	W=91, p=0.363
Rhodococcus	1.1	1.8	W=77.5, p=0.146
Olsenella	**0.0**	**0.0**	**W=92, p=0.033**
Bacteroides	12.2	11.2	W=115, p=1
Barnesiella	1.2	0.7	W=128.5, p=0.610
Porphyromonadaceae uncultured	1.5	0.6	W=136, p=0.421
Alistipes	2.3	2.0	W=122, p=0.799
Christensenellaceae uncultured	1.9	2.1	W=113.5, p=0.968
Clostridium sensu stricto1	0.9	2.0	W=138, p=0.378
Parvimonas	**0.0**	**0.0**	**W=79.5, p=0.041**

Anaerostipes	1.6	1.7	W=95, p=0.451
Blautia	4.9	4.1	W=121, p=0.829
Coprococcus	1.2	1.6	W=109, p=0.829
Lachnospiraceae Incertae Sedis	10.7	9.3	W=140, p=0.337
Pseudobutyrivibrio	3.2	4.3	W=93, p=0.406
Roseburia	1.6	1.5	W=119.5, p=0.875
Lachnospiraceae uncultured	10.5	9.5	W=123, p=0.772
Peptostreptococcaceae Incertae Sedis	0.7	1.8	W=94.5, p=0.433
Peptostreptococcaceae uncultured	0.9	1.6	W=103, p=0.652
Faecalibacterium	6.3	6.0	W=125, p=0.709
Ruminococcaceae Incertae Sedis	1.6	1.9	W=91, p=0.363
Ruminococcus	2.0	0.8	W=157, p=0.104
Subdoligranulum	3.1	3.6	W=100, p=0.576
Ruminococcaceae uncultured	7.1	7.0	W=118, p=0.923
Phascolarctobacterium	1.4	1.6	W=102, p=0.613
Dialister	1.7	1.0	W=145.5, p=0.237
Megamonas	1.0	1.9	W=79.5, p=0.111
Akkermansia	2.3	2.8	W=125.5, p=0.695

Supplementary table 14: Relative abundance of bacterial OTUs in the *3rd trimester* maternal gut by *maternal cortisol* in the *3rd trimester*. Listed are all OTUs with an abundance of greater than 1% and significant OTUs with an abundance of less than 1%. Low cortisol (CAR < 562349; N=23), high cortisol (PCAR > 562350; N=10). Unpaired students t-test and Mann-Whitney-Wilcoxon test. Abbreviations: Operational taxonomic unit (OTU), Cortisol Awakening Response (CAR).

Table 15: Maternal vaginal microbiome by 2nd trimester stress

OTU	**Relative Abundance (%)**		**p-value**
Vaginal	**Low PSS**	**High PSS**	
Chao1 Index	**235.5**	**199.6**	***t*(50) = 1.88, *p*=0.064**
Simpson Index	0.4	0.5	*t*(50) = -1.16, *p*=0.250
Shannon Index	1.8	2.3	*t*(50) = -1.09, *p*=0.278
Phylogenetic diversity	8.4	9.0	*t*(50) = -0.58, *p*=0.558
Observed Species	163	139.9	*t*(50) = 1.09, *p*=0.280
Actinobacteria	9.6	11.6	W=271, p=0.313
Bacteroidetes	3.0	1.4	W=270.5, p=0.309
Firmicutes	82.0	68.6	W=403, p=0.152
Fusobacteria	0.5	1.5	W=317, p=0.876
Proteobacteria	2.6	14.4	W=254, p=0.187

Otherphyla	0.7	1.3	W=366, p=0.455
Bifidobacteriaceae	6.5	6.1	W=311, p=0.793
Corynebacteriaceae	2.0	3.8	W=283, p=0.433
Prevotellaceae	2.6	1.0	W=286.5, p=0.472
Staphylococcaceae	1.8	1.4	W=313, p=0.822
Lactobacillaceae	67.1	61.7	W=360, p=0.529
Streptococcaceae	**6.2**	**0.8**	**W=415, p=0.096**
ClostridialesFamilyXI	4.4	1.8	W=331.5, p=0.918
Veillonellaceae	0.7	1.6	W=320, p=0.925
Leptotrichiaceae	0.5	1.5	W=264, p=0.186
Caulobacteraceae	0.2	1.9	W=266, p=0.270
Sphingomonadaceae	0.5	3.4	W=246.5, p=0.143
Moraxellaceae	0.6	3.3	W=240, p=0.112
Pseudomonadaceae	0.4	2.0	W=249.5, p=0.159
Otherfamily	0.7	1.3	W=366, p=0.455
Gardnerella	6.4	5.6	W=301, p=0.653
Corynebacterium	1.7	3.4	W=294, p=0.562
Rhodcoccus	**0.1**	**0.7**	**W=179, p=0.006**
Prevotella	2.6	1.0	W=286.5, p=0.472
Staphylococcus	1.8	1.4	W=312, p=0.808
Lactobacillus	67.1	61.7	W=360, p=0.529
Streptococcus	6.2	0.8	W=215, p=0.096
Anaerococcus	2.1	0.6	W=352, p=0.627
Sneathia	0.5	1.5	W=264, p=0.186
Brevundimonas	0.2	1.7	W=262, p=0.239
Sphingobium	**0.2**	**1.6**	**W=235.5, p=0.094**
Sphingomonas	0.3	1.8	W=256.5, p=0.201
Acinetobacter	0.6	3.3	W=240, p=0.112
Pseudomonas	0.4	2.0	W=249.5, p=0.159
OtherGenus	0.7	1.3	W=366, p=0.455

Supplementary table 15: Relative abundance of bacterial OTUs in the *maternal vagina* by *maternal stress* in the *2nd trimester*. Listed are all OTUs with an abundance of greater than 1% and significant OTUs with an abundance of less than 1%. Low stress (PSS < 13; N=21), high stress (PSS > 14; N=31). Unpaired students t-test and Mann-Whitney-Wilcoxon test. Abbreviations: Operational taxonomic unit (OTU), Perceived Stress Scale (PSS).

Table 16: Maternal vaginal microbiome by 2nd trimester anxiety

OTU	Relative Abundance (%)	p-value

Vaginal	Low STAI20W	High STAI20W	
Chao1 Index	217.2	227.4	$t(50) = -0.34$, $p=0.730$
Simpson Index	0.5	0.4	$t(50) = 0.67$, $p=0.501$
Shannon Index	2.2	1.8	$t(50) = 0.78$, $p=0.438$
Phylogenetic diversity	9.0	8.6	$t(50) = 0.36$, $p=0.716$
Observed Species	148.8	149.9	$t(50) = -0.05$, $p=0.959$
Actinobacteria	10.1	11.8	W=277, p=0.370
Bacteroidetes	2.0	2.1	W=311, p=0.794
Firmicutes	72.9	75.6	W=340, p=0.795
Fusobacteria	1.4	0.6	W=361, p=0.496
Proteobacteria	11.3	7.2	W=360, p=0.529
Otherphyla	1.4	0.5	W=299, p=0.627
Bifidobacteriaceae	5.7	7.2	W=311.5, p=0.801
Corynebacteriaceae	2.8	3.4	W=322.5, p=0.962
Prevotellaceae	1.5	1.9	W=305.5, p=0.715
Staphylococcaceae	2.1	0.8	W=380, p=0.313
Lactobacillaceae	60.4	69.0	W=300, p=0.643
Streptococcaceae	**4.8**	**0.3**	**W=490.5, p=0.002**
ClostridialesFamilyXI	3.6	1.6	W=357.5, p=0.556
Veillonellaceae	0.5	2.3	W=317, p=0.881
Leptotrichiaceae	1.4	0.6	W=303.5, p=0.641
Caulobacteraceae	1.5	0.8	W=369, p=0.422
Sphingomonadaceae	2.6	1.7	W=369.5, p=0.417
Moraxellaceae	2.6	1.6	W=345, p=0.723
Pseudomonadaceae	1.6	1.0	W=361, p=0.513
Otherfamily	1.4	0.5	W=299, p=0.627
Gardnerella	5.1	7.0	W=286, p=0.466
Corynebacterium	2.6	2.8	W=333.5, p=0.888
Prevotella	1.5	1.9	W=305.5, p=0.715
Staphylococcus	2.1	0.8	W=380, p=0.313
Lactobacillus	60.4	69.0	W=300, p=0.643
Streptococcus	**4.8**	**0.3**	**W=490.5, p=0.002**
Anaerococcus	1.7	0.5	W=364, p=0.477
Shuttleworthia	**0.0**	**0.0**	**W=279, p=0.033**
Sneathia	1.4	0.6	W=303.5, p=0.641
Brevundimonas	1.4	0.8	W=373.5, p=0.374
Sphingobium	1.2	0.8	W=372, p=0.390

Sphingomonas	1.4	0.8	W=360, p=0.525
Acinetobacter	2.6	1.5	W=344.5, p=0.730
Pseudomonas	1.6	1.0	W=361, p=0.513
OtherGenus	1.4	0.5	W=299, p=0.627

Supplementary Table 16: Relative abundance of bacterial OTUs in the *maternal vagina* by *maternal anxiety* in the *2nd trimester*. Listed are all OTUs with an abundance of greater than 1% and significant OTUs with an abundance of less than 1%. Low anxiety (PSS < 4; N=31), high anxiety (PSS > 5; N=21). Unpaired students t-test and Mann-Whitney-Wilcoxon test. Abbreviations: Operational taxonomic unit (OTU), State Trait Anxiety Inventory (STAI).

Table 17: Maternal vaginal microbiome by 2nd trimester depressive symptoms

OTU	**Relative Abundance (%)**		**p-value**
Vaginal	**Low EPDS**	**High EPDS**	
Chao1 Index	219.6	226.6	$t(50) = -0.20$, $p=0.835$
Simpson Index	0.4	0.4	$t(50) = -0.16$, $p=0.866$
Shannon Index	2.0	2.2	$t(50) = -0.23$, $p=0.811$
Phylogenetic diversity	8.4	9.9	$t(50) = -1.28$, $p=0.203$
Observed Species	147.7	153.8	$t(50) = -0.25$, $p=0.801$
Actinobacteria	9.2	15.5	W=172, p=0.086
Bacteroidetes	2.2	1.7	W=201.5, p=0.276
Firmicutes	76.9	65.6	W=334, p=0.091
Fusobacteria	0.3	3.6	W=230.5, p=0.620
Proteobacteria	9.3	10.7	W=204, p=0.304
Otherphyla	1.3	0.3	W=293.5, p=0.403
Bifidobacteriaceae	5.1	9.7	W=208.5, p=0.346
Corynebacteriaceae	2.6	4.5	W=189.5, p=0.179
Prevotellaceae	1.8	1.3	W=200.5, p=0.266
Staphylococcaceae	2.0	0.4	W=252.5, p=0.991
Lactobacillaceae	65.8	58.0	W=287, p=0.490
Streptococcaceae	3.8	0.5	W=270.5, p=0.727
ClostridialesFamilyXI	3.0	2.4	W=223, p=0.526
Veillonellaceae	0.7	3.0	W=195, p=0.219
Leptotrichiaceae	0.2	3.5	W=229, p=0.556
Caulobacteraceae	1.2	1.3	W=216.5, p=0.440
Sphingomonadaceae	2.1	2.5	W=212, p=0.386
Moraxellaceae	2.2	2.3	W=200.5, p=0.267
Pseudomonadaceae	1.3	1.5	W=208.5, p=0.346
Otherfamily	1.3	0.3	W=293.5, p=0.403

Gardnerella	4.7	9.6	W=194.5, p=0.215
Corynebacterium	2.4	3.6	W=205, p=0.309
Atopobium	**0.2**	**0.5**	**W=155, p=0.033**
Prevotella	1.8	1.3	W=200.5, p=0.266
Staphylococcus	2.0	0.4	W=252.5, p=0.991
Lactobacillus	65.8	58.0	W=287, p=0.490
Streptococcus	3.8	0.5	W=270.5, p=0.727
Anaerococcus	1.3	0.8	W=216, p=0.433
Sneathia	0.2	3.5	W=229, p=0.556
Brevundimonas	1.1	1.2	W=215.5, p=0.427
Sphingobium	1.0	1.2	W=208, p=0.341
Sphingomonas	1.1	1.3	W=219.5, p=0.478
Acinetobacter	2.1	2.3	W=199.5, p=0.258
Pseudomonas	1.3	1.5	W=208.5, p=0.346
OtherGenus	1.3	0.3	W=293.5, p=0.403

Supplementary Table 17: Relative abundance of bacterial OTUs in the *maternal vagina* by *maternal depressive symptoms* in the *2nd trimester*. Listed are all OTUs with an abundance of greater than 1% and significant OTUs with an abundance of less than 1%. Low depressive (EPDs < 8; N=39), high depressive (EPDS > 9; N=13). Unpaired students t-test and Mann-Whitney-Wilcoxon test. Abbreviations: Operational taxonomic unit (OTU), Edinburgh Postnatal Depression Scale (EPDS).

Table 18: Maternal vaginal microbiome by 2nd trimester cortisol

OTU	**Relative Abundance (%)**		**p-value**
Vaginal	**Low CAR**	**High CAR**	
Chao1 Index	**256.2**	**181.9**	***t*(46) = 2.52, *p*=0.014**
Simpson Index	0.4	0.4	*t*(46) = 0.51, *p*=0.610
Shannon Index	2.1	1.8	*t*(46) = 0.56, *p*=0.575
Phylogenetic diversity	9.3	8.1	*t*(46) = 1.14, *p*=0.257
Observed Species	**170.5**	**124.2**	***t*(46) = 2.11, *p*=0.039**
Actinobacteria	13.2	7.4	W=322, p=0.332
Bacteroidetes	2.1	1.9	W=202, p=0.569
Firmicutes	72.6	80.0	W=240, p=0.464
Fusobacteria	0.1	0.6	W=275.5, p=1
Proteobacteria	9.5	7.6	W=300, p=0.616
Bifidobacteriaceae	7.8	3.8	W=266, p=0.849
Corynebacteriaceae	4.1	2.0	W=330, p=0.254
Prevotellaceae	1.6	1.7	W=284.5, p=0.857

Staphylococcaceae	1.4	1.4	W=317.5, p=0.381
Lactobacillaceae	61.6	71..5	W=217, p=0.223
Streptococcaceae	3.5	2.7	W=267, p=0.866
ClostridialesFamilyXI	2.9	2.6	W=310, p=0.473
Veillonellaceae	1.4	0.9	W=303, p=0.568
Leptotrichiaceae	0.1	0.5	W=248.5, p=0.519
Caulobacteraceae	1.2	1.0	W=286, p=0.832
Sphingomonadaceae	2.2	1.8	W=298.5, p=0.635
Moraxellaceae	2.3	1.7	W=301, p=0.598
Pseudomonadaceae	1.4	1.0	W=307.5, p=0.506
Gardnerella	7.6	3.1	W=260, p=0.751
Corynebacterium	3.7	1.8	W=323.5, p=0.316
Prevotella	1.6	1.7	W=284.5, p=0.857
Staphylococcus	1.4	1.4	W=316.5, p=0.393
Lactobacillus	61.6	71.5	W=217, p=0.223
Streptococcus	3.5	2.7	W=267, p=0.866
Anaerococcus	1.0	1.2	W=304.5, p=0.547
Blautia	**0.0**	**0.0**	**W=370.5, p=0.008**
Faecalibacterium	**0.0**	**0.0**	**W=370, p=0.018**
Sneathia	0.1	0.5	W=248.5, p=0.519
Brevundimonas	1.1	0.9	W=279, p=0.949
Sphingobium	1.0	0.8	W=308.5, p=0.493
Sphingomonas	1.1	0.9	W=290.5, p=0.759
Acinetobacter	2.2	1.6	W=301.5, p=0.590
Pseudomonas	1.4	1.0	W=307.5, p=0.506

Supplementary Table 18: Relative abundance of bacterial OTUs in the *maternal vagina* by *maternal cortisol* in the *2nd trimester*. Listed are all OTUs with an abundance of greater than 1% and significant OTUs with an abundance of less than 1%. Low cortisol (CAR < 545131; N=29), high cortisol (CAR > 545132; N=19). Unpaired students t-test and Mann-Whitney-Wilcoxon test. Abbreviations: Operational taxonomic unit (OTU), Cortisol Awakening Response (CAR).

Table 19: Maternal vaginal microbiome by 3rd trimester stress

OTU	**Relative Abundance (%)**		**p-value**
Vaginal	**Low PSS**	**High PSS**	
Chao1 Index	**267.7**	**198.9**	***t*(41) = 1.96, *p*=0.055**
Simpson Index	0.4	0.5	*t*(41) = -0.85, *p*=0.398
Shannon Index	2.2	2.4	*t*(41) = -0.33, *p*=0.738

Phylogenetic diversity	9.4	8.8	*t*(41) = 0.52, *p*=0.599
Observed Species	**181.9**	**137.6**	***t*(41) = 1.73, *p*=0.091**
Actinobacteria	11.4	13.2	W=218, p=0.763
Bacteroidetes	2.7	1.4	W=208.5, p=0.592
Firmicutes	74.7	68.3	W=258, p=0.523
Fusobacteria	0.0	0.1	W=251.5, p=0.612
Proteobacteria	8.3	14.7	W=207, p=0.571
Otherphyla	2.2	0.3	W=283, p=0.210
Bifidobacteriaceae	7.4	6.9	W=277.5, p=0.263
Corynebacteriaceae	2.8	5.2	W=211.5, p=0.643
Prevotellaceae	2.2	1.0	W=197.5, p=0.422
Staphylococcaceae	2.1	2.3	W=203, p=0.503
Lactobacillaceae	59.0	59.0	W=234, p=0.952
Streptococcaceae	5.7	1.6	W=261, p=0.473
ClostridialesFamilyXI	5.5	1.9	W=247.5, p=0.697
Veillonellaceae	0.5	1.8	W=229, p=0.970
Leptotrichiaceae	0.0	0.1	W=259.5, p=0.439
Caulobacteraceae	1.1	1.9	W=216, p=0.724
Sphingomonadaceae	1.9	3.4	W=203.5, p=0.511
Moraxellaceae	1.9	3.3	W=199, p=0.444
Pseudomonadaceae	1.1	2.1	W=211.5, p=0.644
Otherfamily	2.2	0.3	W=283, p=0.210
Gardnerella	6.6	6.8	W=271, p=0.336
Corynebacterium	2.5	4.6	W=223.5, p=0.864
Prevotella	2.2	1.0	W=197.5, p=0.422
Staphylococcus	2.1	2.3	W=203, p=0.503
Lactobacillus	59.0	59.0	W=234, p=0.952
Streptococcus	5.7	1.6	W=261, p=0.473
Anaerococcus	2.3	0.7	W=245.5, p=0.733
Sneathia	0.0	0.1	W=259.5, p=0.439
Brevundimonas	1.0	1.7	W=214.5, p=0.697
Sphingobium	0.9	1.6	W=215.5, p=0.715
Sphingomonas	1.0	1.8	W=189.5, p=0.318
Acinetobacter	1.9	3.3	W=200.5, p=0.466
Pseudomonas	1.1	2.1	W=211.5, p=0.644
OtherGenus	2.2	0.3	W=283, p=0.210

Supplementary Table 19: Relative abundance of bacterial OTUs in the *maternal vagina* by *maternal stress* in the *3rd trimester*. Listed are all OTUs with an abundance of greater than

1% and significant OTUs with an abundance of less than 1%. Low stress (PSS < 11; N=22), high stress (PSS > 12; N=21). Unpaired students t-test and Mann-Whitney-Wilcoxon test. Abbreviations: Operational taxonomic unit (OTU), Perceived Stress Scale (PSS).

Table 20: Maternal vaginal microbiome by 3rd trimester anxiety

OTU	**Relative Abundance (%)**		**p-value**
Vaginal	**Low STAI**	**High STAI**	
Chao1 Index	240.6	228.4	*t*(41) = 0.33, *p*=0.740
Simpson Index	0.5	0.4	*t*(41) = 0.71, *p*=0.477
Shannon Index	2.5	2.1	*t*(41) = 0.83, *p*=0.407
Phylogenetic diversity	9.4	8.8	*t*(41) = 0.49, *p*=0.626
Observed Species	169.1	152.6	*t*(41) = 0.62, *p*=0.534
Actinobacteria	11.2	13.2	W=233, p=0.952
Bacteroidetes	2.9	1.4	W=239, p=0.836
Firmicutes	72.1	70.8	W=245, p=0.726
Fusobacteria	0.0	0.1	W=256, p=0.517
Proteobacteria	10.8	11.9	W=195, p=0.404
Otherphyla	2.1	0.5	W=255, p=0.550
Bifidobacteriaceae	5.8	8.4	W=293.5, p=0.124
Corynebacteriaceae	3.9	4.1	W=225.5, p=0.922
Prevotellaceae	2.2	1.1	W=237, p=0.874
Staphylococcaceae	2.6	1.8	W=225.5, p=0.922
Lactobacillaceae	55.3	62.2	W=216, p=0.744
Streptococcaceae	7.0	0.9	W=287.5, p=0.165
ClostridialesFamilyXI	4.7	2.9	W=245.5, p=0.714
Veillonellaceae	0.7	1.6	W=259.5, p=0.479
Leptotrichiaceae	0.0	0.1	W=277.5, p=0.193
Caulobacteraceae	1.4	1.6	W=207, p=0.583
Sphingomonadaceae	2.6	2.8	W=189, p=0.324
Moraxellaceae	2.6	2.7	W=181, p=0.237
Pseudomonadaceae	1.5	1.7	W=190, p=0.336
Otherfamily	2.1	0.5	W=255, p=0.550
Bifidobacterium	**0.8**	**0.0**	**W=321.5, p=0.026**
Gardnerella	4.9	8.3	W=281, p=0.218
Corynebacterium	3.5	3.5	W=230, p=1
Prevotella	2.2	1.1	W=237, p=0.874
Staphylococcus	2.6	1.8	W=225.5, p=0.922

Lactobacillus	55.3	62.2	W=216, p=0.744
Streptococcus	7.0	0.9	W=287.5, p=0.165
Anaerococcus	2.1	1.0	W=234, p=0.931
Sneathia	0.0	0.1	W=277.5, p=0.193
Brevundimonas	1.3	1.4	W=206, p=0.566
Sphingobium	1.2	1.3	W=190, p=0.336
Sphingomonas	1.3	1.4	W=185, p=0.278
Acinetobacter	2.5	2.6	W=182, p=0.247
Pseudomonas	1.5	1.7	W=190, p=0.336
OtherGenus	2.1	0.5	W=255, p=0.550

Supplementary Table 20: Relative abundance of bacterial OTUs in the *maternal vagina* by *maternal anxiety* in the *3rd trimester*. Listed are all OTUs with an abundance of greater than 1% and significant OTUs with an abundance of less than 1%. Low anxiety (STAI < 4; N=20), high anxiety (STAI > 5; N=23). Unpaired students t-test and Mann-Whitney-Wilcoxon test. Abbreviations: Operational taxonomic unit (OTU), State Trait Anxiety Inventory (STAI).

