The effect of parenting on epigenetic regulation of stress-related genes in infants

Jessica Cole

MSc 2023

The effect of parenting on epigenetic regulation of stress-related genes in infants

Jessica Cole

A thesis in fulfilment of the requirements of Manchester Metropolitan University for the degree of Master of Science (by Research)

Department of Science and Engineering

Manchester Metropolitan University

2023

Acknowledgement

I would like to acknowledge the help and support from my supervisors, Dr Chris Murgatroud and Dr Liz Braithwaite.

Abstract.

Early parental care in childhood is of key importance to ensure that children develop well socially, emotionally and cognitively, with evidence indicating that caregiver inconsistency, neglect, and a lack of love may lead to mental health problems, alongside reduced happiness and unrealised overall potential.

Recent evidence indicates that 12.5% of young people and children had a mental disorder, with prevalence increasing; with approximately 5 to 10% of young children have a behavioural disorder. Therefore, since parental care is key in improving their children's behaviour, optimising care will likely lead to better outcomes for children, lowering the risk of relationship challenges, failure at school and poor physical and mental health.

Experience of trauma in early years has been shown to cause dysfunction in the genes for the Oxytocin Receptor (*OXTR*) glucocorticoid receptor (*NR3C1*) and FK506 binding protein 5 (*FKBP5*) genes associating with dysregulation of the hypothalamus–pituitary–adrenal (HPA) axis. This is linked to anxiety and depression-related disorders. Recent therapies using video feedback interventions to provide guidance for parents of children with behavioural problems have been shown to help improve children's mental health. The NR3C1 gene is important in regulating stress and calming measures such as grooming and stroking have been found to reduce DNA methylation of NR3C1-1F. Changes in DNA methylation at the OXTR promoter have been linked to callous unemotional traits, internalisation problems and depression. Furthermore, studies have indicated that poor maternal care in a child's early years leads to increased methylation of OXTR expressed in adulthood. Early-life stress has also been linked to changes in DNA methylation at the FKBP5 that are further linked to depression.

The aim of this paper was to investigate the effect of epigenetic regulation of the above genes *OXTR*, *FKBP5* and *NR3C1* in children as part of the Health Start Happy Start (HSHS) video

feedback intervention. Two hundred and twenty five samples from an original three hundred saliva samples taken from children whose parents/carers had participated in the HSHS intervention were analysed. DNA was extracted from each of the samples and the concentration of DNA was measured using a nano-drop UV-Vis spectrophotometer. This was followed by sodium bisulfite treatment and subsequently PCR cycling for DNA amplification. Pyromark- sequencing was then employed to analyse DNA methylation in regulatory regions at each of the genes.

No clear differences in methylation at OXTR, NR3C1 and FKBP5 between VIPP and control groups or correlations in methylation at any of the genes with changes in behaviour were found following the HSHS video intervention. We did however find a significant association with NR3C1 methylation and sex-specific behavioural changes, alongside a negative association between OXTR methylation and primary caregiver's educational attainment.
This suggests that parental behaviour does not affect DNA methylation outcomes, however, there is significance in the correlation between OXTR methylation and primary caregivers' level of education, and NR3C1 methylation and sex-specific behavioural changes.

Contents

Acknowledgement 2

Abstract. 4

Abbreviations........ 10

1. Introduction 12

1.1 Epigenetic mechanisms........ 14

1.2 DNA methylation 15

1.2.1 *OXTR*........16

1.2.2. *FKBP5* 16

1.2.3. *NR3C1* 17

1.3 The HPA axis 17

1.4 Impacts of childhood adversity on epigenetic modification. 19

1.4.1 Methylation of *NR3C1* gene and childhood adversity 19

1.4.2. Methylation of *FKBP5* gene and childhood adversity 21

1.4.3 Methylation of *OXTR* gene and childhood adversity 22

1.5 Methylation changes and parenting........ 23

1.6 Aims and hypothesis........ 23

2 Methods: The HSHS study 24

2.1 Ethics 26

2.2 Extraction of DNA from infant passive drool samples 26

2.3 Sodium bisulphite modification of DNA 27

2.4 Bisulphite PCR........ 28

2.5 Bisulphite Pyrosequencing........ 30

2.6 Statistical analysis........ 31

2.6.1 methods used on SPSS IBM version 26........32

3. RESULTS 33

3.1 Testing and establishing methods for saliva DNA extraction........ 33

3.2 DNA extraction from the Happy Start child passive drool samples 34

3.3 establishment of DNA methylation analyses using bisulphite sequencing 34

3.3.1 DNA methylation of *FKBP5* in child DNA........34

3.3.2 Establishment of *FKBP5* bisulphite sequencing 35

Figure 4 PCR of the region of the *FKBP5* promoter harbouring CpG1 and CpG2 representative agarose gel of four samples and a negative (-ve) and a 50bp DNA ladder (L).3.3.3 *FKBP5* methylation levels across the samples 35