Table 21: Maternal vaginal microbiome by 3rd trimester depressive symptoms

OTU	**Relative Abundance (%)**		**p-value**
Vaginal	**Low EPDS**	**High EPDS**	
Chao1 Index	231.6	245.1	*t*(41) = -0.28, *p*=0.775
Simpson Index	0.5	0.5	*t*(41) = -0.55, *p*=0.583
Shannon Index	2.3	2.5	*t*(41) = -0.42, *p*=0.671
Phylogenetic diversity	9.0	9.7	*t*(41) = -0.44, *p*=0.659
Observed Species	158.0	170.1	*t*(41) = -0.35, *p*=0.724
Actinobacteria	10.5	20.0	W=114, p=0.433
Bacteroidetes	2.3	1.1	W=130, p=0.766
Firmicutes	74.1	59.6	W=171, p=0.348
Fusobacteria	0.0	0.3	W=139, p=0.987
Proteobacteria	10.7	14.4	W=123, p=0.613
Otherphyla	1.4	0.5	W=124.5, p=0.639
Bifidobacteriaceae	5.8	13.0	W=142.5, p=0.950
Corynebacteriaceae	3.6	5.7	W=114, p=0.425
Prevotellaceae	1.8	0.6	W=123, p=0.606
Staphylococcaceae	2.5	0.7	W=118.5, p=0.512
Lactobacillaceae	60.4	53.0	W=149, p=0.794
Streptococcaceae	4.2	1.9	W=121.5, p=0.574
ClostridialesFamilyXI	4.0	2.8	W=127, p=0.696
Veillonellaceae	1.4	0.3	W=129, p=0.742

Leptotrichiaceae	0.0	0.3	W=134, p=0.845
Caulobacteraceae	1.4	1.8	W=127.5, p=0.707
Sphingomonadaceae	2.5	3.4	W=123.5, p=0.617
Moraxellaceae	2.5	3.2	W=124, p=0.628
Pseudomonadaceae	1.5	2.0	W=131, p=0.790
Otherfamily	1.4	0.5	W=124.5, p=0.639
Gardnerella	5.3	12.9	W=135, p=0.888
Corynebacterium	3.0	5.6	W=103.5, p=0.260
Prevotella	1.8	0.6	W=123, p=0.606
Staphylococcus	2.5	0.7	W=118.5, p=0.512
Lactobacillus	60.4	53.0	W=149, p=0.794
Streptococcus	4.2	1.9	W=121.5, p=0.574
Anaerococcus	1.7	0.8	W=142, p=0.962
Sneathia	0.0	0.3	W=134, p=0.845
Brevundimonas	1.3	1.6	W=125, p=0.650
Sphingobium	1.1	1.6	W=126.5, p=0.684
Sphingomonas	1.3	1.8	W=121.5, p=0.574
Acinetobacter	2.5	3.1	W=124, p=0.628
Pseudomonas	1.5	2.0	W=131, p=0.790
OtherGenus	1.4	0.5	W=124.5, p=0.639

Supplementary Table 21: Relative abundance of bacterial OTUs in the *maternal vagina* by *maternal depressive symptoms* in the *3rd trimester*. Listed are all OTUs with an abundance of greater than 1% and significant OTUs with an abundance of less than 1%. Low depressive symptoms (EPDS < 8; N=35), high depressive symptoms (EPDS > 9; N=8). Unpaired students t-test and Mann-Whitney-Wilcoxon test. Abbreviations: Operational taxonomic unit (OTU), Edinburgh Postnatal Depression Scale (EPDS).

Table 22: Maternal vaginal microbiome by 3rd trimester cortisol

OTU	**Relative Abundance (%)**		**p-value**
Vaginal	**Low CAR**	**High CAR**	
Chao1 Index	245.5	203.1	$t(37) = 1.04$, $p=0.301$
Simpson Index	0.4	0.5	$t(37) = -0.52$, $p=0.604$
Shannon Index	2.1	2.4	$t(37) = -0.48$, $p=0.627$
Phylogenetic diversity	8.7	9.0	$t(37) = -0.20$, $p=0.835$
Observed Species	163.9	140.3	$t(37) = 0.81$, $p=0.420$
Actinobacteria	9.0	16.8	W=162, p=0.717
Bacteroidetes	2.6	0.9	W=182, p=0.849
Firmicutes	78.5	66.5	W=194, p=0.592

Fusobacteria	0.4	0.1	W=207.5, p=0.325
Proteobacteria	7.9	12.1	W=140, p=0.316
Bifidobacteriaceae	4.4	10.3	W=155.5, p=0.577
Corynebacteriaceae	3.6	5.2	W=169.5, p=0.883
Prevotellaceae	2.1	0.5	W=197, p=0.528
Staphylococcaceae	1.5	3.9	W=155.5, p=0.577
Lactobacillaceae	64.2	53.8	W=182, p=0.851
Streptococcaceae	4.5	3.3	W=165.5, p=0.792
ClostridialesFamilyXI	3.9	4.1	W=184.5, p=0.792
Veillonellaceae	2.2	0.2	W=196, p=0.548
Leptotrichiaceae	0.4	0.1	W=202.5, p=0.373
Caulobacteraceae	1.0	1.6	W=136, p=0.259
Sphingomonadaceae	1.8	2.8	W=135.5, p=0.253
Moraxellaceae	1.8	2.8	W=142, p=0.341
Pseudomonadaceae	1.1	1.7	W=137.5, p=0.278
Gardnerella	4.2	10.1	W=130.5, p=0.196
Corynebacterium	0.5	0.4	W=164, p=0.758
Prevotella	2.1	0.5	W=197, p=0.528
Staphylococcus	1.5	3.9	W=155.5, p=0.577
Lactobacillus	64.2	53.8	W=182, p=0.851
Streptococcus	4.5	3.3	W=165.5, p=0.792
Anaerococcus	1.5	1.9	W=175.5, p=1
Sneathia	0.4	0.1	W=202.5, p=0.373
Brevundimonas	0.9	1.5	W=132, p=0.212
Sphingobium	0.8	1.3	W=135.5, p=0.253
Sphingomonas	0.9	1.5	W=140, p=0312
Acinetobacter	1.8	2.8	W=141.5, p=0.333
Pseudomonas	1.1	1.7	W=137.5, p=0.278

Supplementary table 22: Relative abundance of bacterial OTUs in the *maternal vagina* by *maternal cortisol* in the *3rd trimester*. Listed are all OTUs with an abundance of greater than 1% and significant OTUs with an abundance of less than 1%. Low cortisol (CAR < 562349; N=25), high cortisol (CAR > 562350; N=14). Unpaired students t-test and Mann-Whitney-Wilcoxon test. Abbreviations: Operational taxonomic unit (OTU), Cortisol Awakening Response (CAR).

Table 23: Infant gut at 1 week old by 2nd trimester stress

OTU		**Relative Abundance (%)**	
1 week old	**Low PSS**	**High PSS**	**p-value**

Chao1 Index	79.7	76.4	*t*(39) = 0.52, *p*=0.599
Simpson Index	0.6	0.7	*t*(39) = -0.65, *p*=0.514
Shannon Index	2.3	2.4	*t*(39) = -0.31, *p*=0.757
Phylogenetic diversity	4.1	3.9	*t*(39) = 0.90, *p*=0.372
Observed Species	55.6	50.7	*t*(39) = 1.44, *p*=0.157
Actinobacteria	49.4	46.3	W=217, p=0.846
Bacteroidetes	16.4	9.1	W=223.5, p=0.714
Firmicutes	31.9	41.0	W=153, p= 0.148
Proteobacteria	2.0	3.4	W=247.5, p=0.320
Bifidobacteriaceae	32.3	31.7	W=206, p=0.947
Nocardiaceae	15.4	12.5	W=231.5, p=0.565
Coriobacteriaceae	1.2	1.5	W=220.5,p= 0.770
Bacteroidaceae	12.6	8.2	W=225, p=0.685
Porphyromonadaceae	**3.8**	**0.9**	**W=274.5,** p=**0.084**
Staphylococcaceae	5.7	4.7	W=211, p=0.969
Enterococcaceae	1.9	2.5	W=249.5, p=0.293
Lactobacillaceae	1.9	3.3	W=216.5, p=0.853
Streptococcaceae	8.5	7.2	W=213, p=0.927
Clostridiaceae1	5.9	8.7	W=176, p=0.395
Lachnospiraceae	**5.3**	**3.6**	**W=279,** p=**0.069**
Veillonellaceae	1.3	9.6	W=150.5, p=0.129
Enterobacteriaceae	1.8	1.8	W=240.5, p=0.416
Bifidobacterium	32.3	31.7	W=206, p=0.947
Rhodococcus	15.4	12.5	W=231.5, p=0.565
Collinsella	1.2	1.4	W=193, p=0.675
Bacteroides	12.6	8.2	W=225, p=0.685
Porphyromonadaceae uncultured	**2.4**	**0.7**	**W=270,** p=**0.092**
Staphylococcus	5.7	4.7	W=211, p= 0.969
Enterococcus	1.9	2.5	W=249.5, p=0.293
Lactobacillus	1.9	3.3	W=216.5, p=0.853
Streptococcus	8.5	7.2	W=213, p=0.927
Clostridium sensu stricto 1	5.9	8.7	W=176, p=0.395
Lachnospiraceae Incertae Sedis	**5.1**	**3.4**	**W=278.5,** p=**0.070**
Veillonella	1.3	8.2	W=167.5, p=0.283
Enterobacter	1.0	0.1	W=249.5, p=0.279
Escherichia Shigella	0.6	1.7	W=238, p=0.453

Supplementary table 23: Relative abundance of bacterial OTUs in the *infant gut at 1 week old* by *maternal stress* in the *2nd trimester*. Listed are all OTUs with an abundance of greater

than 1% and significant OTUs with an abundance of less than 1%. Low stress (PSS < 13; Vaginal; N=19; C-Section; N=6), high stress (PSS > 14; Vaginal; N=22; C-Section; N=5). Unpaired students t-test and Mann-Whitney-Wilcoxon test. Abbreviations: Operational taxonomic unit (OTU), Perceived Stress Scale (PSS).

Table 24: Infant gut at 1 week old by 2nd trimester anxiety

OTU	**Relative Abundance (%)**		
1 week old	**Low STAI**	**High STAI**	**p-value**
Chao1 Index	80.0	75.0	$t(39) = 0.80$, $p=0.427$
Simpson Index	0.6	0.7	$t(39) = -0.99$, $p=0.325$
Shannon Index	2.2	2.5	$t(39) = -1.52$, $p=0.134$
Phylogenetic diversity	3.9	4.1	$t(39) = -0.72$, $p=0.470$
Observed Species	53.0	53.0	$t(39) = 0.01$, $p=0.986$
Actinobacteria	**57.3**	**34.1**	**W=310, p=0.004**
Bacteroidetes	8.2	18.6	W=169.5, p=0.368
Firmicutes	33.0	42.2	W=150, p=0.158
Proteobacteria	1.3	4.9	W=218.5, p=0.711
Bifidobacteriaceae	36.8	25.1	W=265, p=0.109
Nocardiaceae	**18.8**	**6.9**	**W=297.5, p=0.013**
Propionibacteriaceae	0.0	0.0	W=185.5, p=0.402
Coriobacteriaceae	1.4	1.2	W=195.5, p=0.830
Bacteroidaceae	6.2	16.0	W=184, p=0.605
Porphyromonadaceae	2.0	2.5	W=169.5, p=0.361
Staphylococcaceae	6.0	4.1	W=196, p=0.844
Enterococcaceae	**1.2**	**3.8**	**W=139, p=0.086**
Lactobacillaceae	**2.1**	**3.4**	**W=120, p=0.025**
Streptococcaceae	9.0	6.1	W=217.5, p=0.730
Clostridiaceae1	5.8	9.7	W=180, p=0.533
Clostridiales Family XI	**0.0**	**0.0**	**W=112, p=0.010**
Lachnospiraceae	4.3	4.6	W=217.5, p=0.730
Veillonellaceae	3.4	9.1	W=149.5, p=0.152
Enterobacteriaceae	0.6	3.4	W=245.5, p=0.276
Bifidobacterium	36.8	25.1	W=266, p=0.103
Gardnerella	**0.0**	**0.0**	**W=168, p=0.037**
Rhodococcus	**18.8**	**6.9**	**W=297.5, p=0.013**

Propionibacterium	0.0	0.0	W=185.5, p=0.402
Collinsella	1.4	1.1	W=229, p=0.503
Bacteroides	6.2	16.0	W=184, p=0.605
Porphyromonadaceae uncultured	1.1	2.1	W=171, p=0.260
Staphylococcus	6.0	4.1	W=196, p=0.844
Enterococcus	**1.2**	**3.8**	**W=139, p=0.086**
Lactobacillus	**2.3**	**3.4**	**W=120, p=0.025**
Streptococcus	9.0	6.1	W=217.5, p=0.730
Clostridium sensu stricto 1	5.8	9.7	W=180, p=0.533
Anaerococcus	**0.0**	**0.0**	**W=134, p=0.027**
Finegoldia	**0.0**	**0.0**	**W=138, p=0.043**
Blautia	**0.0**	**0.0**	**W=285, p=0.014**
Coprococcus	**0.0**	**0.0**	**W=151, p=0.023**
Lachnospiraceae Incertae Sedis	3.9	4.6	W=202, p=0.968
Faecalibacterium	**0.0**	**0.0**	**W=154, p=0.032**
Veillonella	2.3	9.1	W=148, p=0.141
Enterobacter	0.0	1.2	W=239.5, p=0.338
Escherichia Shigella	**0.6**	**1.9**	**W=274, p=0.064**

Supplementary table 24: Relative abundance of bacterial OTUs in the *infant gut at 1 week old* by *maternal anxiety* in the *2nd trimester*. Listed are all OTUs with an abundance of greater than 1% and significant OTUs with an abundance of less than 1%. Low anxiety (STAI < 4; Vaginal; N=24; C-Section; N=4), high anxiety (PSS > 5; Vaginal; N=17; C-Section; N=7). Unpaired students t-test and Mann-Whitney-Wilcoxon test. Abbreviations: Operational taxonomic unit (OTU), State Trait Anxiety Inventory (STAI).

Table 25: Infant gut at 1 week old by 2nd trimester depressive symptoms

OTU	**Relative Abundance (%)**		
1 week old	**Low EPDS**	**High EPDS**	**p-value**
Chao1 Index	**80.6**	**67.2**	***t*(39) = 1.77, *p*=0.083**
Simpson Index	0.7	0.7	*t*(39) = 0.21, *p*=0.828
Shannon Index	2.4	2.3	*t*(39) = 0.09, *p*=0.921
Phylogenetic diversity	4.1	3.8	*t*(39) = 0.64, *p*=0.521
Observed Species	**54.6**	**46.5**	***t*(39) = 1.95, *p*=0.058**
Actinobacteria	50.7	35.4	W=176, p=0.155
Bacteroidetes	11.9	15.3	W=130, p=0.960
Firmicutes	34.5	46.3	W=100, p=0.306

Proteobacteria	2.7	2.9	W=163, p=0.315
Bifidobacteriaceae	32.9	28.1	W=154, p=0.479
Nocardiaceae	15.6	6.7	W=175, p=0.161
Coriobacteriaceae	1.6	0.5	W=129, p=0.933
Bacteroidaceae	9.7	12.7	W=175, p=0.680
Porphyromonadaceae	2.1	2.5	W=139.5, p=0.815
Staphylococcaceae	5.5	3.6	W=119, p=0.686
Enterococcaceae	1.5	5.3	W=125, p=0.830
Lactobacillaceae	3.3	0.0	W=130, p=0.960
Streptococcaceae	8.4	5.4	W=153, p=0.500
Clostridiaceae1	7.3	7.7	W=131.5, p=1
Lachnospiraceae	3.7	7.5	W=151, p=0.542
Veillonellaceae	**3.3**	**15.8**	**W=68, p=0.036**
Enterobacteriaceae	**2.1**	**0.3**	**W=183, p=0.095**
Campylobacteraceae	**0.0**	**0.0**	**W=115.5, p=0.048**
Helicobacteraceae	**0.0**	**0.0**	**W=115.5, p=0.048**
Oxalobacteraceae	**0.0**	**0.0**	**W=131.5, p=1**
Bifidobacterium	32.9	28.1	W=155, p=0.610
Rhodococcus	15.6	6.7	W=175, p=0.161
Collinsella	1.5	0.4	W=130.5, p=0.972
Bacteroides	9.7	12.7	W=145, p=0.680
Porphyromonadaceae uncultured	1.4	2.1	W=148.5, p=0.575
Staphylococcus	5.5	3.6	W=119, p=0.686
Enterococcus	1.5	5.3	W=125, p=0.830
Lactobacillus	3.3	0.0	W=140, p=0.508
Streptococcus	8.4	5.4	W=153, p=0.500
Clostridium sensu stricto 1	7.3	7.7	W=131.5, p=1
Lachnospiraceae Incertae Sedis	3.4	7.4	W=151, p=0.542
Veillonella	**2.4**	**15.8**	**W=66, p=0.030**
Campylobacter	**0.0**	**0.0**	**W=115.5, p=0.048**
Helicobacter	**0.0**	**0.0**	**W=115.5, p=0.048**
Enterobacter	**0.6**	**1.4**	**W=191.5, p=0.044**
Massilia	0.0	0.0	W=131.5, p=1
Citrobacter	0.0	0.0	W=137, =0.809
Escherichia Shigella	**0.1**	**0.2**	**W=184.5, p=0.085**

Trabulsiella	0.0	0.0	W=130, p=0.947

Supplementary table 25: Relative abundance of bacterial OTUs in the *infant gut at 1 week old* by *maternal depressive symptoms* in the *2nd trimester*. Listed are all OTUs with an abundance of greater than 1% and significant OTUs with an abundance of less than 1%. Low depressive (EPDS < 8; Vaginal; N=33; C-Section; N=9), high depressive (EPDS > 9; Vaginal; N=8; C-Section; N=2). Unpaired students t-test and Mann-Whitney-Wilcoxon test. Abbreviations: Operational taxonomic unit (OTU), Edinburgh Postnatal Depression Scale (EPDS).

Table 26: Infant gut at 1 week old by 2nd trimester cortisol

OTU	**Relative Abundance (%)**		
1 week old	**Low CAR**	**High CAR**	**p-value**
Chao1 Index	70.4	80.7	$t(34) = -1.66$, $p=0.104$
Simpson Index	0.6	0.7	$t(34) = -0.43$, $p=0.664$
Shannon Index	2.3	2.3	$t(34) = -0.07$, $p=0.938$
Phylogenetic diversity	4.0	4.0	$t(34) = 0.02$ $p=0.979$
Observed Species	51.3	52.6	$t(34) = -0.35$, $p=0.724$
Actinobacteria	43.5	45.8	W=155, p=0.839
Bacteroidetes	12.6	11.9	W=161, p=0.987
Firmicutes	41.3	38.3	W=164, p=0.962
Proteobacteria	**2.3**	**3.7**	**W=109.5, p=0.099**
Bifidobacteriaceae	23.0	35.2	W=126, p=0.261
Nocardiaceae	17.8	9.8	W=181.5, p=0.547
Coriobacteriaceae	1.9	0.6	W=207, p=0.150
Bacteroidaceae	11.8	8.7	W=172.5, p=0.751
Porphyromonadaceae	0.8	3.2	W=180, p=0.572
Staphylococcaceae	5.3	6.1	W=143, p=0.562
Enterococcaceae	4.2	0.9	W=198.5, p=0.253
Lactobacillaceae	3.3	2.3	W=198, p=0.257
Streptococcaceae	9.6	6.7	W=180, p=0.579
Clostridiaceae1	7.8	9.0	W=168.5, p=0.849
Lachnospiraceae	3.6	5.3	W=144, p=0.579
Veillonellaceae	6.3	6.4	W=185.5, p=0.466
Enterobacteriaceae	2.0	1.9	W=148.5, p=0.679
Bifidobacterium	22.9	35.2	W=127.5, p=0.281
Rhodococcus	17.8	9.8	W=181.5, p=0.547
Collinsella	1.9	0.5	W=194, p=0.301
Bacteroides	11.8	8.7	W=172.5, p=0.751

Porphyromonadaceae uncultured	0.5	2.0	W=201.5, p=0.186
Staphylococcus	5.3	6.1	W=143, p=0.562
Enterococcus	4.2	0.9	W=198.5, p=0.253
Lactobacillus	3.3	2.3	W=198, p=0.257
Streptococcus	9.6	6.7	W=180, p=0.579
Clostridium sensu stricto 1	7.8	9.0	W=168.5, p=0.849
Lachnospiraceae Incertae Sedis	3.3	5.1	W=166.5, p=0.889
Veillonella	4.6	6.4	W=169.5, p=0.824
Enterobacter	0.1	1.0	W=151.5, p=0.743
Escherichia Shigella	1.9	0.6	W=152, p=0.761

Supplementary table 26: Relative abundance of bacterial OTUs in the *infant gut at 1 week old* by *maternal cortisol* in the *2nd trimester*. Listed are all OTUs with an abundance of greater than 1% and significant OTUs with an abundance of less than 1%. Low cortisol (CAR < 545131; Vaginal; N=18; C-Section; N=8), high cortisol (CAR > 545132; Vaginal; N=18; C-Section; N=2). Unpaired students t-test and Mann-Whitney-Wilcoxon test. Abbreviations: Operational taxonomic unit (OTU), Cortisol Awakening Response (CAR).

Table 27: Infant gut at 1 week old by 3rd trimester stress

OTU	**Relative Abundance (%)**		
1 week old	**Low PSS**	**High PSS**	**p-value**
Chao1 Index	83.1	76.0	$t(41) = 1.08$, $p=0.283$
Simpson Index	0.7	0.7	$t(41) = 0.27$, $p=0.784$
Shannon Index	2.5	2.4	$t(41) = 0.43$, $p=0.668$
Phylogenetic diversity	**4.2**	**3.8**	**$t(41) = 1.70$, $p=0.095$**
Observed Species	**55.6**	**50.0**	**$t(41) = 1.90$, $p=0.063$**
Actinobacteria	**54.7**	**39.6**	**W=302, p=0.086**
Bacteroidetes	10.5	16.4	W=187, p=0.290
Firmicutes	33.7	37.9	W=220, p=0.800
Proteobacteria	0.9	6.0	W=251, p=0.635
Bifidobacteriaceae	34.0	28.9	W=247, p=0.706
Nocardiaceae	18.1	9.3	W=294, p=0.128
Coriobacteriaceae	2.3	0.6	W=288, p=0.164
Bacteroidaceae	8.3	14.2	W=179.5, p=0.215
Porphyromonadaceae	2.2	2.1	W=229.5, p=0.980
Staphylococcaceae	6.1	4.2	W=220, p=0.800
Enterococcaceae	1.7	3.4	W=238.5, p=0.864

Lactobacillaceae	1.6	3.3	W=192.5, p=0.349
Streptococcaceae	10.0	7.1	W=230, p=0.990
Clostridiaceae1	5.5	10.2	W=184, p=0.258
Lachnospiraceae	4.6	4.8	W=275, p=0.290
Veillonellaceae	2.3	3.5	W=213.5, p=0.679
Caulobacteraceae	**0.0**	**0.0**	**W=297, p=0.008**
Enterobacteriaceae	0.8	5.2	W=270.5, p=0.342
Bifidobacterium	34.0	28.9	W=247, p=0.706
Rhodococcus	18.1	9.3	W=294, p=0.128
Atopobium	**0.0**	**0.0**	**W=275, p=0.035**
Collinsella	2.2	0.5	W=291.5, p=0.132
Bacteroides	8.3	14.2	W=179.5, p=0.215
Porphyromonadaceae uncultured	1.1	1.8	W=203, p=0.477
Staphylococcus	6.1	4.2	W=220, p=0.800
Enterococcus	1.7	3.4	W=238.5, p=0.864
Lactobacillus	1.6	3.3	W=192.5, p=0.349
Streptococcus	10.0	7.1	W=230, p=0.990
Clostridium sensu stricto 1	5.5	10.2	W=184, p=0.258
Finegoldia	**0.0**	**0.0**	**W=132.5, p=0.005**
Blautia	**0.1**	**0.0**	**W=317.5, p=0.017**
Lachnospiraceae Incertae Sedis	4.0	4.5	W=254, p=0.583
Lachnospiraceae uncultured	**0.2**	**0.0**	**W=321.5, p=0.017**
Veillonella	2.2	3.4	W=219, p=0.779
Brevundimonas	**0.0**	**0.0**	**W=297, p=0.008**
Enterobacter	0.1	0.8	W=244.5, p=0.745
Escherichia Shigella	0.6	1.7	W=282.5, p=0.212

Supplementary table 27: Relative abundance of bacterial OTUs in the *infant gut at 1 week old* by *maternal stress* in the *3rd trimester*. Listed are all OTUs with an abundance of greater than 1% and significant OTUs with an abundance of less than 1%. Low stress (PSS < 11; Vaginal; N=21; C-Section; N=4), high stress (PSS > 12; Vaginal; N=22; C-Section; N=5). Unpaired students t-test and Mann-Whitney-Wilcoxon test. Abbreviations: Operational taxonomic unit (OTU), Perceived Stress Scale (PSS).

Table 28: Infant gut at 1 week old by 3rd trimester anxiety

OTU	**Relative Abundance (%)**		
1 week old	**Low STAI**	**High STAI**	**p-value**
Chao1 Index	83.6	74.7	$t(41) = 1.38$, $p=0.173$

Simpson Index	0.7	0.7	$t(41) = 0.11$, $p=0.907$
Shannon Index	2.4	2.4	$t(41) = -0.16$, $p=0.868$
Phylogenetic diversity	4.1	3.8	$t(41) = 1.55$, $p=0.127$
Observed Species	54.9	50.2	$t(41) = 1.58$, $p=0.120$
Actinobacteria	53.1	39.9	W=294, p=0.122
Bacteroidetes	14.4	12.5	W=250, p=0.634
Firmicutes	31.4	40.9	W=178, p=0.212
Proteobacteria	0.9	6.5	W=247, p=0.687
Bifidobacteriaceae	33.7	28.7	W=254, p=0.567
Nocardiaceae	17.0	9.7	W=289, p=0.154
Coriobacteriaceae	2.1	0.7	W=281.5, p=0.208
Bacteroidaceae	11.6	11.0	W=245.5, p=0.714
Porphyromonadaceae	2.7	1.5	W=251, p=0.612
Staphylococcaceae	6.5	3.6	W=251, p=0.621
Enterococcaceae	2.1	3.1	W=278, p=0.245
Lactobacillaceae	**0.8**	**4.4**	**W=152.5, p=0.057**
Streptococcaceae	9.8	7.1	W=237.5, p=0.864
Clostridiaceae1	**4.6**	**11.7**	**W=143, p=0.035**
Lachnospiraceae	**4.8**	**4.5**	**W=303.5, p=0.075**
Veillonellaceae	1.8	4.1	W=175.5, p=0.188
Enterobacteriaceae	0.8	5.6	W=259, p=0.486
Pasteurellaceae	**0.0**	**0.0**	**W=308, p=0.037**
Bifidobacterium	33.7	28.7	W=254, p=0.567
Rhodococcus	17.0	9.7	W=289, p=0.154
Collinsella	2.0	0.6	W=292, p=0.122
Bacteroides	11.6	11.0	W=245.5, p=0.714
Porphyromonadaceae uncultured	1.7	1.1	W=217, p=0.746
Staphylococcus	6.5	3.6	W=251, p=0.621
Enterococcus	2.1	3.1	W=278, p=0.245
Lactobacillus	**0.8**	**4.4**	**W=152.5, p=0.057**
Streptococcus	9.8	7.1	W=237.5, p=0.864
Clostridium sensu stricto 1	**4.6**	**11.7**	**W=143, p=0.035**
Blautia	**0.0**	**0.1**	**W=302, p=0.047**
Lachnospiraceae Incertae Sedis	4.1	4.4	W=295, p=0.115
Peptostreptococcaceae Incertae Sedis	**0.0**	**0.8**	**W=155.5, p=0.044**

Veillonella	1.8	4.0	W=178.5, p=0.213
Enterobacter	0.1	0.6	W=263.5, p=0.408
Escherichia Shigella	0.9	1.7	W=282, p=0.206
Trabulsiella	**0.0**	**2.7**	**W=154.5, p=0.024**
Haemophilus	**0.0**	**0.0**	**W=308, p=0.037**

Supplementary table 28: Relative abundance of bacterial OTUs in the *infant gut at 1 week old* by *maternal anxiety* in the *3rd trimester*. Listed are all OTUs with an abundance of greater than 1% and significant OTUs with an abundance of less than 1%. Low anxiety (STAI < 4; Vaginal; N=23; C-Section; N=5), high anxiety (STAI > 5; Vaginal; N=20; C-Section; N=4). Unpaired students t-test and Mann-Whitney-Wilcoxon test. Abbreviations: Operational taxonomic unit (OTU), State Trait Anxiety Inventory.

Table 29: Infant gut at 1 week old by 3rd trimester depressive symptoms

OTU	**Relative Abundance (%)**		
1 week old	**Low EPDS**	**High EPDS**	**p-value**
Chao1 Index	**83.1**	**71.0**	***t*(41) = 1.75, *p*=0.086**
Simpson Index	0.7	0.6	*t*(41) = 0.83, *p*=0.410
Shannon Index	2.5	2.3	*t*(41) = 0.91, *p*=0.365
Phylogenetic diversity	**4.1**	**3.6**	***t*(41) = 2.28, *p*=0.027**
Observed Species	**54.7**	**48.2**	***t*(41) = 2.05, *p*=0.045**
Actinobacteria	50.1	39.7	W=241, p=0.232
Bacteroidetes	14.7	10.7	W=224, p=0.450
Firmicutes	33.8	40.5	W=166, p=0.456
Proteobacteria	1.2	8.8	W=191, p=0.926
Bifidobacteriaceae	32.9	27.9	W=211, p=0.681
Nocardiaceae	14.7	11.2	W=218.5, p=0.542
Coriobacteriaceae	1.9	0.4	W=243, p=0.203
Bacteroidaceae	12.2	9.2	W=219.5, p=0.525
Porphyromonadaceae	2.4	1.5	W=181.5, p=0.727
Bacillales Family XI	**0.0**	**0.0**	**W=273.5, p=0.031**
Staphylococcaceae	6.4	2.4	W=252, p=0.136
Enterococcaceae	2.1	3.5	W=222.5, p=0.473
Lactobacillaceae	2.6	2.3	W=158.5, p=0.334
Streptococcaceae	9.8	5.6	W=242, p=0.218
Clostridiaceae1	**5.2**	**14.2**	**W=114.5, p=0.034**
Lachnospiraceae	4.3	5.7	W=247.5, p=0.168

Veillonellaceae	1.7	5.6	W=167, p=0.466
Aeromonadaceae	**0.0**	**0.1**	**W=165, p=0.032**
Enterobacteriaceae	0.7	8.5	W=198, p=0.947
Bifidobacterium	32.9	27.9	W=211, p=0.681
Rhodococcus	14.7	11.2	W=218.5, p=0.542
Collinsella	**1.8**	**0.3**	**W=261, p=0.074**
Bacteroides	12.2	9.2	W=219.5, p=0.525
Porphyromonadaceae uncultured	1.6	1.0	W=159.5, p=0.324
Gemella	**0.0**	**0.0**	**W=273.5, p=0.031**
Staphylococcus	6.4	2.4	W=252, p=0.136
Enterococcus	2.1	3.5	W=222.5, p=0.473
Lactobacillus	2.6	2.3	W=158.5, p=0.334
Streptococcus	9.8	5.6	W=242, p=0.218
Clostridium sensu stricto 1	**5.3**	**14.2**	**W=114.5, p=0.034**
Lachnospiraceae Incertae Sedis	3.6	5.7	W=231.5, p=0.340
Lachnospiraceae uncultured	**0.0**	**0.0**	**W=277, p=0.018**
Veillonella	1.7	5.6	W=161, p=0.374
Aeromonas	**0.0**	**0.1**	**W=165, p=0.032**
Enterobacter	0.1	1.4	W=202, p=0.859
Escherichia Shigella	0.5	2.6	W=210.5, p=0.689

Supplementary table 29: Relative abundance of bacterial OTUs in the *infant gut at 1 week old* by *maternal depressive symptoms* in the *3rd trimester*. Listed are all OTUs with an abundance of greater than 1% and significant OTUs with an abundance of less than 1%. Low depressive (EPDS < 8; Vaginal; N=30; C-Section; N=7), high depressive (EPDS > 9; Vaginal; N=13; C-Section; N=2). Unpaired students t-test and Mann-Whitney-Wilcoxon test. Abbreviations: Operational taxonomic unit (OTU), Edinburgh Postnatal Depression Scale (EPDS).

Table 30: Infant gut at 1 week old by 3rd trimester cortisol

OTU	**Relative Abundance (%)**		
1 week old	**Low CAR**	**High CAR**	**p-value**
Chao1 Index	83.1	76.2	$t(35) = 0.93$, $p=0.357$
Simpson Index	0.7	0.7	$t(35) = 0.36$, $p=0.717$
Shannon Index	2.4	2.3	$t(35) = 0.53$, p=0.597
Phylogenetic diversity	4.0	3.8	$t(35) = 0.82$, $p=0.415$
Observed Species	54.1	50.5	$t(35) = 1.06$, $p=0.293$
Actinobacteria	43.7	56.4	W=124, p=0.184
Bacteroidetes	16.4	10.6	W=186.5, p=0.580

Firmicutes	35.8	32.1	W=193, p=0.457
Proteobacteria	3.9	0.6	W=215.5, p=0.149
Bifidobacteriaceae	**23.8**	**40.8**	**W=112, p=0.088**
Nocardiaceae	17.1	14.2	W=194.5, p=0.425
Coriobacteriaceae	2.1	1.1	W=184, p=0.629
Bacteroidaceae	13.9	9.1	W=182, p=0.678
Porphyromonadaceae	2.4	1.5	W=213.5, p=0.163
Staphylococcaceae	3.7	5.6	W=115, p=0.108
Enterococcaceae	2.5	3.6	W=172, p=0.914
Lactobacillaceae	3.9	0.6	W=192.5, p=0.458
Streptococcaceae	7.3	7.5	W=160, p=0.820
Clostridiaceae1	8.2	6.8	W=209, p=0.217
Lachnospiraceae	5.0	4.0	W=210, p=0.203
Veillonellaceae	3.8	3.0	W=196.5, p=0.390
Sphingomonadales Ellin 6055	**0.0**	**0.0**	**W=136.5, p=0.044**
Enterobacteriaceae	**3.1**	**0.5**	**W=227.5, p=0.069**
Bifidobacterium	**23.8**	**40.8**	**W=112, p=0.088**
Rhodococcus	17.1	14.2	W=194.5, p=0.425
Collinsella	2.1	1.0	W=192, p=0.457
Bacteroides	13.9	9.1	W=182, p=0.678
Porphyromonadaceae uncultured	1.1	1.3	W=200.5, p=0.301
Staphylococcus	3.7	5.6	W=115, p=0.108
Enterococcus	2.5	3.6	W=172, p=0.914
Lactobacillus	3.9	0.6	W=192.5, p=0.458
Streptococcus	7.3	7.5	W=160, p=0.820
Clostridium sensu stricto 1	8.2	6.8	W=209, p=0.217
Lachnospiraceae Incertae Sedis	**4.4**	**3.7**	**W=225, p=0.082**
Veillonella	2.3	3.0	W=180.5, p=0.712
Sphingomonadales Ellin 6055 uncultured	**0.0**	**0.0**	**W=136.5, p=0.044**
Enterobacter	1.0	0.0	W=220.5, p=0.100
Escherichia Shigella	1.9	0.5	W=206, p=0.246

Supplementary table 30: Relative abundance of bacterial OTUs in the *infant gut at 1 week old* by *maternal cortisol* in the *3rd trimester*. Listed are all OTUs with an abundance of greater than 1% and significant OTUs with an abundance of less than 1%. Low cortisol (CAR < 562349; Vaginal; N=21; C-Section; N=7), high cortisol (CAR > 562350; Vaginal; N=16; C-Section; N=7). Unpaired students t-test and Mann-Whitney-Wilcoxon test. Abbreviations: Operational taxonomic unit (OTU), Cortisol Awakening Response (CAR).

Table 31: Infant gut at 2 weeks old by 2nd trimester stress

OTU	**Relative Abundance (%)**		
2 weeks old	**Low PSS**	**High PSS**	**p-value**
Chao1 Index	83.3	80.1	*t*(36) = 0.43, *p*=0.663
Simpson Index	**0.7**	**0.7**	***t*(36) =-1.69, *p*=0.099**
Shannon Index	2.4	2.7	*t*(36) =-1.51,p= 0.138
Phylogenetic diversity	4.3	4.3	*t*(36) = 0.15,p= 0.874
Observed Species	55.8	56.9	*t*(36) =-0.29, p=0.768
Actinobacteria	52.8	54.0	W=177, p=0.942
Bacteroidetes	11.9	7.7	W=174.5, p=0.883
Firmicutes	31.3	36.4	W=156, p=0.496
Proteobacteria	3.8	1.7	W=205, p=0.478
Bifidobacteriaceae	41.8	36.6	W=199, p=0.592
Nocardiaceae	9.3	14.3	W=145.5, p=0.320
Coriobacteriaceae	1.3	2.4	W=154.5, p=0.458
Bacteroidaceae	10.7	5.8	W=177.5, p=0.953
Porphyromonadaceae	1.1	1.8	W=196, p=0.636
Staphylococcaceae	5.1	2.8	W=196, p=0.654
Enterococcaceae	0.6	2.1	W=153, p=0.437
Lactobacillaceae	1.7	1.2	W=171.5, p=0.814
Streptococcaceae	6.3	4.9	W=196.5, p=0.639
Clostridiaceae1	5.8	9.0	W=135, p=0.193
Lachnospiraceae	5.0	5.5	W=169.5, p=0.769
Veillonellaceae	5.9	7.9	W=128, p=0.131
Enterobacteriaceae	3.3	1.7	W=174.5, p=0.883
Pseudomonadaceae	**0.0**	**0.0**	**W=239.5, p=0.044**
Xanthomonadaceae	**0.0**	**0.0**	**W=220, p=0.029**
Bifidobacterium	41.8	36.6	W=199, p=0.592
Rhodococcus	9.3	14.4	W=145.5, p=0.320
Collinsella	1.1	2.3	W=155, p=0.446
Bacteroides	10.7	5.8	W=177.5, p=0.953
Staphylococcus	5.1	2.8	W=196, p=0.654
Enterococcus	0.6	2.1	W=153, p=0.437
Lactobacillus	1.7	1.2	W=171.5, p=0.814
Streptococcus	6.3	4.9	W=196.5, p=0.639

Clostridium sensu stricto 1	5.8	9.0	W=135, p=0.193
Blautia	**0.0**	**0.2**	**W=117, p=0.006**
Lachnospiraceae Incertae Sedis	4.9	2.6	W=184.5, p=0.906
Dialister	**0.0**	**0.0**	**W=240, p=0.006**
Veillonella	5.1	5.6	W=140, p=0.248
Sphingomonas	**0.0**	**0.0**	**W=220, p=0.030**
Enterobacter	2.0	0.3	W=152, p=0.416
Pseudomonas	**0.0**	**0.0**	**W=239.5, p=0.044**
Stenotrophomonas	**0.0**	**0.0**	**W=220, p=0.029**

Supplementary table 31: Relative abundance of bacterial OTUs in the *infant gut at 1 week old* by *maternal stress* in the *2nd trimester*. Listed are all OTUs with an abundance of greater than 1% and significant OTUs with an abundance of less than 1%. Low stress (PSS < 13; Vaginal; N=18; C-Section; N=8), high stress (PSS > 14; Vaginal; N=20; C-Section; N=4). Unpaired students t-test and Mann-Whitney-Wilcoxon test. Abbreviations: Operational taxonomic unit (OTU), Perceived Stress Scale (PSS).

Table 32: Infant gut at 2 weeks old by 2nd trimester anxiety

OTU	**Relative Abundance (%)**		
2 weeks old	**Low STAI**	**High STAI**	**p-value**
Chao1 Index	79.5	85.8	*t*(36) =-0.82, *p*=0.412
Simpson Index	0.7	0.7	*t*(36) =-0.82, *p*=0.417
Shannon Index	2.5	2.7	*t*(36) =-0.70,p=0.482
Phylogenetic diversity	4.3	4.3	*t*(36) =-0.18,p=0.853
Observed Species	56.5	56.3	*t*(36) =0.06, p=0.952
Actinobacteria	54.1	52.1	W=178, p=0.648
Bacteroidetes	7.8	13.3	W=135.5, p=0.414
Firmicutes	35.2	31.6	W=184, p=0.523
Proteobacteria	2.7	2.8	W=148, p=0.670
Bifidobacteriaceae	38.0	41.0	W=139, p=0.484
Nocardiaceae	13.8	8.3	W=196.5, p=0.302
Coriobacteriaceae	1.9	1.8	W=127.5, p=0.281
Bacteroidaceae	6.1	12.1	W=128, p=0.295
Porphyromonadaceae	1.6	1.1	W=153, p=0.773
Staphylococcaceae	4.0	3.6	W=169, p=0.855
Enterococcaceae	1.2	1.7	W=135.5, p=0.414
Lactobacillaceae	0.4	3.4	W=131.5, p=0.345

Streptococcaceae	6.5	3.7	W=210.5, p=0.143
Clostridiaceae1	7.7	7.2	W=175, p=0.711
Lachnospiraceae	5.7	4.5	W=140, p=0.498
Veillonellaceae	8.4	4.1	W=186.5, p=0.469
Shewanellaceae	**0.0**	**0.0**	**W= 118.5, p=0.022**
Enterobacteriaceae	2.3	2.7	W=137, p=0.441
Bifidobacterium	38.0	41.0	W=139, p=0.484
Rhodococcus	13.8	8.3	W=196.5, p=0.302
Collinsella	1.8	1.6	W=134, p=0.359
Bacteroides	6.1	12.1	W=128, p=0.295
Staphylococcus	4.0	3.6	W=169, p=0.855
Enterococcus	1.22	1.7	W=135.5, p=0.414
Lactobacillus	0.4	3.4	W=131.5, p=0.345
Streptococcus	6.5	3.7	W=210.5, p=0.143
Clostridium sensu stricto 1	7.7	7.2	W=175, p=0.711
Lachnospiraceae Incertae Sedis	3.5	4.1	W=140, p=0.497
Veillonella	6.1	4.1	W=165.5, p=0.938
Shewanella	**0.0**	**0.0**	**W=118.5, p=0.022**
Enterobacter	1.1	1.1	W=111, p=0.113

Supplementary table 32: Relative abundance of bacterial OTUs in the *infant gut at 2 weeks old* by *maternal anxiety* in the *2nd trimester*. Listed are all OTUs with an abundance of greater than 1% and significant OTUs with an abundance of less than 1%. Low anxiety (STAI < 4; Vaginal; N=25; C-Section; N=6), high anxiety (STAI > 5; Vaginal; N=13; C-Section; N=6). Unpaired students t-test and Mann-Whitney-Wilcoxon test. Abbreviations: Operational taxonomic unit (OTU), State Trait Anxiety Inventory (STAI).