3.3.4 DNA methylation at *NR3C1* in child DNA 36

3.3.5 Establishment of *NR3C1* bisulphite sequencing....37

3.3.6 *NR3C1* methylation levels across the samples....37

3.3.7 DNA methylation of *OXTR* in child DNA 38

3.2.8 Establishment of *OXTR* bisulphite sequencing....39

3.3.9 *OXTR* methylation levels across the samples....39

3.4. Inspecting and testing distribution of DNA methylation data 40

3.5 Primary caregiver and child demographics 46

3.6 Testing for differences in DNA methylation between children who have received the VIPP intervention compared with those who have not 47

3.7 Testing whether the degree of child behaviour change from pre-intervention (TP1) to 2-year follow up (TP2) associated with levels of DNA methylation 51

4. Discussion 57

5 Limitations 62

6. Further research 64

7. Conclusion 65

8. References.... 65

9. Appendices.... 85

Tables

TABLE 1: PCR PROGRAMME FOR SODIUM BISULPHATE CONVERSION 27

TABLE 2: THE TEMPERATURE AND TIMINGS OF PCR CYCLES FOR *NR3C1*, *FKBP5* AND *OXTR* GENES 29

TABLE 3: PRIMERS USED FOR FKBP5, OXTR AND NR3C1, LOCATION AND SEQUENCE ANALYSED....28

TABLE 4: MASTER MIX PREPARATION 30

TABLE 5 SEQUENCING PRIMERS AND SEQUENCES TO BE ANALYSED 31

TABLE 6 RESULTD OF TESTING SALIVA EXTRACTION PROTOCOLS 33

TABLE 7 AVERAGE RESULTS OF EXTRACTION DNA FROM SALIVA SAMPLES....34

TABLE 8 DESCRIPTIVE STATISTICS OF METHYLATED CPGS AT OXTR, NR3C1, AND FKBP5 INCLUDING MEAN, STANDARD DEVIATION, SKEWNESS AND KURTOSIS....40

TABLE 9 DESCRIPTIVE STATISTICS FOR LOG TRANSFORMED METHYLATION DATA FOR OXTR, NR3C1 AND FKBP5 INCLUDING MEAN, STANDARD DEVIATION, SKEWNESS AND KURTOSIS, YELLOW HIGHLIGHTED DATA SHOW KURTOSED VALUES. 41

TABLE 10 DESCRIPTIVE STATISTICS FOR SQUARE ROOT TRANSFORMED METHYLATION DATA FOR OXTR, NR3C1 AND FKBP5 INCLUDING MEAN, STANDARD DEVIATION, SKEWNESS AND KURTOSIS. YELLOW HIGHLIGHTED DATA SHOWN KURTOSED VALUES....41

TABLE 11 EXTREME VALUES FOR DATA POINTS FOR CPG VALUES FROM OXTR, FKBP5 AND NR3C1 EXHIBITING THOSE CASES WITH EXTREME VALUES THAT NEEDED TO BE REMOVED (GREEN)....43

TABLE 12 DESCRIPTIVE STATISTICS FOR SQUARE ROOT TRANSFORMED METHYLATION FOR OXTR, NR3C1 AND FKBP5 FOLLOWING REMOVAL OF OUTLIERS. GREEN HIGHLIGHTED DATA SHOW SKEWED AND KURTOSED DATA. 46

TABLE 13 PRIMARY CAREGIVER DEMOGRAPHICS ....**ERROR! BOOKMARK NOT DEFINED.**

TABLE 14 CHILD DEMOGRAPHICS....47

TABLE 15 DESCRIPTIVE STATISTICS FOR AVERAGE DNA METHYLATION FOR NR3C1 AND FKBP5 AND OXTR CPGS1 AND 2 48

TABLE 16 INDEPENDENT SAMPLES T-TESTD FOR DIFFERENCES IN AVERAGE DNA METHYLATION FOR NR3C1 AND FKBP5 AND OXTR CPGS 1 AND 2 BETWEEN VIPP AND CONTROL GROUPS 48