Table 33: Infant gut at 2 weeks old by 2nd trimester depressive symptoms

OTU	**Relative Abundance (%)**		
2 weeks old	**Low EPDS**	**High EPDS**	**p-value**
Chao1 Index	82.6	78.5	$t(36) = 0.48$, $p=0.628$
Simpson Index	0.5	0.7	$t(36) = -0.71$, $p=0.477$
Shannon Index	2.5	2.75	$t(36) = -0.74$, $p=0.462$
Phylogenetic diversity	4.3	4.4	$t(36) = -0.51$, $p=0.606$
Observed Species	56.0	57.8	$t(36) = -0.43$, $p=0.665$
Actinobacteria	**56.9**	**42.2**	**W=184, p=0.068**

Bacteroidetes	9.9	9.0	W=115, p=0.606
Firmicutes	30.2	46.2	W=84, p=0.115
Proteobacteria	2.8	2.4	W=127, p=0.919
Bifidobacteriaceae	41.3	31.7	W=161, p=0.308
Nocardiaceae	13.0	8.4	W=166.5, p=0.222
Coriobacteriaceae	2.2	1.0	W=111.5, p=0.519
Bacteroidaceae	8.2	8.1	W=108, p=0.449
Porphyromonadaceae	1.6	0.9	W=119.5, p=0.707
Staphylococcaceae	4.1	2.9	W=122, p=0.787
Enterococcaceae	1.2	2.1	W=106.5, p=0.418
Lactobacillaceae	1.8	0.3	W=136.5, p=0.849
Streptococcaceae	6.0	4.1	W=156.5, p=0.381
Clostridiaceae1	5.5	14.2	W=88.5, p=0.154
Lachnospiraceae	5.3	5.2	W=147.5, p=0.570
Veillonellaceae	5.4	11.9	W=86, p=0.130
Enterobacteriaceae	2.5	2.3	W=119, p=0.633
Bifidobacterium	41.3	31.7	W=161, p=0.308
Rhodococcus	13.0	8.4	W=166.5, p=0.222
Collinsella	2.0	0.7	W=112, p=0.511
Bacteroides	8.2	8.1	W=108, p=0.449
Staphylococcus	4.1	2.9	W=122, p=0.787
Enterococcus	1.2	2.1	W=106.5, p=0.418
Lactobacillus	1.8	0.3	W=136.5, p=0.849
Streptococcus	6.0	4.1	W=156.5, p=0.381
Clostridium sensu stricto 1	5.5	14.2	W=88.5, p=0.164
Lachnospiraceae Incertae Sedis	4.1	2.3	W=150, p=0.513
Megamonas	**0.0**	**0.0**	**W=80.5, p=0.007**
Veillonella	3.9	10.3	W=95, p=0.229
Enterobacter	1.3	0.5	W=83.5, p=0.106

Supplementary table 33: Relative abundance of bacterial OTUs in the *infant gut at 2 weeks old* by *maternal depressive symptoms* in the *2nd trimester*. Listed are all OTUs with an abundance of greater than 1% and significant OTUs with an abundance of less than 1%. Low depressive (EPDS < 8; Vaginal; N=29; C-Section; N=11), high depressive (EPDS > 9; Vaginal; N=9; C-Section; N=1). Unpaired students t-test and Mann-Whitney-Wilcoxon test. Abbreviations: Operational taxonomic unit (OTU), Edinburgh Postnatal; Depression Scale (EPDS).

Table 34: Infant gut at 2 weeks old by 2nd trimester cortisol

OTU	Relative Abundance (%)		
2 weeks old	**Low CAR**	**High CAR**	**p-value**
Chao1 Index	86.6	82.7	*t*(29) = 0.44, *p*=0.606
Simpson Index	**0.7**	**0.6**	***t*(29) = 2.29, *p*=0.028**
Shannon Index	**2.8**	**2.3**	***t*(29) = 2.63,p=0.013**
Phylogenetic diversity	4.6	4.2	*t*(29) = 1.42,p=0.164
Observed Species	59.8	54.6	*t*(29) = 1.19, p=0.240
Actinobacteria	**44.3**	**55.5**	**W=76, p=0.091**
Bacteroidetes	**16.5**	**7.2**	**W=170.5, p=0.042**
Firmicutes	36.8	35.1	W=117, p=0.953
Proteobacteria	2.1	2.0	W=107, p=0.652
Bifidobacteriaceae	31.6	41.4	W=93, p=0.916
Nocardiaceae	9.2	13.6	W=86.5, p=0.204
Coriobacteriaceae	**2.7**	**0.0**	**W=199.5, p=0.001**
Bacteroidaceae	**12.4**	**7.1**	**W=161, p=0.099**
Porphyromonadaceae	**4.0**	**0.1**	**W=169.5, p=0.040**
Rikenellaceae	**0.0**	**0.0**	**W=155.5, p=0.037**
Staphylococcaceae	3.3	3.8	W=105, p=0.797
Enterococcaceae	1.7	1.4	W=132, p=0.619
Lactobacillaceae	1.4	1.5	W=122, p=0.920
Streptococcaceae	3.7	7.3	W=89, p=0.241
Clostridiaceae1	7.9	7.7	W=96.5, p=0.382
Lachnospiraceae	4.6	6.1	W=149, p=0.241
Aeromonadaceae	0.0	0.0	W=118.5, p=1
Veillonellaceae	11.1	5.3	W=144, p=0.330
Enterobacteriaceae	1.6	1.6	W=113, p=0.827
Bifidobacterium	31.6	41.4	W=93, p=0.316
Rhodococcus	9.2	13.6	W=86.5, p=0.204
Collinsella	**2.6**	**0.0**	**W=180, p=0.008**
Bacteroides	**12.4**	**7.1**	**W=161, p=0.099**
Parabacteroides	**3.1**	**0.1**	**W=167, p=0.038**
Alistipes	**0.0**	**0.0**	**W=155.5, p=0.037**
Staphylococcus	3.3	3.8	W=105, p=0.597
Enterococcus	1.7	1.4	W=132, p=0.619

Lactobacillus	1.4	1.5	W=122, p=0.920
Streptococcus	3.7	7.3	W=89, p=0.241
Clostridium sensu stricto 1	7.9	7.7	W=96.5, p=0.382
Lachnospiraceae Incertae Sedis	**4.5**	**2.9**	**W=161.5, p=0.094**
Phascolarctobacterium	**1.2**	**0.0**	**W=161.5, p=0.009**
Veillonella	8.9	4.4	W=142.5, p=0.361
Aeromonas	0.0	0.0	W=118.5, p=1
Enterobacter	0.3	0.8	W=144.5, p=0.315

Supplementary table 34: Relative abundance of bacterial OTUs in the *infant gut at 2 weeks old* by *maternal cortisol* in the *2nd trimester*. Listed are all OTUs with an abundance of greater than 1% and significant OTUs with an abundance of less than 1%. Good sleep quality (CAR < 545131; Vaginal; N=14; C-Section; N=8), poor sleep quality (CAR > 545132; Vaginal; N=17; C-Section; N=3). Unpaired students t-test and Mann-Whitney-Wilcox test. Abbreviations: Operational taxonomic unit (OTU), Cortisol Awakening Response (CAR).

Table 35: Infant gut at 2 weeks old by 3rd trimester stress

OTU	**Relative Abundance (%)**		
2 weeks old	**Low PSS**	**High PSS**	**p-value**
Chao1 Index	86.6	76.6	$t(33) = 1.23$, $p=0.224$
Simpson Index	0.7	0.7	$t(33) =1.30$, $p=0.201$
Shannon Index	2.7	2.5	$t(33) = 1.18$,p=0.245
Phylogenetic diversity	4.5	4.2	$t(33) = 1.09$,p=0.281
Observed Species	59.6	54.7	$t(33) = 1.23$, p=0.226
Actinobacteria	53.5	51.2	W=158, p=0.805
Bacteroidetes	10.7	14.4	W=131, p=0.537
Firmicutes	31.0	32.5	W=147, p=0.934
Proteobacteria	4.7	1.8	W=171.5, p=0.483
Bifidobacteriaceae	36.6	43.8	W=122, p=0.364
Nocardiaceae	**13.8**	**6.3**	**W=202.5, p=0.083**
Coriobacteriaceae	**2.7**	**0.2**	**W=212.5, p=0.036**
Bacteroidaceae	9.7	13.3	W=133, p=0.582
Porphyromonadaceae	0.8	1.1	W=138.5, p=0.707
Staphylococcaceae	3.6	3.4	W=137, p=0.680
Enterococcaceae	1.0	1.6	W=124.5, p=0.403
Lactobacillaceae	1.1	2.5	W=113, p=0.222
Streptococcaceae	5.1	5.6	W=178, p=0.364
Clostridiaceae1	4.5	8.1	W=125, p=0.414
Lachnospiraceae	7.6	5.6	W=190.5, p=0.182

Veillonellaceae	**6.5**	**3.1**	**W=205, p=0.069**
Enterobacteriaceae	4.4	1.5	W=178.5, p=0.350
Bifidobacterium	36.6	43.8	W=122, p=0.362
Rhodococcus	**13.8**	**6.3**	**W=202.5, p=0.083**
Collinsella	**2.5**	**0.0**	**W=199.5, p=0.078**
Bacteroides	9.7	13.3	W=133, p=0.582
Staphylococcus	3.6	3.4	W=137, p=0.680
Enterococcus	1.0	1.6	W=124.5, p=0.403
Lactobacillus	1.1	2.5	W=113, p=0.222
Streptococcus	5.2	5.6	W=178, p=0.364
Clostridium sensu stricto 1	4.5	8.1	W=125, p=0.414
Finegoldia	**0.0**	**0.8**	**W=83, p=0.020**
Lachnospiraceae Incertae Sedis	4.8	3.9	W=188.5, p=0.204
Dialister	**0.0**	**0.0**	**W=195, p=0.023**
Negativicoccus	**0.0**	**0.0**	**W-110, p=0.044**
Veillonella	5.0	3.1	W=193.5, p=0.151
Enterobacter	1.3	0.3	W=142.5, p=0.813

Supplementary table 35: Relative abundance of bacterial OTUs in the *infant gut at 2 weeks old* by *maternal pregnancy stress* in the *3rd trimester*. Listed are all OTUs with an abundance of greater than 1% and significant OTUs with an abundance of less than 1%. Low stress (PSS < 11; Vaginal; N=20; C-Section; N=6), high stress (PSS > 15; Vaginal; N=14; C-Section; N=5). Unpaired students t-test and Mann-Whitney-Wilcoxon test. Abbreviations: Operational taxonomic unit (OTU), Perceived Stress Scale (PSS).

Table 36: Infant gut at 2 weeks old by 3rd trimester anxiety

OTU	**Relative Abundance (%)**		
2 weeks old	**Low STAI**	**High STAI**	**p-value**
Chao1 Index	86.9	73.2	$t(33) = 1.68$, $p=0.101$
Simpson Index	0.7	0.7	$t(33) = -0.52$, $p=0.606$
Shannon Index	2.6	2.7	$t(33) = -0.61$,p=0.541
Phylogenetic diversity	4.4	4.3	$t(33) = 0.58$,p=0.559
Observed Species	58.6	55.4	$t(33) = 0.76$, p=0.449
Actinobacteria	54.1	49.4	W=160, p=0.460
Bacteroidetes	11.5	13.7	W=122, p=0.590
Firmicutes	30.3	34.0	W=131, p=0.823
Proteobacteria	3.8	2.7	W=133.5, p=0.889
Bifidobacteriaceae	39.1	40.9	W=132, p=0.850
Nocardiaceae	12.4	7.1	W=177.5, p=0.175

Coriobacteriaceae	2.2	0.5	W=138.5, p=1
Bacteroidaceae	10.7	12.3	W=121.5, p=0.577
Porphyromonadaceae	0.7	1.3	W=106.5, p=0.269
Staphylococcaceae	4.1	2.4	W=158, p=0.503
Enterococcaceae	1.1	1.6	W=133.5, p=0.889
Lactobacillaceae	**0.8**	**3.4**	**W=83.5, p=0.059**
Streptococcaceae	5.5	5.1	W=165, p=0.362
Clostridiaceae1	4.0	9.9	W=105, p=0.258
Lachnospiraceae	7.7	5.0	W=151.5, p=0.651
Veillonellaceae	5.7	3.8	W=165, p=0.356
Shewanellaceae	**0.0**	**0.0**	**W=100, p=0.031**
Enterobacteriaceae	3.5	2.6	W=120.5, p=0.554
Pasteurellaceae	**0.2**	**0.0**	**W=198.5, p=0.024**
Actinomyces	**0.3**	**0.5**	**W=162.5, p=0.354**
Bifidobacterium	39.1	40.9	W=132, p=0.850
Rhodococcus	12.4	7.1	W=177.5, p=0.175
Collinsella	2.0	0.2	W=141.5, p=0.910
Bacteroides	10.7	12.3	W=121.5, p=0.577
Staphylococcus	4.1	2.4	W=158, p=0.503
Enterococcus	1.1	1.6	W=133.5, p=0.889
Lactobacillus	**0.8**	**3.4**	**W=83.5, p=0.059**
Streptococcus	5.5	5.1	W=165, p=0.362
Clostridium sensu stricto 1	4.0	9.9	W=105, p=0.258
Lachnospiraceae Incertae Sedis	4.2	4.9	W=146.5, p=0.780
Veillonella	4.4	3.7	W=157, p=0.520
Shewanella	**0.0**	**0.0**	**W=100, p=0.031**
Enterobacter	**0.9**	**1.0**	**W=88.5, p=0.085**
Haemophilus	**0.2**	**0.0**	**W=199, p=0.023**

Supplementary table 36: Relative abundance of bacterial OTUs in the *infant gut at 2 weeks old* by *maternal pregnancy anxiety* in the *3rd trimester*. Listed are all OTUs with an abundance of greater than 1% and significant OTUs with an abundance of less than 1%. Low anxiety (STAI < 4; Vaginal; N=23; C-Section; N=5), high anxiety (STAI > 5; Vaginal; N=12; C-Section; N=6). Unpaired students t-test and Mann-Whitney-Wilcoxon test. Abbreviations: Operational taxonomic unit (OTU), State Trait Anxiety Inventory (STAI).

Table 37: Infant gut at 2 weeks old by 3rd trimester depressive symptoms

OTU	Relative Abundance (%)		
2 weeks old	**Low EPDS**	**High EPDS**	**p-value**
Chao1 Index	**86.7**	**67.0**	***t*(33) = 2.20, *p*=0.034**
Simpson Index	0.7	0.7	*t*(33) =-0.50, *p*=0.616
Shannon Index	2.6	2.7	*t*(33) =-0.21,p=0.834
Phylogenetic diversity	4.5	4.0	*t*(33) = 1.68,p=0.101
Observed Species	**59.9**	**49.3**	***t*(33) = 2.40, p=0.022**
Actinobacteria	55.4	42.6	W=146, p=0.143
Bacteroidetes	12.4	11.9	W=112, p=0.890
Firmicutes	**28.2**	**43.2**	**W=59, p=0.055**
Proteobacteria	3.8	2.2	W=127.5, p=0.455
Bifidobacteriaceae	40.3	37.7	W=117, p=0.743
Nocardiaceae	**12.6**	**3.8**	**W=167.5, p=0.020**
Propionibacteriaceae	**0.1**	**0.0**	**W=152, p=0.037**
Coriobacteriaceae	2.1	0.0	W=148.5, p=0.111
Bacteroidaceae	11.4	10.9	W=111.5, p=0.906
Porphyromonadaceae	0.9	1.0	W=104.5, p=0.903
Staphylococcaceae	3.9	2.1	W=127, p=0.475
Enterococcaceae	1.0	2.3	W=103.5, p=0.874
Lactobacillaceae	0.9	4.4	W=70, p=0.139
Streptococcaceae	5.2	5.7	W=130, p=0.405
Clostridiaceae1	3.5	14.5	W=73, p=0.175
Lachnospiraceae	6.9	6.3	W=131.5, p=0.366
Veillonellaceae	4.9	5.4	W=108, p=1
Aeromonadaceae	**0.0**	**0.0**	**W=64, p=0.014**
Shewanellaceae	**0.0**	**0.0**	**W=77, p=0.048**
Enterobacteriaceae	3.5	2.1	W=119.5, p=0.665
Pasteurellaceae	**0.1**	**0.0**	**W=165, p=0.016**
Bifidobacterium	40.3	37.7	W=117, p=0.743
Rhodococcus	12.6	3.8	W=167.5, p=0.020
Collinsella	1.8	0.0	W=133.5, p=0.340
Bacteroides	11.4	10.9	W=111.5, p=0.906

Staphylococcus	3.9	2.1	W=127, p=0.475
Enterococcus	1.0	2.3	W=103.5, p=0.874
Lactobacillus	0.9	4.4	W=70, p=0.139
Streptococcus	5.2	5.7	W=130, p=0.405
Clostridium sensu stricto 1	3.5	14.5	W=73, p=0.175
Lachnospiraceae Incertae Sedis	3.9	6.3	W=124.5, p=0.529
Veillonella	3.8	5.4	W=97.5, p=0.694
Aeromonas	**0.0**	**0.0**	**W=77, p=0.048**
Shewanell	**0.0**	**0.0**	**W=77, p=0.048**
Enterobacter	1.0	0.5	W=82, p=0.311
Haemophilus	**0.1**	**0.0**	**W=165, p=0.016**

Supplementary table 37: Relative abundance of bacterial OTUs in the *infant gut at 2 weeks old* by *maternal pregnancy depressive symptoms* in the *3rd trimester*. Listed are all OTUs with an abundance of greater than 1% and significant OTUs with an abundance of less than 1%. Low depressive (EPDS < 8; Vaginal; N=27; C-Section; N=9), high depressive (EPDS > 9; Vaginal; N=8; C-Section; N=2). Unpaired students t-test and Mann-Whitney-Wilcoxon test. Abbreviations: Operational taxonomic unit (OTU), Edinburgh Postnatal Depression Scale (EPDS).

Table 38: Infant gut at 2 weeks old by 3rd trimester cortisol

OTU	**Relative Abundance (%)**		
2 weeks old	**Low CAR**	**High CAR**	**p-value**
Chao1 Index	85.9	76.0	$t(33) = 1.22$, $p=0.228$
Simpson Index	0.7	0.7	$t(33) = 1.12$, $p=0.267$
Shannon Index	2.7	2.4	$t(33) = 1.36$,p=0.180
Phylogenetic diversity	4.4	4.0	$t(33) = 1.31$,p=0.196
Observed Species	58.3	52.4	$t(33) = 1.66$, p=0.104
Actinobacteria	46.6	55.2	W=122, p=0.318
Bacteroidetes	17.9	9.3	W=167, p=0.655
Firmicutes	33.5	33.5	W=159, p=0.857
Proteobacteria	1.7	1.8	W=164.5, p=0.716
Bifidobacteriaceae	**29.2**	**44.9**	**W=96, p=0.061**
Nocardiaceae	12.8	8.4	W=192, p=0.203
Coriobacteriaceae	**4.3**	**1.0**	**W=222.5, p=0.020**

Bacteroidaceae	16.0	8.7	W=166.5, p=0.667
Porphyromonadaceae	1.8	0.5	W=200.5, p=0.110
Staphylococcaceae	2.9	4.8	W=104, p=0.110
Enterococcaceae	1.0	1.7	W=147, p=0.855
Lactobacillaceae	3.1	0.5	W=196.5, p=0.153
Streptococcaceae	5.4	7.4	W=129, p=0.442
Clostridiaceae1	7.5	6.0	W=174, p=0.498
Lachnospiraceae	5.2	5.7	W=179.5, p=0.390
Acidaminococcaceae	**1.1**	**0.1**	**W=215.5, P=0.007**
Veillonellaceae	6.1	6.2	W=151.5, p=0.973
Enterobacteriaceae	1.1	1.6	W=177, p=0.437
Bifidobacterium	**29.2**	**44.9**	**W=96, p=0.061**
Rhodococcus	12.8	8.4	W=192, p=0.203
Collinsella	4.2	0.9	W=188, p=0.225
Bacteroides	16.0	8.7	W=166.5, p=0.667
Staphylococcus	2.9	4.8	W=104, p=0.110
Enterococcus	1.0	1.7	W=147, p=0.855
Lactobacillus	3.1	0.5	W=196.5, p=0.153
Streptococcus	5.4	7.4	W=129, p=0.442
Clostridium sensu stricto 1	7.5	6.0	W=174, p=0.498
Coprococcus	**0.0**	**0.0**	**W=189, p=0.033**
Lachnospiraceae Incertae Sedis	3.6	4.1	W=190.5, p=0.221
Phascolarctobacterium	**1.1**	**0.1**	**W=215.5, p=0.007**
Veillonella	4.3	6.2	W=140.5, p=0.691
Enterobacter	0.4	0.5	W=177.5, p=0.421

Supplementary table 38: Relative abundance of bacterial OTUs in the *infant gut at 2 weeks old* by *maternal cortisol* in the *3rd trimester*. Listed are all OTUs with an abundance of greater than 1% and significant OTUs with an abundance of less than 1%. Low cortisol (CAR < 562349; Vaginal; N=17; C-Section; N=5), high cortisol (CAR > 562350; Vaginal; N=18; C-Section; N=6). Unpaired students t-test and Mann-Whitney-Wilcoxon test. Abbreviations: Operational taxonomic unit (OTU), Cortisol Awakening Response (CAR).

Table 39: Infant gut at 3 weeks old by 2nd trimester stress

OTU	Relative Abundance (%)		
3 weeks old	**Low PSS**	**High PSS**	**p-value**
Chao1 Index	83.8	86.3	*t*(42) =-0.32, *p*=0.746
Simpson Index	**0.7**	**0.8**	***t*(42) =-2.56, *p*=0.013**
Shannon Index	**2.6**	**2.9**	***t*(42) =-1.86,p=0.069**
Phylogenetic diversity	4.4	4.4	*t*(42) = 0.01,p=0.988
Observed Species	60.6	59.3	*t*(42) = 0.40, p=0.689
Actinobacteria	51.4	41.9	W=299, p=0.182
Bacteroidetes	13.6	8.2	W=305.5, p=0.135
Firmicutes	**31.8**	**45.4**	**W=149, p=0.029**
Proteobacteria	3.1	4.3	W=235, p=0.887
Bifidobacteriaceae	**39.1**	**26.3**	**W=315, p=0.086**
Nocardiaceae	10.4	13.2	W=223, p=0.672
Coriobacteriaceae	1.6	1.8	W=255.5, p=0.749
Bacteroidaceae	**11.5**	**7.1**	**W=326, p=0.048**
Porphyromonadaceae	**2.1**	**1.0**	**W=347.7, p=0.012**
Staphylococcaceae	2.5	1.2	W=250, p=0.850
Enterococcaceae	1.0	2.3	W=204, p=0.383
Lactobacillaceae	3.5	1.6	W=286, p=0.300
Streptococcaceae	7.5	4.5	W=272, p=0.484
Clostridiaceae1	**6.5**	**14.2**	**W=159.5, p=0.055**
Lachnospiraceae	5.1	9.7	W=203.5, p=0.378
Veillonellaceae	**4.6**	**10.1**	**W=144, p=0.021**
Enterobacteriaceae	2.7	3.5	W=234, p=0.869
Bifidobacterium	**39.0**	**26.3**	**W=315, p=0.086**
Rhodococcus	10.3	13.2	W=223, p=0.672
Collinsella	1.4	1.7	W=217, p=0.558
Bacteroides	**11.5**	**7.1**	**W=326, p=0.048**
Parabacteroides	**1.0**	**1.0**	**W=337.5, p=0.020**
Porphyromonadaceae uncultured	**1.0**	**0.0**	**W=345, p=0.007**
Staphylococcus	2.5	1.2	W=250, p=0.850
Enterococcus	1.0	2.3	W=204, p=0.383

Lactobacillus	3.5	1.6	W=286, p=0.300
Streptococcus	7.5	4.5	W=272, p=0.484
Clostridiumsensustricto1	**6.4**	**14.2**	**W=157, p=0.048**
LachnospiraceaeIncertaeSedis	5.1	6.6	W=243.5, p=0.971
Lachnospiraceaeuncultured	0.0	2.2	W=225.5, p=0.698
Veillonella	**3.6**	**9.2**	**W=157, p=0.048**
Sphingomonas	0.0	0.0	W=218, p=0.497
Enterobacter	1.44	0.97	W=221.5, p=0.643
EscherichiaShigella	0.8	2.3	W=240.5, p=0.990

Supplementary table 39: Relative abundance of bacterial OTUs in the *infant gut at 3 weeks old* by *maternal stress* in the *2nd trimester*. Listed are all OTUs with an abundance of greater than 1% and significant OTUs with an abundance of less than 1%. Low stress (PSS < 13; Vaginal; N=23; C-Section; N=7), high stress (PSS > 14; Vaginal; N=21; C-Section; N=5). Unpaired students t-test and Mann-Whitney-Wilcoxon test. Abbreviations: Operational taxonomic unit (OTU), Perceived Stress Scale (PSS).

Table 40: Infant gut at 3 weeks old by 2nd trimester anxiety

OTU	**Relative Abundance (%)**		
3 weeks old	**Low STAI**	**High STAI**	**p-value**
Chao1 Index	82.7	90.5	*t*(42) =-0.95, *p*=0.344
Simpson Index	0.7	0.7	*t*(42) =-0.69, *p*=0.492
Shannon Index	2.7	2.8	*t*(42) =-0.25,p=0.797
Phylogenetic diversity	4.4	4.6	*t*(42) =-0.93,p=0.357
Observed Species	59.5	61.3	*t*(42) =-0.51, p=0.606
Actinobacteria	48.7	42.5	W=241, p=0.320
Bacteroidetes	8.5	17.1	W=117.5, p=0.545
Firmicutes	39.1	36.4	W=205, p=0.939
Proteobacteria	3.5	3.8	W=182, p=0.625
Bifidobacteriaceae	33.7	31.1	W=198, p=0.939
Nocardiaceae	12.6	9.5	W=249.5, p=0.221
Coriobacteriaceae	1.9	1.1	W=199.5, p=0.969
Bacteroidaceae	6.9	15.3	W=167.5, p=0.388
Porphyromonadaceae	1.5	1.7	W=221.5, p=0.612
Rikenellaceae	**0.0**	**0.0**	**W=170.5, p=0.029**

Staphylococcaceae	1.8	2.1	W=212.5, p=0.787
Enterococcaceae	1.6	1.8	W=189, p=0.757
Lactobacillaceae	2.4	3.0	W=176.5, p=0.527
Streptococcaceae	6.4	5.4	W=217, p=0.703
Clostridiaceae1	10.2	9.9	W=215, p=0.738
Clostridiales Family XIII	**0.0**	**0.0**	**W=170.5, p=0.029**
Lachnospiraceae	7.1	7.7	W=171, p=0.440
Ruminococcaceae	0.2	0.4	W=203, p=0.977
Veillonellaceae	8.1	5.1	W=246, p=0.261
Enterobacteriaceae	2.9	3.3	W=191, p=0.797
Bifidobacterium	33.7	31.1	W=198, p=0.939
Scardovia	**0.0**	**0.0**	**W=170.5, p=0.029**
Corynebacteriaceae uncultured	**0.0**	**0.0**	**W=146.5, p=0.010**
Rhodococcus	12.6	9.5	W=249.5, p=0.221
Collinsella	1.8	0.9	W=194.5, p=0.862
Alistipes	**0.0**	**0.0**	**W=170.5, p=0.029**
Bacteroides	6.9	15.3	W=167.5, p=0.388
Staphylococcus	1.8	2.1	W=212.5, p=0.787
Enterococcus	1.6	1.8	W=189, p=0.757
Lactobacillus	2.4	3.0	W=176.5, p=0.527
Streptococcus	6.4	5.4	W=217, p=0.703
Clostridiumsensustricto1	10.2	9.9	W=213.5, p=0.767
Cellulosilyticum	**0.0**	**0.0**	**W=170.5, p=0.029**
LachnospiraceaeIncertaeSedis	5.6	6.2	W=179.5, p=0.580
Stomatobaculum	**0.0**	**0.0**	**W=170.5, p=0.029**
Lachnospiraceaeuncultured	0.9	1.3	W=158.5, p=0.245
Veillonella	6.7	5.1	W=219.5, p=0.652
Enterobacter	1.2	1.0	W=182, p=0.621
EscherichiaShigella	1.4	2.1	W=223, p=0.588

Supplementary table 40: Relative abundance of bacterial OTUs in the *infant gut at 3 weeks old* by *maternal anxiety* in the *2nd trimester*. Listed are all OTUs with an abundance of greater than 1% and significant OTUs with an abundance of less than 1%. Low anxiety (STAI < 4; Vaginal; N=31; C-Section; N=5), high anxiety (STAI > 5; Vaginal;

N=13; C-Section; N=7). Unpaired students t-test and Mann-Whitney-Wilcoxon test. Abbreviations: Operational taxonomic unit (OTU), State Trait Anxiety Inventory (STAI).

Table 41: Infant gut at 3 weeks old by 2nd trimester depressive symptoms

OTU	**Relative Abundance (%)**		
3 weeks old	**Low EPDS**	**High EPDS**	**p-value**
Chao1 Index	86.4	78.5	$t(42) = 0.81$, $p=0.422$
Simpson Index	0.7	0.7	$t(42) = -0.63$, $p=0.525$
Shannon Index	2.7	2.8	$t(42) = -0.44$,p=0.660
Phylogenetic diversity	4.4	4.7	$t(42) = -0.90$,p=0.371
Observed Species	59.9	60.5	$t(42) = -0.13$, p=0.892
Actinobacteria	48.9	37.7	W=187, p=0.200
Bacteroidetes	10.1	15.4	W=123.5, p=0.542
Firmicutes	37.7	41.1	W=129, p=0.665
Proteobacteria	3.2	5.7	W=105.5, p=0.247
Bifidobacteriaceae	34.3	27.0	W=163, p=0.580
Nocardiaceae	12.3	9.1	W=168.5, p=0.465
Coriobacteriaceae	1.9	0.6	W=129.5, p=0.668
Bacteroidaceae	8.1	15.0	W=112.5, p=0.345
Porphyromonadaceae	1.9	0.1	W=167, p=0.489
Candidate division TM7 uncultured	**0.0**	**0.0**	**W=98, P=0.011**
Staphylococcaceae	2.0	1.3	W=129, p=0.659
Enterococcaceae	**1.3**	**3.0**	**W=80, p=0.052**
Lactobacillaceae	**3.1**	**0.1**	**W=204, p=0.069**
Streptococcaceae	6.5	4.0	W=164, p=0.559
Clostridiaceae1	9.5	12.8	W=151.5, p=0.831
Lachnospiraceae	6.6	10.6	W=128, p=0.637
Veillonellaceae	7.2	7.3	W=123, p=0.539
Enterobacteriaceae	2.7	4.8	W=134, p=0.772
Trueperella	**0.0**	**0.0**	**W=126, P=0.039**
Bifidobacterium	34.3	27.0	W=163, p=0.580
Rhodococcus	12.3	9.1	W=168.5, p=0.465
Collinsella	1.8	0.4	W=133, p=0.740

Slackia	**0.0**	**0.0**	**W=126, P=0.039**
Bacteroides	8.1	15.0	W=112.5, p=0.345
Paraprevotella	**0.0**	**0.2**	**W=126, P=0.039**
Staphylococcus	2.0	1.3	W=129, p=0.659
Enterococcus	**1.3**	**3.0**	**W=80, p=0.052**
Lactobacillus	**3.1**	**0.1**	**W=204, p=0.069**
Streptococcus	6.5	4.0	W=164, p=0.559
Clostridiumsensustricto1	9.5	12.8	W=150, p=0.867
Mogibacterium	**0.0**	**0.0**	**W=126, P=0.039**
Blautia	**0.0**	**0.6**	**W=102.5, P=0.049**
LachnospiraceaeIncertaeSedis	5.3	7.9	W=133, p=0.464
Pseudobutyrivibrio	**0.0**	**0.0**	**W=126, P=0.039**
Lachnospiraceaeuncultured	1.1	0.7	W=131.5, p=0.698
Veillonella	6.1	7.0	W=119, p=0.455
Neisseria	**0.0**	**0.0**	**W=126, P=0.039**
Enterobacter	1.2	1.0	W=118.5, p=0.442
EscherichiaShigella	1.2	3.1	W169.5=, p=0.446

Supplementary table 41: Relative abundance of bacterial OTUs in the *infant gut at 3 weeks old* by *maternal depressive symptoms* in the *2nd trimester*. Listed are all OTUs with an abundance of greater than 1% and significant OTUs with an abundance of less than 1%. Low depressive (EPDS < 8; Vaginal; N=36; C-Section; N=12), high depressive (EPDS > 9; Vaginal; N=8; C-Section; N=0). Unpaired students t-test and Mann-Whitney-Wilcoxon test. Abbreviations: Operational taxonomic unit (OTU), Edinburgh Postnatal; Depression Scale (EPDS).

Table 42: Infant gut at 3 weeks old by 2nd trimester cortisol

OTU	**Relative Abundance (%)**		
3 weeks old	**Low CAR**	**High CAR**	**p-value**
Chao1 Index	81.3	89.0	t(36) =-0.95, p=0.347
Simpson Index	0.7	0.7	t(36) = 1.13, p=0.262
Shannon Index	2.8	2.7	t(36) = 0.94, p=0.348
Phylogenetic diversity	4.4	4.5	t(36) =-0.23,p=0.815
Observed Species	59.0	62.2	t(36) =-0.90, p=0.369
Actinobacteria	40.9	51.8	W=132, p=0.162

Bacteroidetes	11.2	8.9	W=187, p=0.860
Firmicutes	44.4	34.9	W=209, p=0.418
Proteobacteria	3.3	4.2	W=192, p=0.748
Bifidobacteriaceae	**24.8**	**39.8**	**W=122, p=0.090**
Nocardiaceae	12.8	11.3	W=183, p=0.953
Coriobacteriaceae	2.7	0.1	W=243, p=0.069
Bacteroidaceae	8.7	7.7	W=179, p=0.976
Porphyromonadaceae	2.3	1.1	W=178,5, p=0.964
Staphylococcaceae	2.0	2.1	W=147, p=0.335
Enterococcaceae	**2.4**	**1.0**	**W=149, p=0.046**
Lactobacillaceae	2.6	3.1	W=137, p=0.207
Streptococcaceae	6.5	6.4	W=187, p=0.862
Clostridiaceae1	13.1	10.2	W=170.5, p=0.781
Lachnospiraceae	**9.9**	**5.6**	**W=246, p=0.057**
Acidaminococcaceae	**0.0**	**0.0**	**W=228, p=0.042**
Veillonellaceae	7.0	5.4	W=195, p=0.686
Enterobacteriaceae	2.7	3.5	W=187, p=0.860
Bifidobacterium	**24.8**	**39.8**	**W=122, p=0.090**
Rhodococcus	12.8	11.3	W=183, p=0.953
Collinsella	2.5	0.0	W=222.5, p=0.212
Bacteroides	8.7	7.7	W=179, p=0.976
Staphylococcus	2.0	2.1	W=147, p=0.335
Enterococcus	**2.4**	**1.0**	**W=249, p=0.046**
Lactobacillus	1.6	3.1	W=137, p=0.207
Streptococcus	6.5	6.4	W=187, p=0.862
Clostridiumsensustricto1	13.1	10.2	W=173, p=0.838
LachnospiraceaeIncertaeSedis	**8.9**	**3.4**	**W=253, p=0.035**
Lachnospiraceaeuncultured	0.8	1.3	W=,200 p=0.559
Peptostreptococcus	**0.0**	**0.0**	**W=142.5, p=0.039**
Phascolarctobacterium	**0.3**	**0.0**	**W=228, p=0.042**
Veillonella	6.1	5.3	W=185, p=0.908
Bilophila	**0.0**	**0.0**	**W=218.5, p=0.039**

Enterobacter	0.7	1.5	W=183, p=0.953
EscherichiaShigella	1.6	1.8	W=160.5, p=0.568

Supplementary table 42: Relative abundance of bacterial OTUs in the *infant gut at 3 weeks old* by *maternal cortisol* in the *2nd trimester*. Listed are all OTUs with an abundance of greater than 1% and significant OTUs with an abundance of less than 1%. Low cortisol (CAR < 545131; Vaginal; N=19; C-Section; N=7), high cortisol (CAR > 545132; Vaginal; N=19; C-Section; N=4). Unpaired students t-test and Mann-Whitney-Wilcoxon test. Abbreviations: Operational taxonomic unit (OTU), Cortisol Awakening Response (CAR).

Table 43: Infant gut at 3 weeks old by 3rd trimester stress

OTU	**Relative Abundance (%)**		
3 weeks old	**Low PSS**	**High PSS**	**p-value**
Chao1 Index	77.2	87.0	*t*(38) =-1.25, *p*=0.218
Simpson Index	0.7	0.7	*t*(38) =-0.45, *p*=0.653
Shannon Index	2.7	2.7	*t*(38) =-0.00,p=0.996
Phylogenetic diversity	4.5	4.3	*t*(38) = 0.57,p=0.570
Observed Species	59.9	58.5	*t*(38) = 0.44, p=0.660
Actinobacteria	**51.9**	**39.0**	**W=266, p=0.076**
Bacteroidetes	8.7	13.9	W=184.5, p=0.684
Firmicutes	37.1	42.0	W=175, p=0.511
Proteobacteria	2.1	4.8	W=167.5, p=0.386
Bifidobacteriaceae	33.1	30.8	W=203, p=0.946
Corynebacteriaceae	0.0	0.0	W=228.5, p=0.437
Nocardiaceae	**16.5**	**6.1**	**W=282, p=0.027**
Coriobacteriaceae	1.9	1.0	W=238.5, p=0.301
Bacteroidaceae	7.1	12.9	W=178, p=0.560
Porphyromonadaceae	1.4	0.9	W=216, p=0.673
Staphylococcaceae	3.0	1.8	W=218, p=0.635
Enterococcaceae	1.3	1.5	W=175.5, p=0.514
Lactobacillaceae	1.7	2.8	W=164.5, p=0.342
Streptococcaceae	7.9	5.3	W=217, p=0.658
Clostridiaceae1	8.7	12.4	W=208, p=0.839
Lachnospiraceae	5.9	10.1	W=170, p=0.424

Veillonellaceae	6.7	6.5	W=216, p=0.678
Enterobacteriaceae	1.9	3.8	W=180, p=0.597
Bifidobacterium	33.1	30.8	W=203, p=0.946
Corynebacterium	0.0	0.0	W=243, p=0.233
Rhodococcus	**16.5**	**6.1**	**W=282, p=0.027**
Collinsella	1.6	1.0	W=232, p=0.377
Bacteroides	7.1	12.9	W=178, p=0.560
Staphylococcus	3.0	1.8	W=218, p=0.635
Enterococcus	1.3	1.5	W=175.5, p=0.514
Lactobacillus	1.7	2.8	W=164.5, p=0.342
Streptococcus	7.9	5.3	W=217, p=0.658
Clostridiumsensustricto1	8.6	12.4	W=205.5, p=0.892
LachnospiraceaeIncertaeSedis	3.9	8.2	W=168, p=0.393
Lachnospiraceaeuncultured	1.2	0.8	W=182.5, p=0.625
Dialister	**0.0**	**0.0**	**W=260, P=0.009**
Veillonella	5.3	6.5	W=198, p=0.967
Enterobacter	**0.4**	**1.6**	**W=137.5, p=0.090**
EscherichiaShigella	1.4	1.8	W=242.5, p=0.255

Supplementary table 43: Relative abundance of bacterial OTUs in the *infant gut at 3 weeks old* by *maternal stress* in the *3rd trimester*. Listed are all OTUs with an abundance of greater than 1% and significant OTUs with an abundance of less than 1%. Low stress (PSS < 13; Vaginal; N=20; C-Section; N=5), high stress (PSS > 14; Vaginal; N=20; C-Section; N=5). Unpaired students t-test and Mann-Whitney-Wilcoxon test. Abbreviations: Operational taxonomic unit (OTU), Perceived Stress Scale (PSS)

Table 44: Infant gut at 3 weeks old by 3rd trimester anxiety

OTU	**Relative Abundance (%)**		
3 weeks old	**Low STAI**	**High STAI**	**p-value**
Chao1 Index	82.3	81.9	$t(38) = 0.05$, $p=0.955$
Simpson Index	0.7	0.7	$t(38) = -0.94$, $p=0.353$
Shannon Index	2.7	2.8	$t(38) = -0.46$,p=0.647
Phylogenetic diversity	4.4	4.4	$t(38) = -0.16$,p=0.870
Observed Species	60.8	57.2	$t(38) = 1.12$, p=0269
Actinobacteria	50.6	39.1	W=259, p=0.100

Bacteroidetes	9.5	13.5	W=203, p=0.902
Firmicutes	36.2	43.6	W=171, p=0.475
Proteobacteria	3.4	3.6	W=201, p=0.945
Bifidobacteriaceae	34.4	29.0	W=210, p=0.757
Corynebacteriaceae	**0.0**	**0.0**	**W=285.5, 0.015**
Nocardiaceae	**14.2**	**7.7**	**W=265, p=0.070**
Coriobacteriaceae	1.2	1.8	W=209.5, p=0.763
Bacteroidaceae	7.8	12.8	W=194, p=0.924
Porphyromonadaceae	1.7	0.5	W=231.5, p=0.366
Staphylococcaceae	3.4	1.2	W=224.5, p=0.479
Enterococcaceae	1.6	1.3	W=203, p=0.902
Lactobacillaceae	1.6	3.1	W=138.5, p=0.107
Streptococcaceae	7.3	5.8	W=225, p=0.475
Clostridiaceae1	9.0	12.4	W=186.5, p=0.764
Lachnospiraceae	6.4	10.0	W=150.5, p=0.201
Veillonellaceae	5.7	7.7	W=147, p=0.171
Enterobacteriaceae	3.3	2.4	W=239, p=0.270
Bifidobacterium	34.4	29.0	W=210, p=0.757
Corynebacterium	**0.0**	**0.0**	**W=298.5, p=0.004**
Rhodococcus	**14.2**	**7.7**	**W=265, p=0.070**
Collinsella	1.0	1.7	W=178.5, p=0.592
Bacteroides	7.8	12.8	W=194, p=0.924
Staphylococcus	3.4	1.2	W=224.5, p=0.479
Enterococcus	1.6	1.3	W=203, p=0.902
Lactobacillus	1.6	3.1	W=138.5, p=0.107
Streptococcus	7.3	5.8	W=225, p=0.475
Clostridiumsensustricto1	9.0	12.4	W=184, p=0.713
LachnospiraceaeIncertaeSedis	3.7	8.9	W=142, p=0.131
Lachnospiraceaeuncultured	1.1	0.9	W=156, p=0.230
Veillonella	**4.5**	**7.7**	**W=128, p=0.058**
Enterobacter	1.3	0.5	W=190.5, p=0.847
EscherichiaShigella	**1.7**	**1.5**	**W=280, p=0.026**

Supplementary table 44: Relative abundance of bacterial OTUs in the *infant gut at 3 weeks old* by *maternal anxiety* in the *3rd trimester*. Listed are all OTUs with an abundance of greater than 1% and significant OTUs with an abundance of less than 1%. Low anxiety (STAI < 4; Vaginal; N=22; C-Section; N=6), high anxiety (STAI > 5; Vaginal; N=18; C-Section; N=4). Unpaired students t-test and Mann-Whitney-Wilcoxon test. Abbreviations: Operational taxonomic unit (OTU), State Trait Anxiety Inventory (STAI).