TABLE 17 PEARSON'S CORRELATION FOR DNA METHYLATION AND CHANGE IN SDQ SCORES FROM TIMEPOINT 1 AND 3 51

TABLE 18 STEPWISE REGRESSION FOR AVERAGE NR3C1 DNA METHYLATION AND SDQ CHANGE SCORE 52

TABLE 19 STEPWISE REGRESSION FOR AVERAGE FKBP5 DNA METHYLATION AND SDQ CHANGE SCORE....53

TABLE 20 STEPWISE REGRESSION FOR AVERAGE OXTR CPG 1 DNA METHYLSTION SDQ CHANGE SCORE. 54

TABLE 21 STEPWISE REGRESSION FOR AVERAGE OXTR CPG2 DNA METHYLATION AND SDQ CHANGES SCORE....55

TABLE 22 CHILD DEMOGRAPHICS USING SPSS IBM 27....25

TABLE 23 PRIMARY CAREGIVER USING SPSS IBM 27....26

Figures

FIGURE 1: SCHEMATIC OF THE WASH STEPS FOR THE PYROMARK Q24 30

FIGURE 2 AN EXAMPLE OF CHILD PASSIVE DROOL SALIVA SAMPLES FROM THE HAPPY START COLLECTION DEMONSTRATING SOME VARIATION ON VOLUMES AND COLOURS....34

FIGURE 3 REGION OF THE HUMAN FKBP5 PROMOTER TESTED AT CPG1 AND CPG2 35

FIGURE 4 PCR OF THE REGION OF THE FHBP5 PROMOTER HARBOURING CPG1 AND CPG2 REPRESENTATIVE AGAROSE GEL OF FOUR SAMPLES AND A NEGATIVE (-VE) AND A 50BP DNA LADDER (L). 35

FIGURE 5 A BAR CHART SHOWING LEVELS OF DNA METHYLATION AT CPG1 AND CPG2 AT THE FKBP5 PROMOTER AND THE AVERAGE METHYLATION OF BOTH CPG SITE (N=211) 36

FIGURE 6 REGION OF THE HUMAN NR3C1 PROMOTER TESTED FOR DNA METHYLATION AT CPG1, CPG2, CPG3 AND CPG4....37

FIGURE 7 PCR OF THE REGION OF THE HUMAN NR3C1 PROMOTER HARBOURING CPG1 AND CPG2. REPRESENTATIVE AGAROSE GEL OF FOUR SAMPLES AND A NEGATIVE (-VE) AND A 50BP DNA LADDER (L).....37

FIGURE 8 A BAR CHART SHOWING LEVELS OF DNA METHYLATION OF CPG1 AND CPG2 AT THE FKBP5 PROMOTER AND THE AVERAGE OF BOTH CPG SITES (N=139) 38

FIGURE 9 REGIONS OF THE HUMAN OXTR PROMOTER TESTED FOR DNA METHYLATION AT CPG1 AND CPG2.........38

FIGURE 10 PCR OF THE REGION OF THE HUMAN OXTR PROMOTER HARBOURING CPG1 AND CPG2. REPRESENTATIVE AGAROSE GEL OF FOUR AND A NEGATIVE (-VE) AND A 50BP DNA LADDER (L). 39

FIGURE 11 A BAR CHART SHOWING LEVELS OF DNA METHYLATION AT CPG1 AND CPG2 AT THE OXTR PROMOTER AND THE AVERAGE METHYLATION OF BOTH CPG SITES (N=211). 39

FIGURE 12 HISTOGRAM OF SQUARE ROOT TRANSFORMED DATA OXTR CPG1 (A) AND CPG (C) AND CPG2 (D), AND NR3C1 CPG1 (E) AND CPG2 (F)..42

FIGURE 13 A BAR CHART SHOWING LEVELS OF DNA METHYLATION ACROSS ALL CPG SITES AT THE NR3C1 PROMOTER BETWEEN VIPP (N=6.38) AND CONTROL (N=6.42) GROUPS AT AVERAGE ACROSS ALL SITES. 50

FIGURE 14 A BAR CHART SHOWING LEVELS OF DNA METHYLATION AT CPG1 AND CPG2 AT THE FKBP5 PROMOTER BETWEEN VIPP (N=88.22) AND CONTROL (N= 88.25) GROUPS 50