Table 45: Infant gut at 3 weeks old by 3rd trimester depressive symptoms

OTU	**Relative Abundance (%)**		
3 weeks old	**Low EPDS**	**High EPDS**	**p-value**
Chao1 Index	83.3	79.3	*t*(38) = 0.43, *p*=0.664
Simpson Index	0.7	0.7	*t*(38) =-0.44, *p*=0.657
Shannon Index	2.7	2.8	*t*(38) =-0.21,p=0.830
Phylogenetic diversity	4.5	4.2	*t*(38) = 0.85,p=0.395
Observed Species	60.4	55.9	*t*(38) = 1.26, p=0.211
Actinobacteria	**49.2**	**35.6**	**W=221, p=0.064**
Bacteroidetes	8.8	17.8	W=127, p=0.332
Firmicutes	38.6	42.0	W=145, p=0.676
Proteobacteria	3.1	4.4	W=149, p=0.762
Bifidobacteriaceae	33.2	28.6	W=171, p=0.742
Corynebacteriaceae	**0.0**	**0.0**	**W=226, p=0.040**
Nocardiaceae	**14.0**	**4.3**	**W=222.5, p=0.058**
Coriobacteriaceae	1.2	2.1	W=159.5, p=1
Bacteroidaceae	7.6	16.5	W=119.5, p=0.231
Porphyromonadaceae	1.2	1.0	W=126, p=0.314
Staphylococcaceae	2.9	1.2	W=171, p=0.738
Enterococcaceae	1.3	1.7	W=137.5, p=0.513
Lactobacillaceae	2.2	2.3	W=168.5, p=0.796
Streptococcaceae	7.5	4.4	W=202, p=0.206
Clostridiaceae1	9.0	14.8	W=164, p=0.903
Lachnospiraceae	7.7	9.0	W=150, p=0.785
Veillonellaceae	6.5	6.9	W=130, p=0.385
Enterobacteriaceae	2.6	3.4	W=190, p=0.363
Bifidobacterium	33.2	28.6	W=171, p=0.742

Corynebacterium	**0.0**	**0.0**	**W=235.5, p=0.017**
Rhodococcus	**14.0**	**4.3**	**W=222.5, p=0.058**
Collinsella	1.0	2.0	W=156, p=0.925
Bacteroides	7.6	16.5	W=119.5, p=0.231
Coprobacter	**0.0**	**0.0**	**W=130.5, p=0.022**
Staphylococcus	2.9	1.2	W=171, p=0.738
Enterococcus	1.3	1.7	W=137.5, p=0.513
Lactobacillus	2.2	2.3	W=168.5, p=0.796
Streptococcus	7.5	4.4	W=202, p=0.206
Clostridiumsensustricto1	8.9	14.8	W=163, p=0.927
LachnospiraceaeIncertaeSedis	5.0	8.9	W=133.5, p=0.439
Lachnospiraceaeuncultured	1.4	0.0	W=149, p=0.747
Veillonella	5.6	6.9	W=117, p=0.203
Enterobacter	1.0	0.8	W=179, p=0.560
EscherichiaShigella	**1.4**	**2.1**	**W=233, p=0.026**

Supplementary table 45: Relative abundance of bacterial OTUs in the *infant gut at 3 weeks old* by *maternal depressive symptoms* in the *3rd trimester*. Listed are all OTUs with an abundance of greater than 1% and significant OTUs with an abundance of less than 1%. Low depressive (EPDS < 8; Vaginal; N=29; C-Section; N=9), high depressive (EPDS > 9; Vaginal; N=11; C-Section; N=1). Unpaired students t-test and Mann-Whitney-Wilcoxon test. Abbreviations: Operational taxonomic unit (OTU), Edinburgh Postnatal Depression Scale (EPDS).

Table 46: Infant gut at 3 weeks old by 3rd trimester cortisol

OTU	**Relative Abundance (%)**		
3 weeks old	**Low CAR**	**High CAR**	**p-value**
Chao1 Index	79.3	90.3	$t(35) = -1.29$, $p=0.202$
Simpson Index	0.7	0.7	$t(35) = -0.62$, $p=0.535$
Shannon Index	2.7	2.9	$t(35) = -0.83$, $p=0.407$
Phylogenetic diversity	4.2	4.5	$t(35) = -0.87$, $p=0.384$
Observed Species	56.3	61.2	$t(35) = 1.02$, $p=0.313$
Actinobacteria	48.7	44.3	W=191, p=0.557
Bacteroidetes	7.5	14.0	W=149, p=0.513
Firmicutes	41.7	35.7	W=197, p=0.443

Proteobacteria	1.9	5.8	W=135, p=0.284
Bifidobacteriaceae	29.7	34.7	W=159, p=0.729
Nocardiaceae	13.6	8.5	W=205.5, p=0.301
Coriobacteriaceae	4.7	0.3	W=156.5, p=0.669
Bacteroidaceae	6.5	12.0	W=141, p=0.369
Porphyromonadaceae	0.9	1.9	W=187, p=0.634
Staphylococcaceae	2.6	2.5	W=147.5, p=0.484
Enterococcaceae	1.8	1.2	W=197, p=0.437
Lactobacillaceae	3.2	1.1	W=185.5, p=0.669
Streptococcaceae	5.3	7.3	W=141, p=0.374
Clostridiaceae1	14.4	8.5	W=190, p=0.573
Lachnospiraceae	7.3	6.6	W=178, p=0.843
Veillonellaceae	5.2	7.4	W=143.5, p=0.411
Enterobacteriaceae	1.0	5.3	W=146, p=0.456
Bifidobacterium	29.7	34.7	W=159, p=0.729
Rhodococcus	13.6	8.5	W=205.5, p=0.301
Collinsella	4.6	0.2	W=163.5, p=0.828
Bacteroides	6.5	12.0	W=141, p=0.369
Staphylococcus	2.6	2.5	W=147.5, p=0.484
Enterococcus	1.8	1.2	W=197, p=0.437
Lactobacillus	3.2	1.1	W=185.5, p=0.669
Lactococcus	**0.0**	**0.0**	**W=225, p=0.010**
Streptococcus	5.3	7.3	W=141, p=0.374
Clostridiumsensustricto1	14.4	8.5	W=191, p=0.553
LachnospiraceaeIncertaeSedis	5.6	5.2	W=187, p=0.637
Lachnospiraceaeuncultured	0.5	1.4	W=152, p=0.552
Megamonas	**0.0**	**0.0**	**W=153.5, p=0.034**
Veillonella	4.3	7.4	W=125, p=0.166
Enterobacter	0.4	1.8	W=127, p=0.181
EscherichiaShigella	0.5	2.9	W=171.5, p=1

Supplementary table 46: Relative abundance of bacterial OTUs in the *infant gut at 3 weeks old* by *maternal cortisol* in the *3rd trimester*. Listed are all OTUs with an abundance of greater than 1% and significant OTUs with an abundance of less than 1%. Low cortisol (CAR < 562349; Vaginal; N=19; C-Section; N=5), high cortisol (CAR > 562350;

Vaginal; N=18; C-Section; N=6). Unpaired students t-test and Mann-Whitney-Wilcoxon test. Abbreviations: Operational taxonomic unit (OTU), Cortisol Awakening Response (CAR).

Table 47: Infant gut at 3 months old by 2nd trimester stress

OTU	Relative Abundance (%)		
3 months old	**Low PSS**	**High PSS**	**p-value**
Chao1 Index	**76.5**	**92.9**	***t*(30) =-2.36, *p*=0.024**
Simpson Index	**0.7**	**0.8**	***t*(30) =-2.23, *p*=0.032**
Shannon Index	**2.6**	**3.2**	***t*(30) =-2.31,p=0.028**
Phylogenetic diversity	4.7	5.1	*t*(30) =-1.17,p=0.250
Observed Species	60.0	67.0	*t*(30) =-1.49, p=0.313
Actinobacteria	**58.3**	**39.5**	**W=180, p=0.051**
Bacteroidetes	11.2	10.3	W=139, p=0.692
Candidate division TM7	**0.0**	**0.0**	**W=86, P=0.030**
Firmicutes	**27.6**	**47.3**	**W=66, p=0.018**
Proteobacteria	1.2	2.6	W=105.5, p=0.406
Bifidobacteriaceae	**52.4**	**30.1**	**W=184, p=0.036**
Nocardiaceae	3.5	7.6	W=100, p=0.304
Coriobacteriaceae	2.1	1.2	W=145.5, p=0.520
Bacteroidaceae	9.1	8.8	W=142, p=0.615
Porphyromonadaceae	2.0	1.5	W=135.5, p=0.787
Candidate division TM7 uncultured	**0.0**	**0.0**	**W=86, P=0.030**
Enterococcaceae	0.7	1.6	W=144, p=0.558
Lactobacillaceae	**2.3**	**0.5**	**W=203, p=0.004**
Streptococcaceae	2.1	1.9	W=104, p=0.375
Clostridiaceae1	1.5	7.7	W=103.5, p=0.365
Lachnospiraceae	10.6	20.1	W=89, p=0.148
Erysipelotrichaceae	**0.2**	**4.6**	**W=55.5, p=0.005**
Veillonellaceae	8.5	7.4	W=156, p=0.300
Enterobacteriaceae	1.0	2.3	W=105, p=0.396
Actinobaculum	**0.0**	**0.0**	**W=88, P=0.018**
Bifidobacterium	**52.4**	**30.1**	**W=184, p=0.036**

Rhodococcus	3.5	7.6	W=100, p=0.304
Collinsella	1.8	1.0	W=138, p=0.701
Bacteroides	9.1	8.8	W=142, p=0.615
Prevotella	0.0	0.0	W=135, p=0.698
Carnobacterium	**0.0**	**0.0**	**W=74, P=0.023**
Enterococcus	0.7	1.6	W=144, p=0.558
Lactobacillus	**2.3**	**0.5**	**W=203, p=0.004**
Streptococcus	2.1	1.9	W=104, p=0.375
Clostridiumsensustricto1	1.5	7.7	W=103.5, p=0.365
Anaerococcus	**0.0**	**0.1**	**W=79.5, p=0.027**
Blautia	1.5	5.1	W=94.5, p=0.210
Coprococcus	**0.0**	**0.3**	**W=96, p=0.038**
LachnospiraceaeIncertaeSedis	8.6	12.0	W=100, p=0.163
Lachnospiraceaeuncultured	**0.0**	**1.4**	**W=74.5, p=0.043**
Flavonifractor	**0.0**	**0.5**	**W-80, p=0.034**
Erysipelotrichaceae Incertae Sedis	**0.2**	**4.6**	**W=67, p=0.017**
Veillonella	8.5	6.5	W=169, p=0.126
Enterobacter	0.4	0.6	W=111, p=0.531

Supplementary table 47: Relative abundance of bacterial OTUs in the *infant gut at 3 months old* by *maternal stress* in the *2nd trimester*. Listed are all OTUs with an abundance of greater than 1% and significant OTUs with an abundance of less than 1%. Low stress (PSS < 13; Vaginal; N=16; C-Section; N=7), high stress (PSS > 14; Vaginal; N=26; C-Section; N=3). Unpaired students t-test and Mann-Whitney-Wilcoxon test. Abbreviations: Operational taxonomic unit (OTU), Perceived Stress Scale (PSS).

Table 48: Infant gut at 3 months old by 2nd trimester anxiety

OTU	**Relative Abundance (%)**		
3 months old	**Low STAI**	**High STAI**	**p-value**
Chao1 Index	85.6	82.4	$t(30) = 0.39$, $p=0.696$
Simpson Index	0.7	0.7	$t(30) = -0.81$, $p=0.420$
Shannon Index	2.8	3.0	$t(30) = -0.79$, $p=0.434$
Phylogenetic diversity	5.0	4.7	$t(30) = 0.66$, $p=0.510$
Observed Species	63.9	62.4	$t(30) = 0.28$, $p=0.779$
Actinobacteria	50.2	45.6	W=113, p=0.711

Bacteroidetes	12.3	6.8	W=123, p=0.425
Candidate division TM7	0.0	0.0	W=87.5, p=0.369
Firmicutes	34.4	45.0	W=79, p=0.320
Proteobacteria	1.7	2.4	W=81, p=0.356
Bifidobacteriaceae	42.2	38.8	W=106.5, p=0.916
Nocardiaceae	**6.3**	**3.5**	**W=151, p=0.047**
Propionibacteriaceae	0.0	0.0	W=100.5, p=0.835
Coriobacteriaceae	1.1	2.9	W=68, p=0.141
Bacteroidaceae	10.1	6.0	W=118, p=0.564
Porphyromonadaceae	2.1	0.8	W=125, p=0.368
Candidate division TM7 uncultured	0.0	0.0	W=87.5, p=0.369
Enterococcaceae	**0.9**	**1.8**	**W=62.5, p=0.088**
Lactobacillaceae	1.1	2.2	W=108.5, p=0.849
Streptococcaceae	2.4	1.0	W=132, p=0.240
Clostridiaceae1	**3.4**	**7.7**	**W=52, p=0.032**
Lachnospiraceae	15.2	15.9	W=96, p=0.773
Peptostreptococcaceae	**0.5**	**1.2**	**W=54, p=0.037**
Erysipelotrichaceae	0.6	7.0	W=90.5, p=0.591
Veillonellaceae	8.5	6.4	W=110.5, p=0.785
Enterobacteriaceae	1.4	2.3	W=74.5, p=0.232
Bifidobacterium	42.2	38.8	W=106.5, p=0.916
Rhodococcus	**6.3**	**3.5**	**W=151, p=0.047**
Collinsella	1.0	2.5	W=113, p=0.686
Eggerthella	**0.1**	**0.4**	**W=56.5, p=0.041**
Bacteroides	10.1	6.0	W=118, p=0.564
Enterococcus	**0.9**	**1.8**	**W=62.5, p=0.088**
Lactobacillus	1.1	2.2	W=108.5, p=0.849
Streptococcus	2.4	1.0	W=132, p=0.240
Clostridiumsensustricto1	**3.4**	**7.7**	**W=52, p=0.032**
Blautia	2.1	6.5	W=79, p=0.311
Coprococcus	**0.0**	**0.4**	**W=73, p=0.028**
LachnospiraceaeIncertaeSedis	11.2	8.0	W=106, p=0.934

Lachnospiraceaeuncultured	0.8	0.4	W=103.5, p=1
Ruminococcus	**0.1**	**0.0**	**W=144, p=0.034**
Erysipelotrichaceae Incertae Sedis	0.6	7.0	W=94, p=0.693
Veillonella	8.0	6.4	W=103.5, p=1
Enterobacter	0.4	0.8	W=70.5, p=0.170

Supplementary table 48: Relative abundance of bacterial OTUs in the *infant gut at 3 months old* by *maternal anxiety* in the *2nd trimester*. Listed are all OTUs with an abundance of greater than 1% and significant OTUs with an abundance of less than 1%. Low anxiety (STAI < 4; Vaginal; N=23; C-Section; N=4), high anxiety (STAI > 5; Vaginal; N=9; C-Section; N=6). Unpaired students t-test and Mann-Whitney-Wilcoxon test. Abbreviations: Operational taxonomic unit (OTU), State Trait Anxiety Inventory (STAI).

Table 49: Infant gut at 3 months old by 2nd trimester depressive symptoms

OTU	**Relative Abundance (%)**		
3 months old	**Low EPDS**	**High EPDS**	**p-value**
Chao1 Index	83.4	91.9	*t*(30) =-0.82, *p*=0.413
Simpson Index	0.7	0.7	*t*(30) = 0.59, *p*=0.554
Shannon Index	2.9	2.8	*t*(30) = 0.34,p=0.735
Phylogenetic diversity	4.9	5.0	*t*(30) =-0.11,p=0.910
Observed Species	63.3	64.6	*t*(30) =-0.19, p=0.850
Actinobacteria	50.1	42.2	W=72, p=0.840
Bacteroidetes	11.5	6.8	W=73, p=0.795
Firmicutes	35.5	47.9	W=50, p=0.389
Proteobacteria	1.7	2.9	W=45.5, p=0.264
Bifidobacteriaceae	41.8	38.7	W=69.5, p=0.937
Nocardiaceae	6.2	2.2	W=88, p=0.309
Coriobacteriaceae	1.8	0.6	W=46.5, p=0.286
Bacteroidaceae	9.3	6.8	W=68, p=1
Porphyromonadaceae	2.1	0.0	W=94.5, p=0.159
Enterococcaceae	0.9	2.6	W=47, p=0.298
Lactobacillaceae	1.7	0.1	W=94.5, p=0.166
Streptococcaceae	1.8	3.4	W=66, p=0.958
Clostridiaceae1	4.8	3.7	W=69, p=0.958
Lachnospiraceae	15.8	12.9	W=79, p=0.579

Erysipelotrichaceae	0.5	12.5	W=43, p=0.202
Veillonellaceae	7.5	10.4	W=56.5, p=0.585
Neisseriaceae	**0.0**	**0.0**	**W=54, p=0.025**
Moraxellaceae	**0.0**	**0.0**	**W=27.5, p=0.013**
Xanthomonadaceae	**0.0**	**0.0**	**W=54, p=0.025**
Enterobacteriaceae	1.4	2.7	W=50, p=0.377
Bifidobacterium	41.8	38.7	W=69.5, p=0.937
Scardovia	**0.0**	**0.0**	**W=54, p=0.025**
Corynebacterium	**0.0**	**0.0**	**W=30.5, p=0.021**
Rhodococcus	6.2	2.2	W=88, p=0.309
Collinsella	1.7	0.0	W=75.5, p=0.676
Coriobacteriaceae uncultured	**0.0**	**0.0**	**W=54, p=0.025**
Bacteroides	9.3	6.8	W=68, p=1
Enterococcus	0.9	2.6	W=47, p=0.298
Lactobacillus	1.7	0.1	W=94.5, p=0.166
Streptococcus	1.8	3.4	W=66, p=0.958
Clostridiumsensustricto1	4.8	3.7	W=69, p=0.958
Blautia	3.1	4.4	W=60.5, p=0.734
Coprococcus	**0.0**	**0.8**	**W=43.5, p=0.033**
LachnospiraceaeIncertaeSedis	10.9	6.9	W=83, p=0.448
Marvinbryantia	**0.0**	**0.0**	**W=54, p=0.025**
Lachnospiraceaeuncultured	0.7	0.5	W=46.5, p=0.282
Erysipelotrichaceae Incertae Sedis	0.5	12.5	W=52, p=0.415
Turicibacter	**0.0**	**0.0**	**W=54, p=0.025**
Veillonella	7.0	10.4	W=54.5, p=0.516
Neisseria	**0.0**	**0.0**	**W=54, p=0.025**
Enterobacter	0.5	0.7	W=59.5, p=0.695
Stenotrophomonas	**0.0**	**0.0**	**W=54, p=0.025**

Supplementary table 49: Relative abundance of bacterial OTUs in the *infant gut at 3 months old* by *maternal depressive symptoms* in the *2nd trimester*. Listed are all OTUs with an abundance of greater than 1% and significant OTUs with an abundance of less than 1%. Low depressive (EPDS < 8; Vaginal; N=27; C-Section; N=10), high depressive (EPDS > 9; Vaginal; N=5; C-Section; N=0). Unpaired students t-test and Mann-Whitney-Wilcoxon test. Abbreviations: Operational taxonomic unit (OTU), Edinburgh Postnatal Depression Scale (EPDS).

Table 50: Infant gut at 3 months old by 2nd trimester cortisol

OTU	Relative Abundance (%)		
3 months old	**Low CAR**	**High CAR**	**p-value**
Chao1 Index	89.3	80.5	*t*(26) = 1.02, *p*=0.313
Simpson Index	0.7	0.7	*t*(26) = 0.46, *p*=0.646
Shannon Index	2.9	2.9	*t*(26) =-0.02,p=0.978
Phylogenetic diversity	5.2	4.9	*t*(26) = 0.81,p=0.424
Observed Species	66.8	62.1	*t*(26) = 0.88, p=0.384
Actinobacteria	51.4	47.8	W=107, p=0.631
Bacteroidetes	9.1	10.3	W=112, p=0.443
Firmicutes	37.6	38.2	W=102, p=0.801
Proteobacteria	1.1	2.6	W=62, p=0.119
Bifidobacteriaceae	39.8	44.2	W=89, p=0.762
Nocardiaceae	6.9	2.8	W=111, p=0.507
Coriobacteriaceae	**4.3**	**0.3**	**W=148.5, p=0.015**
Bacteroidaceae	7.9	8.2	W=120, p=0.280
Porphyromonadaceae	1.2	2.1	W=98, p=0.943
Enterococcaceae	1.2	1.1	W=83.5, p=0.577
Lactobacillaceae	1.1	1.9	W=70.5, p=0.241
Streptococcaceae	2.2	2.3	W=86, p=0.659
Clostridiaceae1	5.1	5.0	W=81, p=0.500
Lachnospiraceae	12.5	16.9	W=93, p=0.909
Erysipelotrichaceae	3.7	2.1	W=107, p=0.614
Veillonellaceae	10.0	5.9	W=124, p=0.205
Enterobacteriaceae	0.9	2.2	W=66.5, p=0.177
Bifidobacterium	39.8	44.2	W=89, p=0.762
Rhodococcus	6.9	2.8	W=111, p=0.507
Collinsella	**4.0**	**0.1**	**W=142, p=0.027**
Bacteroides	7.9	8.2	W=120, p=0.280
Enterococcus	1.2	1.2	W=83.5, p=0.577
Lactobacillus	1.1	1.9	W=70.5, p=0.241
Streptococcus	2.2	2.3	W=86, p=0.659
Clostridiumsensustricto1	5.1	5.0	W=81, p=0.500

Blautia	4.3	3.7	W=83, p=0.559
LachnospiraceaeIncertaeSedis	7.3	11.0	W=86, p=0.664
Lachnospiraceaeuncultured	0.4	0.8	W=103, p=0.760
Erysipelotrichaceae Incertae Sedis	3.7	2.1	W=106, p=0.641
Veillonella	8.9	5.9	W=109, p=0.567
Enterobacter	0.3	0.6	W=71.5, p=0.262

Supplementary table 50: Relative abundance of bacterial OTUs in the *infant gut at 3 months old* by *maternal cortisol* in the *2nd trimester*. Listed are all OTUs with an abundance of greater than 1% and significant OTUs with an abundance of less than 1%. Low cortisol (CAR < 545131; Vaginal; N=12; C-Section; N=6), high cortisol (CAR > 545132; Vaginal; N=16; C-Section; N=3). Unpaired students t-test and Mann-Whitney-Wilcoxon test. Abbreviations: Operational taxonomic unit (OTU), Cortisol Awakening Response (CAR).

Table 51: Infant gut at 3 months old by 3rd trimester stress

OTU	**Relative Abundance (%)**		
3 months old	**Low PSS**	**High PSS**	**p-value**
Chao1 Index	90.5	82.8	$t(28) = 0.72$, $p=0.472$
Simpson Index	0.7	0.7	$t(28) = 0.81$, $p=0.423$
Shannon Index	2.9	2.7	$t(28) = 0.78$,p=0.48
Phylogenetic diversity	4.8	4.8	$t(28) = 0.10$,p=0.913
Observed Species	63.2	58.6	$t(28) = 0.93$, p=0.357
Actinobacteria	47.2	51.8	W=93, p=0.482
Bacteroidetes	13.1	9.6	W=109.5, p=0.983
Firmicutes	35.5	34.1	W=112, p=0.967
Proteobacteria	3.0	3.2	W=89.5, p=0.390
Bifidobacteriaceae	37.7	46.5	W=93, p=0.476
Nocardiaceae	**7.1**	**3.6**	**W=171, p=0.010**
Coriobacteriaceae	2.0	1.3	W=136.5, p=0.284
Bacteroidaceae	11.0	8.5	W=111, p=1
Porphyromonadaceae	2.0	1.1	W=114.5, p=0.881
Enterococcaceae	**0.2**	**2.2**	**W=64.5, p=0.055**
Lactobacillaceae	1.0	1.6	W=84.5, p=0.282
Streptococcaceae	2.2	3.3	W=102, p=0.741

Clostridiaceae1	5.8	4.5	W=112, p=0.966
Lachnospiraceae	15.1	9.8	W=127, p=0.508
Erysipelotrichaceae	0.4	3.5	W=98.5, p=0.610
Veillonellaceae	8.7	6.4	W=137, p=0.276
Enterobacteriaceae	2.7	2.9	W=99, p=0.645
Bifidobacterium	37.7	46.5	W=93, p=0.476
Rhodococcus	**7.1**	**3.6**	**W=171, p=0.010**
Collinsella	1.8	1.1	W=136, p=0.262
Bacteroides	11.0	8.5	W=111, p=1
Enterococcus	**0.2**	**2.2**	**W=64.5, p=0.055**
Lactobacillus	1.0	1.6	W=84.5, p=0.282
Streptococcus	2.2	3.3	W=102, p=0.741
Clostridiumsensustricto1	5.8	4.5	W=112, p=0.966
Blautia	3.4	0.9	W=113.5, p=0.915
LachnospiraceaeIncertaeSedis	9.5	8.4	W=125, p=0.563
Lachnospiraceaeuncultured	0.9	0.2	W=117, p=0.797
Peptostreptococcus	**0.0**	**0.0**	**W=85, p=0.044**
Erysipelotrichaceae Incertae Sedis	0.4	3.5	W=116.5, p=0.801
Megamonas	**0.0**	**0.0**	**W=85, p=0.044**
Megasphaera	**0.0**	**0.0**	**W=149.5, p=0.021**
Veillonella	8.7	6.4	W=137, p=0.276
Enterobacter	1.3	1.6	W=96.5, p=0.570

Supplementary table 51: Relative abundance of bacterial OTUs in the *infant gut at 3 months old* by *maternal stress* in the *3rd trimester*. Listed are all OTUs with an abundance of greater than 1% and significant OTUs with an abundance of less than 1%. Low stress (PSS < 11; Vaginal; N=17; C-Section; N=4), high stress (PSS > 12; Vaginal; N=13; C-Section; N=4). Unpaired students t-test and Mann-Whitney-Wilcoxon test. Abbreviations: Operational taxonomic unit (OTU), Perceived Stress Scale (PSS).

Table 52: Infant gut at 3 months old by 3rd trimester anxiety

OTU	**Relative Abundance (%)**		
3 months old	**Low STAI**	**High STAI**	**p-value**
Chao1 Index	86.6	88.4	*t*(28) =-0.16, *p*=0.871
Simpson Index	0.7	0.7	*t*(28) =-0.38, *p*=0.704

Shannon Index	2.8	2.8	*t*(28) =-0.14,p=0.884
Phylogenetic diversity	4.8	5.0	*t*(28) =-0.54,p=0.587
Observed Species	61.4	61.0	*t*(28) = 0.08, p=0.932
Actinobacteria	48.2	51.1	W=94, p=0.812
Bacteroidetes	12.3	10.2	W=90.5, p=0.692
Firmicutes	34.7	35.3	W=89, p=0.649
Proteobacteria	3.7	1.9	W=104.5, p=0.860
Bifidobacteriaceae	40.3	44.0	W=98, p=0.947
Nocardiaceae	5.8	5.2	W=113, p=0.588
Coriobacteriaceae	1.8	1.5	W=102.5, p=0.929
Bacteroidaceae	10.2	9.3	W=89, p=0.649
Porphyromonadaceae	2.1	0.8	W=106.5, p=0.787
Enterococcaceae	0.8	1.5	W=72.5, p=0.233
Lactobacillaceae	1.1	1.5	W=90.5, p=0.690
Streptococcaceae	3.1	1.9	W=126, p=0.267
Clostridiaceae1	5.0	5.7	W=99, p=0.982
Lachnospiraceae	13.4	11.7	W=99, p=0.982
Erysipelotrichaceae	**0.3**	**4.6**	**W=63, p=0.088**
Veillonellaceae	8.4	6.3	W=121, p=0.367
Enterobacteriaceae	3.4	1.6	W=107, p=0.774
Trueperella	**0.0**	**0.0**	**W=80, p=0.047**
Bifidobacterium	40.3	44.0	W=98, p=0.947
Rhodococcus	5.8	5.2	W=113, p=0.588
Collinsella	**1.6**	**1.2**	**W=138, p=0.077**
Bacteroides	10.2	9.3	W=89, p=0.649
Enterococcus	0.8	1.5	W=72.5, p=0.233
Lactobacillus	1.1	1.5	W=90.5, p=0.690
Streptococcus	3.1	1.9	W=126, p=0.267
Clostridiumsensustricto1	5.0	5.7	W=99, p=0.982
Anaerococcus	**0.1**	**0.0**	**W=50, p=0.011**
Blautia	2.8	1.2	W=100.5, p=1
LachnospiraceaeIncertaeSedis	8.7	9.7	W=98, p=0.948
Lachnospiraceaeuncultured	0.7	0.2	W=96.5, p=0.892

Erysipelotrichaceae Incertae Sedis	0.3	4.6	W=70.5, p=0.164
Veillonella	8.4	6.3	W=121, p=0.367
Enterobacter	1.9	0.7	W=120, p=0.388

Supplementary table 52: Relative abundance of bacterial OTUs in the *infant gut at 3 months old* by *maternal anxiety* in the *3rd trimester*. Listed are all OTUs with an abundance of greater than 1% and significant OTUs with an abundance of less than 1%. Low anxiety (STAI < 4; Vaginal; N=20; C-Section; N=5), high anxiety (STAI > 5; Vaginal; N=10; C-Section; N=3). Unpaired students t-test and Mann-Whitney-Wilcoxon test. Abbreviations: Operational taxonomic unit (OTU), State Trait Anxiety Inventory (STAI).

Table 53: Infant gut at 3 months old by 3rd trimester depressive symptoms

OTU	**Relative Abundance (%)**		
3 months old	**Low EPDS**	**High EPDS**	**p-value**
Chao1 Index	88.4	84.4	$t(28) = 0.34$, $p=0.735$
Simpson Index	0.7	0.7	$t(28) = 0.82$, $p=0.415$
Shannon Index	2.9	2.6	$t(28) = 1.03$,p=0.311
Phylogenetic diversity	4.8	4.8	$t(28) = 0.12$,p=0.899
Observed Species	62.9	57.4	$t(28) = 1.03$, p=0.307
Actinobacteria	46.8	54.9	W=72, p=0.325
Bacteroidetes	11.9	10.7	W=85.5, p=0.700
Firmicutes	36.7	30.8	W=104, p=0.689
Proteobacteria	3.6	1.9	W=95.5, p=0.981
Bifidobacteriaceae	39.0	47.5	W=79, p=0.497
Nocardiaceae	5.6	5.5	W=109, p=0.533
Coriobacteriaceae	1.7	1.7	W=94.5, p=1
Bacteroidaceae	9.7	10.3	W=76, p=0.422
Porphyromonadaceae	2.2	0.4	W=112.5, p=0.419
Enterococcaceae	0.7	1.9	W=62, p=0.145
Lactobacillaceae	1.2	1.5	W=95.5, p=0.981
Streptococcaceae	2.9	2.1	W=111, p=0.476
Clostridiaceae1	6.4	2.4	W=121, p=0.239
Lachnospiraceae	14.8	8.2	W=111, p=0.476
Erysipelotrichaceae	0.5	4.7	W=86.5, p=0.719
Veillonellaceae	7.4	8.4	W=94, p=1

Enterobacteriaceae	3.3	1.7	W=100.5, p=0.803
Bifidobacterium	39.0	47.5	W=79, p=0.497
Rhodococcus	5.6	5.5	W=109, p=0.533
Collinsella	1.5	1.4	W=115, p=0.332
Bacteroides	9.7	10.3	W=76, p=0.422
Enterococcus	0.7	1.9	W=62, p=0.145
Lactobacillus	1.2	1.5	W=95.5, p=0.981
Streptococcus	2.9	2.1	W=111, p=0.476
Clostridiumsensustricto1	6.4	2.4	W=121, p=0.239
Blautia	2.8	1.2	W=120, p=0.252
LachnospiraceaeIncertaeSedis	10.2	6.2	W=112, p=0.448
Lachnospiraceaeuncultured	0.7	0.2	W=104.5, p=0.659
Erysipelotrichaceae Incertae Sedis	0.5	4.7	W=93, p=0.960
Veillonella	7.4	8.3	W=94, p=1
Enterobacter	1.8	0.7	W=116, p=0.339

Supplementary table 53: Relative abundance of bacterial OTUs in the *infant gut at 3 months old* by *maternal depressive symptoms* in the *3rd trimester*. Listed are all OTUs with an abundance of greater than 1% and significant OTUs with an abundance of less than 1%. Low depressive (EPDS < 8; Vaginal; N=21; C-Section; N=7), high depressive (EPDS > 9; Vaginal; N=9; C-Section; N=1). Unpaired students t-test and Mann-Whitney-Wilcoxon test. Abbreviations: Operational taxonomic unit (OTU), Edinburgh Postnatal Depression Scale (EPDS).

Table 54: Infant gut at 3 months old by 3rd trimester cortisol

OTU	**Relative Abundance (%)**		
3 months old	**Low CAR**	**High CAR**	**p-value**
Chao1 Index	**84.0**	**100.1**	***t*(30) =-1.72, *p*=0.094**
Simpson Index	0.7	0.7	*t*(30) = 1.44, *p*=0.158
Shannon Index	3.0	2.7	*t*(30) = 0.92,p=0.362
Phylogenetic diversity	5.1	4.9	*t*(30) = 0.56,p=0.578
Observed Species	64.5	63.6	*t*(30) = 0.17, p=0.858
Actinobacteria	**47.1**	**57.3**	**W=70, p=0.040**
Bacteroidetes	12.9	7.8	W=154, p=0.249
Firmicutes	36.2	31.8	W=155, p=0.238

Proteobacteria	1.9	2.8	W=105, p=0.489
Bifidobacteriaceae	**36.8**	**50.8**	**W=69, p=0.036**
Nocardiaceae	5.5	4.3	W=147, p=0.382
Coriobacteriaceae	4.4	1.6	W=146, p=0.398
Bacteroidaceae	10.3	6.9	W=146, p=0.404
Porphyromonadaceae	2.5	0.9	W=159.5, p=0.167
Enterococcaceae	0.4	1.9	W=120.5, p=0.923
Lactobacillaceae	1.5	1.1	W=143, p=0.462
Streptococcaceae	2.0	3.8	W=86, p=0.157
Clostridiaceae1	5.6	1.7	W=140.5, p=0.526
Lachnospiraceae	15.0	11.5	W=165, p=0.116
Erysipelotrichaceae	0.3	3.7	W=112.5, p=0.673
Veillonellaceae	9.5	5.2	W=162, p=0.144
Enterobacteriaceae	1.6	2.6	W=106, p=0.514
Actinomyces	**0.1**	**0.2**	**W=69.5, p=0.038**
Bifidobacterium	**36.8**	**50.8**	**W=69, p=0.036**
Rhodococcus	5.5	4.3	W=147, p=0.382
Collinsella	4.2	1.3	W=132, p=0.752
Bacteroides	10.3	6.9	W=146, p=0.404
Odoribacter	**0.2**	**0.0**	**W=169, p=0.016**
Enterococcus	0.4	1.9	W=120.5, p=0.923
Lactobacillus	1.5	1.1	W=143, p=0.462
Streptococcus	2.0	3.8	W=86, p=0.157
Clostridiumsensustricto1	5.6	1.7	W=140.5, p=0.526
Blautia	3.4	2.5	W=130, p=0.816
LachnospiraceaeIncertaeSedis	10.5	6.8	W=161, p=0.157
Lachnospiraceaeuncultured	0.5	0.9	W=108, p=0.557
Erysipelotrichaceae Incertae Sedis	0.3	3.7	W=127.5, p=0.885
Veillonella	8.8	5.1	W=153, p=0.265
Enterobacter	1.0	0.8	W=115, p=0.758

Supplementary table 54: Relative abundance of bacterial OTUs in the *infant gut at 3 months old* by *maternal cortisol* in the *3rd trimester*. Listed are all OTUs with an abundance of greater than 1% and significant OTUs with an abundance of less than 1%. Low cortisol (CAR < 562349; Vaginal; N=19; C-Section; N=5), high cortisol (CAR > 552350;

Vaginal; N=13; C-Section; N=4). Unpaired students t-test and Mann-Whitney-Wilcoxon test. Abbreviations: Operational taxonomic unit (OTU), Cortisol Awakening Response (CAR).

Table 55: Infant gut at 5 months old by 2nd trimester stress

OTU	Relative Abundance (%)		
5 months old	**Low PSS**	**High PSS**	**p-value**
Chao1 Index	**87.5**	**115.4**	***t*(20) =-1.92, *p*=0.069**
Simpson Index	**0.6**	**0.8**	***t*(20) =-3.02, *p*=0.006**
Shannon Index	**2.4**	**3.3**	***t*(20) =-2.60,p=0.016**
Phylogenetic diversity	**5.2**	**6.3**	***t*(20) =-2.13,p=0.045**
Observed Species	**64.1**	**83.5**	***t*(20) =-2.28, p=0.033**
Actinobacteria	59.2	44.8	W=66, p=0.203
Bacteroidetes	12.4	13.8	W=48.5, p=1
Firmicutes	28.0	39.3	W=34, p=0.329
Proteobacteria	**0.1**	**0.8**	**W=21.5, p=0.055**
Bifidobacteriaceae	**52.4**	**34.1**	**W=71, p=0.098**
Nocardiaceae	3.4	9.2	W=40, p=0.590
Coriobacteriaceae	2.2	1.3	W=57, p=0.530
Bacteroidaceae	11.4	11.3	W=50.5, p=0.882
Porphyromonadaceae	1.0	1.3	W=50.5, p=0.882
Lactobacillaceae	**2.9**	**0.5**	**W=78, p=0.027**
Streptococcaceae	3.7	1.1	W=52.5, p=0.768
Clostridiaceae1	**0.1**	**2.3**	**W=17, p=0.024**
Lachnospiraceae	9.1	17.4	W=34, p=0.329
Ruminococcaceae	**0.2**	**1.3**	**W=16, p=0.020**
Erysipelotrichaceae	**0.2**	**3.8**	**W=24.5, p=0.089**
Veillonellaceae	10.2	9.4	W=51, p=0.857
Bifidobacterium	**53.4**	**34.1**	**W=71, p=0.098**
Rhodococcus	3.4	9.2	W=40, p=0.590
Collinsella	1.9	1.1	W=58, p=0.477
Bacteroides	11.4	11.3	W=50.5, p=0.882
Lactobacillus	**2.9**	**0.5**	**W=78, p=0.027**

Streptococcus	3.7	1.1	W=48.5, p=1
Clostridiumsensustricto1	**0.1**	**2.3**	**W=17, p=0.024**
Blautia	1.0	4.4	W=40, p=0.578
LachnospiraceaeIncertaeSedis	7.4	9.6	W=41, p=0.640
Lachnospiraceaeuncultured	**0.5**	**1.5**	**W=23.5, p=0.076**
Ruminococcaceae Incertae Sedis	**0.0**	**0.1**	**W=18, p=0.027**
ErysipelotrichaceaeIncertaeSedis	**0.2**	**3.8**	**W=24, p=0.080**
Citrobacter	**0.0**	**0.0**	**W=15, p=0.009**
Enterobacter	**0.0**	**0.1**	**W=17, p=0.023**
Megasphaera	0.0	3.0	W=44, p=0.754
Veillonella	1.2	6.4	W=60, p=0.407

Supplementary table 55: Relative abundance of bacterial OTUs in the *infant gut at 5 months old* by *maternal stress* in the *2nd trimester*. Listed are all OTUs with an abundance of greater than 1% and significant OTUs with an abundance of less than 1%. Low stress (PSS < 13; Vaginal; N=6; C-Section; N=1), high stress (PSS > 16; Vaginal; N=13; C-Section; N=2). Unpaired students t-test and Mann-Whitney-Wilcoxon test. Abbreviations: Operational taxonomic unit (OTU), Perceived Stress Scale (PSS).