FIGURE 15A BAR CHART SHOWING LEVELS OF DNA METHYLATION OF CPG1 (CONTROL N=47.8; VIPP= 47.37) AND CPG2 (CONTROL N=68.73; VIPP= 69.39) AND THE AVERAGES (CONTROL N=58.07; VIPP N=58.19) 50

Abbreviations

OXTR= oxytocin receptor

NR3C1= nuclear receptor subfamily 3 group C 1

FKBP5= FK506-Binding protein 51

SLC6A4= solute carrier family 6 member 4

BDNF= brain derived neurotrophic factor

CpG= cytosine nucleotide–phosphate–guanine nucleotide

CpT= thymine

CpA= adinine

DNA= deoxyribonucleic acid

RNA= ribonucleic acid

HPA axis= Hypothalamic pituitary adrenal axis

DNMTs= DNA methyltransferases

SAM= S-adenosyl-l-methionine

MDD= major depressive disorders

ELA= early life adversity

ELS= early life stress

EphB2-NR1= ephrin type B receptor 2

NMDA receptor= N-methyl-D-asoarate

GR resistance= glucocorticoid resistance

CRF= corticotrophin releasing factor

CORT= cortisterone

ACTH= adrenocorticotropic hormone

SNP= single nucleotide polymorphism

GXE= Gene X Environment

PTSD= post-traumatic stress disorder

VIPP= video-feedback intervention to promote positive feedback

VIPP-SD= video-feedback to promote positive parenting and sensitive discipline.

HSHS= healthy start happy start

SDQ= strengths and difficulties questionnaire.

PACS / PPACs= preschool parental account of children's symptoms

PHQ= patient health questions

CBCL= child behaviour checklist

GAD= generalised anxiety disorder

ADHD= attention deficit hyperactivity disorder

ASD= autism spectrum disorder

AW2 Buffer= wash buffer 2

BD buffer= buffer descriptor

EB buffer= elution buffer

TBE buffer= tris-botate-EDTA

DNTPs= Deoxynucleotide triphosphates

PCR= polymerase chain reaction

RPM= revolutions per minute

BP= base pairs

TP / T= timepoint

SQRT= square root

MMU= Manchester Metropolitan University

1. Introduction

Behaviour problems are one of the most common mental health disorders in childhood. These include behaviours related to conduct disorder, describing norm-breaking behaviours and violations of the rights of others, and behaviours related to oppositional defiant disorder, describing noncompliant, angry, and defiant behaviours (Hammerton et al., 2019).

Early intervention has been evidenced to improve quality of care and life for foster children, reducing the risks of poor physical and mental health providing foster children paths from crime into meaningful employment (Deidda et al., 2018). Developmental delays and concerns have also been identified in children who are in the foster care system (Mcleigh et al, J., Tunnel, K., and Lazcano, J, 2020). A study by Hiller et al., (2020) from the perspective of foster carers identified that they are unable to support the needs of children in their care, due to a lack of appropriate training to support complex behavioural needs (Hiller et al., 2020).

Longitudinal studies in the UK show that these behavioural problems, particularly conduct disorder are associated with a wide range of adverse outcomes in adulthood (Hammerton et al., 2019). These early behaviour problems and their adverse outcomes have considerable financial and emotional impact on affected young people and their families, as well as on education, mental health and juvenile justice systems, and can undermine children's health and employment outcomes into adulthood (Sumner et al., 2022). Boys are more likely than girls to suffer from behavioural disorders differently dependent on maternal depression. This is pre and postnatal dependent (Braithwaite et al., 2020; Hill et al., 2019).

It is not known exactly why some children develop disruptive behaviour disorders. Many factors may play a role, including biological and social factors. It is known that children are at greater risk when they are exposed to other types of violence and criminal behaviour, when they experience maltreatment or harsh or inconsistent parenting, or when their parents have mental health conditions like substance use disorders, depression, or attention-deficit hyperactivity disorder

(ADHD). The quality of early childhood care can also impact whether a child develops behaviour problems. For example, one study showed a higher level of parenting stress associated with higher levels of reported child behaviour problems. In this study parenting stress positively related to negative parenting styles, and negative parenting styles partially mediated the relationship between parenting stress and child behaviour problems (Mak et al., 2020). Findings from this study suggests that reducing parenting stress, improving parenting behaviours such as parenting styles, and enhancing parent-child relationship through early support (e.g., parenting skills training) are of vital importance and mutual benefits to the parents, children, and family relationships are at large.