Table 56: Infant gut at 5 months old by 2nd trimester anxiety

OTU	**Relative Abundance (%)**		
5 months old	***Low STAI***	***High STAI***	***p-value***
Chao1 Index	103.8	118.3	*t*(20) =-0.94, *p*=0.358
Simpson Index	0.7	0.8	*t*(20) =-0.57, *p*=0.569
Shannon Index	2.9	3.3	*t*(20) =-0.84,p=0.410
Phylogenetic diversity	6.0	6.1	*t*(20) =-0.18,p=0.851
Observed Species	77.3	80.6	*t*(20) =-0.34, p=0.732
Actinobacteria	51.9	40.1	W=60, p=0.407
Bacteroidetes	14.1	11.5	W=50.5, p=0.882
Firmicutes	32.7	45.7	W=31, p=0.230
Proteobacteria	0.5	1.0	W=20.5, p=0.184
Bifidobacteriaceae	42.0	32.4	W=57, p=0.541
Nocardiaceae	8.3	5.8	W=40, p=0.590
Coriobacteriaceae	1.5	1.8	W=47, p=0.970
Bacteroidaceae	13.2	6.3	W=59, p=0.438

Porphyromonadaceae	0.9	2.0	W=39, p=0.528
Lactobacillaceae	1.3	0.7	W=55, p=0.626
Streptococcaceae	1.9	1.7	W=55.5, p=0.605
Clostridiaceae1	**0.7**	**4.3**	**W=21, p=0.050**
Lachnospiraceae	13.8	18.6	W=37, p=0.449
Erysipelotrichaceae	3.0	2.5	W=38.5, p=0.505
Veillonellaceae	7.9	14.3	W=29, p=0.177
Bifidobacterium	42.0	32.4	W=57, p=0.541
Rhodococcus	8.3	5.8	W=40, p=0.590
Collinsella	1.3	1.6	W=47.5, p=1
Bacteroides	13.2	6.3	W=59, p=0.438
Lactobacillus	1.3	0.7	W=55, p=0.626
Streptococcus	1.8	1.7	W=52.5, p=0.768
Clostridiumsensustricto1	**0.7**	**4.3**	**W=22, p=0.060**
Blautia	2.0	7.4	W=45, p=0.852
LachnospiraceaeIncertaeSedis	9.8	6.8	W=45, p=0.857
Lachnospiraceaeuncultured	1.2	1.1	W=41, p=0.630
ErysipelotrichaceaeIncertaeSedis	2.9	2.4	W=37.5, p=0.456
Megasphaera	1.0	5.1	W=35, p=0.264
Veillonella	6.8	9.1	W=39, p=0.541
Sutterella	**0.0**	**0.0**	**W=21, p=0.007**
Escherichia Shigella	**0.1**	**0.5**	**W=18.5, p=0.032**

Supplementary table 56: Relative abundance of bacterial OTUs in the *infant gut at 5 months old* by *maternal anxiety* in the *2nd trimester*. Listed are all OTUs with an abundance of greater than 1% and significant OTUs with an abundance of less than 1%. Low anxiety (STAI < 4; Vaginal; N=16; C-Section; N=1), high anxiety (STAI > 5; Vaginal; N=6; C-Section; N=2). Unpaired students t-test and Mann-Whitney-Wilcoxon test. Abbreviations: Operational taxonomic unit (OTU), State Trait Anxiety Inventory (STAI).

Table 57: Infant gut at 5 months old by 2nd trimester depressive symptoms

OTU	**Relative Abundance (%)**		
5 months old	**Low EPDS**	**High EPDS**	**p-value**
Chao1 Index	107.3	110.1	$t(20) = -0.15$, $p = 0.878$
Simpson Index	0.7	0.8	$t(20) = -0.35$, $p = 0.723$

Shannon Index	3.0	3.0	*t*(20) = 0.00,p=0.996
Phylogenetic diversity	6.1	5.5	*t*(20) = 0.87,p=0.392
Observed Species	80.2	69.2	*t*(20) = 1.02, p=0.316
Actinobacteria	49.3	45.7	W=38, p=0.902
Bacteroidetes	14.3	9.6	W=44, p=0.523
Firmicutes	35.2	41.0	W=32, p=0.774
Proteobacteria	**0.5**	**1.4**	**W=14, p=0.067**
Bifidobacteriaceae	39.4	39.0	W=38, p=0.902
Nocardiaceae	8.1	5.3	W=41, p=0.712
Propionibacteriaceae	**0.0**	**0.0**	**W=27, p=0.045**
Coriobacteriaceae	1.6	1.2	W=24, p=0.327
Bacteroidaceae	12.2	7.3	W=37.5, p=0.932
Porphyromonadaceae	1.0	2.0	W=46, p=0.415
Candidate division TM7 Unknown	**0.0**	**0.0**	**W=27, p=0.045**
Lactobacillaceae	1.3	0.5	W=48, p=0.319
Streptococcaceae	2.0	1.0	W=53.5, p=0.147
Clostridiaceae1	1.8	1.3	W=29, p=0.579
Lachnospiraceae	14.3	18.8	W=36, p=1
Erysipelotrichaceae	2.7	3.3	W=30.5, p=0.669
Veillonellaceae	8.9	12.7	W=32, p=0.774
Shewanellaceae	**0.0**	**0.0**	**W=27, p=0.045**
Bifidobacterium	39.4	39.0	W=38, p=0.902
Rhodococcus	8.1	5.3	W=41, p=0.712
Propionimicrobium	**0.0**	**0.0**	**W=27, p=0.045**
Collinsella	1.4	1.0	W=35, p=0.965
Bacteroides	12.2	7.3	W=37.5, p=0.932
Carnobacterium	**0.0**	**0.0**	**W=12, p=0.036**
Lactobacillus	1.3	0.5	W=48, p=0.319
Streptococcus	2.0	1.0	W=51, p=0.216
Clostridiumsensustricto1	1.8	1.2	W=30, p=0.639
Eubacterium	**0.0**	**0.0**	**W=27, p=0.045**
Blautia	1.8	10.9	W=31, p=0.699

LachnospiraceaeIncertaeSedis	9.6	6.1	W=42, p=0.652
Lachnospiraceaeuncultured	1.3	1.0	W=33, p=0.831
ErysipelotrichaceaeIncertaeSedis	2.7	3.3	W=29.5, p=0.605
Megasphaera	0.9	7.7	W=24.5, p=0.257
Veillonella	8.0	4.9	W=45, p=0.484
Shewanella	**0.0**	**0.0**	**W=27, p=0.045**
Proteus	**0.0**	**0.0**	**W=27, p=0.045**

Supplementary table 57: Relative abundance of bacterial OTUs in the *infant gut at 5 months old* by *maternal depressive symptoms* in the *2nd trimester*. Listed are all OTUs with an abundance of greater than 1% and significant OTUs with an abundance of less than 1%. Low depressive (EPDS < 8; Vaginal; N=18; C-Section; N=3), high depressive (EPDS > 9; Vaginal; N=4; C-Section; N=0). Unpaired students t-test and Mann-Whitney-Wilcoxon test. Abbreviations: Operational taxonomic unit (OTU), Edinburgh Postnatal Depression Scale (EPDS).

Table 58: Infant gut at 5 months old by 2nd trimester cortisol

OTU	**Relative Abundance (%)**		
5 months old	**Low CAR**	**High CAR**	**p-value**
Chao1 Index	121.9	100.9	$t(19) = 1.52$, $p=0.141$
Simpson Index	0.8	0.7	$t(19) = 1.35$, $p=0.191$
Shannon Index	3.2	2.8	$t(19) = 1.12$,p=0.274
Phylogenetic diversity	6.5	5.9	$t(19) = 1.22$,p=0.236
Observed Species	84.6	75.0	$t(19) = 1.12$, p=0.274
Actinobacteria	50.8	45.2	W=56, p=0.917
Bacteroidetes	13.6	12.9	W=63.5, p=0.522
Firmicutes	33.6	40.4	W=51, p=0.862
Proteobacteria	0.9	0.6	W=68.5, p=0.319
Bifidobacteriaceae	42.6	36.2	W=61, p=0.651
Nocardiaceae	5.0	80.5.3	W=31, p=0.111
Coriobacteriaceae	**3.0**	**0.5**	**W=82.5, p=0.046**
Bacteroidaceae	11.4	12.5	W=63, p=0.545
Porphyromonadaceae	2.0	0.4	W=66, p=0.409
Lactobacillaceae	0.3	1.8	W=31, p=0.103
Streptococcaceae	1.3	2.3	W=43.5, p=0.477

Clostridiaceae1	2.3	1.2	W=38.5, p=0.286
Lachnospiraceae	11.9	19.5	W=54, p=1
Erysipelotrichaceae	1.0	4.5	W=49.5, p=0.776
Veillonellaceae	**14.0**	**6.7**	**W=82, p=0.049**
Sphingomonadaceae	**0.0**	**0.0**	**W=72, p=0.040**
Bifidobacterium	42.6	36.2	W=61, p=0.651
Rhodococcus	5.0	8.3	W=31, p=0.111
Collinsella	**2.9**	**0.2**	**W=92, p=0.006**
Bacteroides	11.4	12.5	W=63, p=0.545
Porphyromonadaceae uncultured	**0.9**	**0.0**	**W=81.5, p=0.042**
Lactobacillus	0.3	1.8	W=31, p=0.103
Streptococcus	1.3	2.2	W=46.5, p=0.618
Clostridiumsensustricto1	2.3	1.1	W=37.5, p=0.255
Blautia	2.4	5.3	W=57.5, p=0.830
LachnospiraceaeIncertaeSedis	6.7	11.6	W=53, p=0.972
Lachnospiraceaeuncultured	0.7	1.5	W=56, p=0.914
ErysipelotrichaceaeIncertaeSedis	1.0	4.5	W=50.5, p=0.830
Megasphaera	5.3	0.0	W=70.5, p=0.175
Veillonella	8.6	6.7	W=60, p=0.702

Supplementary table 58: Relative abundance of bacterial OTUs in the *infant gut at 5 months old* by *maternal cortisol* in the *2nd trimester*. Listed are all OTUs with an abundance of greater than 1% and significant OTUs with an abundance of less than 1%. Low cortisol (CAR < 545131; Vaginal; N=9; C-Section; N=3), high cortisol (CAR > 545132; Vaginal; N=12; C-Section; N=0). Unpaired students t-test and Mann-Whitney-Wilcoxon test. Abbreviations: Operational taxonomic unit (OTU), Cortisol Awakening Response (CAR).

Table 59: Infant gut at 5 months old by 3rd trimester stress

OTU	**Relative Abundance (%)**		
5 months old	**Low PSS**	**High PSS**	**p-value**
Chao1 Index	96.8	105.3	$t(19) = -0.57$, $p=0.572$
Simpson Index	0.7	0.8	$t(19) = -0.65$, $p=0.522$
Shannon Index	3.0	3.0	$t(19) = -0.03$,p=0.971
Phylogenetic diversity	5.9	5.9	$t(19) = 0.00$,p=0.993

Observed Species	76.7	74.9	*t*(19) = 0.18, p=0.857
Actinobacteria	52.0	47.6	W=62, p=0.500
Bacteroidetes	11.5	15.8	W=43, p=0.546
Firmicutes	34.8	34.2	W=50, p=0.915
Proteobacteria	**0.3**	**1.5**	**W=26, p=0.063**
Bifidobacteriaceae	42.5	38.5	W=62, p=0.500
Nocardiaceae	7.3	7.6	W=60, p=0.595
Coriobacteriaceae	2.0	1.3	W=63.5, p=0.425
Bacteroidaceae	10.4	14.5	W=41, p=0.446
Porphyromonadaceae	1.0	1.2	W=59.5, p=0.609
Lactobacillaceae	0.9	0.9	W=55, p=0.854
Streptococcaceae	2.6	1.8	W=57.5, p=0.717
Clostridiaceae1	0.7	2.1	W=54.5, p=0.884
Lachnospiraceae	18.8	9.2	W=65, p=0.373
Erysipelotrichaceae	2.2	3.8	W=52, p=1
Veillonellaceae	5.2	12.3	W=31, p=0.140
Bifidobacterium	42.5	38.5	W=62, p=0.500
Rhodococcus	7.3	7.6	W=60, p=0.595
Collinsella	1.7	1.2	W=56, p=0.795
Bacteroides	10.4	14.5	W=41, p=0.446
Lactobacillus	0.9	0.9	W=55, p=0.854
Streptococcus	2.6	1.8	W=57.5, p=0.717
Clostridiumsensustricto1	0.7	2.1	W=55.5, p=0.772
Peptoniphilus	**0.0**	**0.0**	**W=74.5, p=0.031**
Blautia	3.2	0.6	W=57, p=0.741
LachnospiraceaeIncertaeSedis	12.5	6.7	W=61, p=0.546
Lachnospiraceaeuncultured	1.7	0.3	W=64.5, p=0.382
ErysipelotrichaceaeIncertaeSedis	2.2	3.8	W=54, p=0.912
Phascolarctobacterium	**0.0**	**0.1**	**W=28, p=0.032**
Megasphaera	0.0	2.4	W=41.5, p=0.387
Veillonella	5.1	9.9	W=36, p=0.268

Supplementary table 59: Relative abundance of bacterial OTUs in the *infant gut at 5 months old* by *maternal stress* in the *3rd trimester*. Listed are all OTUs with an abundance of greater than 1% and significant OTUs with an abundance of less than 1%. Low stress

(PSS < 11; Vaginal; N=8; C-Section; N=2), high stress (PSS > 12; Vaginal; N=13; C-Section; N=0). Unpaired students t-test and Mann-Whitney-Wilcoxon test. Abbreviations: Operational taxonomic unit (OTU), Perceived Stress Scale (PSS).

Table 60: Infant gut at 5 months old by 3rd trimester anxiety

OTU	**Relative Abundance (%)**		
5 months old	**Low STAI**	**High STAI**	**p-value**
Chao1 Index	96.4	108.3	*t*(19) =-0.82, *p*=0.420
Simpson Index	0.7	0.8	*t*(19) =-1.55, *p*=0.136
Shannon Index	2.8	3.2	*t*(19) =-1.28,p=0.213
Phylogenetic diversity	5.8	6.0	*t*(19) =-0.28,p=0.779
Observed Species	73.8	77.6	*t*(19) =-0.39, p=0.700
Actinobacteria	47.1	51.8	W=53, p=0.917
Bacteroidetes	15.0	13.2	W=56, p=0.972
Firmicutes	35.9	32.8	W=56, p=0.972
Proteobacteria	**1.0**	**1.1**	**W=30, p=0.084**
Bifidobacteriaceae	39.9	40.1	W=61, p=0.704
Nocardiaceae	6.0	9.1	W=53, p=0.917
Coriobacteriaceae	0.9	2.3	W=42.5, p=0.397
Bacteroidaceae	14.5	11.2	W=61, p=0.698
Porphyromonadaceae	0.4	1.9	W=42, p=0.374
Lactobacillaceae	0.8	1.0	W=57.5, p=0.886
Streptococcaceae	3.2	0.9	W=76.5, p=0.139
Clostridiaceae1	0.5	2.8	W=34, p=0.148
Lachnospiraceae	14.9	10.6	W=34, p=0.148
Erysipelotrichaceae	2.9	3.6	W=51, p=0.804
Acidaminococcaceae	**0.0**	**0.2**	**W=31, p=0.037**
Veillonellaceae	9.2	10.0	W=56, p=0.972
Bifidobacterium	39.9	40.1	W=61, p=0.704
Rhodococcus	6.0	9.1	W=53, p=0.917
Collinsella	0.6	2.2	W=35, p=0.160
Bacteroides	14.5	11.2	W=61, p=0.698
Lactobacillus	0.8	1.0	W=57.5, p=0.886

Streptococcus	3.2	0.9	W=76.5, p=0.139
Clostridiumsensustricto1	0.5	2.8	W=36, p=0.192
Blautia	2.3	0.8	W=62.5, p=0.617
LachnospiraceaeIncertaeSedis	10.3	7.3	W=52, p=0.863
Lachnospiraceaeuncultured	1.2	0.4	W=58.5, p=0.831
ErysipelotrichaceaeIncertaeSedis	2.9	3.6	W=49, p=0.695
Megasphaera	0.0	3.1	W=42, p=0.293
Veillonella	9.2	6.8	W=66, p=0.467
Sutterella	**0.0**	**0.0**	**W=33, p=0.027**

Supplementary table 60: Relative abundance of bacterial OTUs in the *infant gut at 5 months old* by *maternal anxiety* in the *3rd trimester*. Listed are all OTUs with an abundance of greater than 1% and significant OTUs with an abundance of less than 1%. Low anxiety (STAI < 4; Vaginal; N=11; C-Section; N=2), high anxiety (STAI > 5; Vaginal; N=10; C-Section; N=0). Unpaired students t-test and Mann-Whitney-Wilcoxon test. Abbreviations: Operational taxonomic unit (OTU), State Trait Anxiety Inventory (STAI).

Table 61: Infant gut at 5 months old by 3rd trimester depressive symptoms

OTU	**Relative Abundance (%)**		
5 months old	**Low EPDS**	**High EPDS**	**p-value**
Chao1 Index	107.0	86.4	$t(30) = 1.24$, $p=0.227$
Simpson Index	0.7	0.7	$t(30) = 0.63$, $p=0.534$
Shannon Index	3.1	2.7	$t(30) = 1.20$,p=0.244
Phylogenetic diversity	6.1	5.3	$t(30) = 1.16$,p=0.257
Observed Species	79.3	63.8	$t(30) = 1.43$, p=0.168
Actinobacteria	44.8	63.7	W=23, p=0.179
Bacteroidetes	14.5	13.0	W=44, p=0.779
Firmicutes	38.2	22.2	W=57, p=0.179
Proteobacteria	1.1	0.7	W=43, p=0.841
Bifidobacteriaceae	**34.9**	**56.4**	**W=19, p=0.091**
Nocardiaceae	8.3	4.9	W=57, p=0.179
Coriobacteriaceae	1.3	2.3	W=27, p=0.301
Bacteroidaceae	13.4	11.5	W=43.5, p=0.804
Porphyromonadaceae	1.0	1.5	W=33, p=0.588
Lactobacillaceae	0.7	1.6	W=25, p=0.225

Streptococcaceae	2.5	1.0	W=51.5, p=0.363
Clostridiaceae1	2.0	0.2	W=58, p=0.148
Lachnospiraceae	15.0	5.7	W=58, p=0.153
Erysipelotrichaceae	**4.2**	**0.1**	**W=69, p=0.018**
Veillonellaceae	9.3	10.5	W=40, p=1
Bifidobacterium	**34.9**	**56.4**	**W=19, p=0.091**
Rhodococcus	8.3	4.9	W=57, p=0.179
Collinsella	1.2	2.1	W=30, p=0.423
Bacteroides	13.4	11.5	W=43.5, p=0.804
Lactobacillus	0.7	1.6	W=25, p=0.225
Streptococcus	2.5	1.0	W=51.5, p=0.363
Clostridiumsensustricto1	2.0	0.2	W=58, p=0.148
Blautia	2.1	0.0	W=58, p=0.143
LachnospiraceaeIncertaeSedis	10.0	5.5	W=52, p=0.353
Lachnospiraceaeuncultured	1.1	0.0	W=57, p=0.170
ErysipelotrichaceaeIncertaeSedis	**4.2**	**0.1**	**W=68.5, p=0.019**
Megasphaera	1.9	0.0	W=34, p=0.588
Veillonella	7.3	10.5	W=35, p=0.719

Supplementary table 61: Relative abundance of bacterial OTUs in the *infant gut at 5 months old* by *maternal depressive symptoms* in the *3rd trimester*. Listed are all OTUs with an abundance of greater than 1% and significant OTUs with an abundance of less than 1%. Low depressive (EPDS < 8; Vaginal; N=16; C-Section; N=2), high depressive (EPDS > 9; Vaginal; N=5; C-Section; N=0). Unpaired students t-test and Mann-Whitney-Wilcoxon test. Abbreviations: Operational taxonomic unit (OTU), Edinburgh Postnatal Depression Scale (EPDS).

Table 62: Infant gut at 5 months old by 3rd trimester cortisol

OTU	**Relative Abundance (%)**		
5 months old	**Low CAR**	**High CAR**	**p-value**
Chao1 Index	103.2	102.3	$t(21) = 0.05$, $p=0.954$
Simpson Index	0.7	0.7	$t(21) = -0.50$, $p=0.617$
Shannon Index	2.9	3.1	$t(21) = -0.59$,p=0.559
Phylogenetic diversity	5.8	5.8	$t(21) = 0.36$,p=0.970
Observed Species	73.0	75.5	$t(21) = -0.27$, p=0.786

Actinobacteria	49.8	53.5	W=55, p=0.643
Bacteroidetes	12.7	14.3	W=66.5, p=0.850
Firmicutes	35.9	29.6	W=84, p=0.201
Proteobacteria	1.3	0.5	W=79, p=0.328
Bifidobacteriaceae	40.0	45.2	W=53, p=0.557
Nocardiaceae	6.8	7.0	W=58, p=0.781
Coriobacteriaceae	2.9	1.0	W=83, p=0.219
Bacteroidaceae	11.2	13.7	W=63.5, p=1
Porphyromonadaceae	1.4	0.4	W=75, p=0.565
Lactobacillaceae	0.8	0.6	W=56.5, p=0.699
Streptococcaceae	2.5	1.8	W=46.45, p=0.313
Clostridiaceae1	1.9	0.7	W=44.5, p=0.256
Lachnospiraceae	11.0	14.1	W=74, p=0.516
Erysipelotrichaceae	3.4	1.4	W=71.5, p=0.613
Veillonellaceae	**13.5**	**6.6**	**W=92, p=0.072**
Bifidobacterium	39.9	45.2	W=53, p=0.557
Rhodococcus	6.8	7.0	W=58, p=0.781
Collinsella	2.7	0.9	W=89, p=0.102
Bacteroides	11.2	13.7	W=63.5, p=1
Lactobacillus	0.8	0.6	W=56.5, p=0.699
Streptococcus	2.5	1.8	W=46, p=0.305
Clostridiumsensustricto1	1.9	0.7	W=44.5, p=0.256
Blautia	1.0	2.1	W=70, p=0.679
LachnospiraceaeIncertaeSedis	8.3	9.5	W=71, p=0.643
Oribacterium	**0.0**	**0.0**	**W=42, p=0.026**
Lachnospiraceaeuncultured	0.3	1.3	W=51.5, p=0.485
Peptostreptococcus	**0.0**	**0.0**	**W=38.5, p=0.035**
ErysipelotrichaceaeIncertaeSedis	3.4	1.4	W=72, p=0.589
Megasphaera	3.4	0.0	W=72.5, p=0.519
Veillonella	10.0	6.5	W=76, p=0.438

Supplementary table 62: Relative abundance of bacterial OTUs in the *infant gut at 5 months old* by *maternal cortisol* in the *3rd trimester*. Listed are all OTUs with an abundance of greater than 1% and significant OTUs with an abundance of less than 1%.

Good sleep quality (CAR < 562349; Vaginal; N=14; C-Section; N=2), poor sleep quality (CAR > 562350; Vaginal; N=9; C-Section; N=1). Unpaired students t-test and Mann-Whitney-Wilcoxon test. Abbreviations: Operational taxonomic unit (OTU), Cortisol Awakening Response (CAR).