An important point is that a number of studies have shown sex differences in outcomes from early life stress. One study showed that boys were at a greater risk of depressive symptoms than girls following exposure to pre-natal and post-natal depression (Braithwaite et al., 2020). Male infants were prone to increased methylation of *NR3C1* (nuclear receptor subfamily 3 group 1) when exposed to prenatal depressive symptoms (Braithwate et al., 2015). Although it has been shown that girls are more affected than boys in *NR3C1* when exposed to pre- and post-natal depression (Hill et al., 2019). In mice, female offspring have higher basal corticosterone (CORT) when they have high *FKBP5* (FK506- Binding protein 5) expressing parents (Criado-marrero et al., 2020). Oxytocin receptor (*OXTR)* has been shown to affect childhood anxious traits in females at cytosine nucleotide–phosphate–guanine (CpG) promoter sites (Gouin et al., 2017).

Treatment options include cognitive behaviour therapy, medication and treatment for associated problems and parent management training. However, there are few effective interventions for early childhood and targeting parenting styles to induce positive parenting is important. Recently, a home-based parenting programme to prevent childhood behaviour problems, which focuses on children when they are still just toddlers, has proven highly successful. This study, the Video-feedback Intervention to promote Positive Parenting and Sensitive Discipline (VIPP-SD), proved successful in reducing behaviour problems in children aged 12 to 36 months as part of the

University of Cambridge and Imperial College London project called 'Healthy Start, Happy Start' (HSHS) (O'Farrelly et al., 2021). This involved a programme of six sessions, each lasting 90 minutes in which carefully prepared feedback is given to parents about how they can build on positive moments when playing and engaging with their child using video clips of everyday interactions, which are filmed by a health professional while visiting their home. As the children were far younger than the age at which interventions for behaviour problems are normally available, these results suggest that providing tailored support for parents of children displaying challenging behaviour at this earlier stage, would significantly reduce the chances of those problems worsening.

Early life stress (ELS) has been shown to influence a change in *FKBP5*, which leads to behavioural changes. This results in lower levels of basal corticosterone in mice and has been shown to have a stronger effect in females; leading to depressive-like behaviours (Criado-Marrero et al., 2020). Female mice with a *FKBP5* knockout have shown a reduction in the basal activity of the HPA axis, with a better recovery when exposed to an acute stressor (Hoeijmakers et al., 2014). The dysregulation of the Hypothalamic pituitary adrenal (HPA) axis due to glucocorticoid receptors has been shown to lead to psychiatric disorders. These disorders include major depressive disorder and generalised anxiety disorder and others (Laryea et al., 2015). Social stressors in infancy affect epigenetic regulation of *NR3C1* (CpG 1-4) altering the endocrine response and result in self-regulation being compromised (Condradt et al., 2015). Oxytocin improves matching facial gestures, social interactions, and prosocial behaviours in infants` development. Facial gestures and social interaction are stunted when infants are subjected to social deprivation, whilst cortisol improves prosocial behaviours (Festante et al., 2021).

1.1 Epigenetic mechanisms

An epigenetic process involves a series of steps that enable changes in heritable phenotypes (Kim, Samaranayake, and Pradhan, 2009). This process modifies gene expression, but it does not alter DNA sequences (Hernando-Herraez et al., 2015). Epigenetic modifications are triggered by

external stimuli such as ELS, chemical exposure, diet, and drug use (Gottschalk and Domschke, 2016). The three main types of epigenetic modifications include DNA methylation, histone modification, and noncoding RNA modification (Gottschalk and Domschke, 2016). Among the epigenetic modifications, DNA methylation is the most studied in literature as it plays a role in activating or silencing stress responses in humans and mice (Meloni, 2014).

1.2 DNA methylation

DNA methylation refers to the attachment of a methyl group to the five positions on the pyrimidine ring to change gene expression (Zhang et al., 2013). This attachment is common with a two DNA base sequence - cytosine followed by a guanine, also known as CpG sites (Tyrka, Ridout, and Parade, 2016). The addition of a methyl group to the cytosine transforms the pyrimidine ring to 5-methylcytosine.

DNA methylation plays a crucial role in the expression of genetic information. Several studies have shown that DNA methylation at CpG islands influences not only gene expression but also tissue-specific and cancer-formation processes, in addition to ELS (Kulski, 2016; Wilkinson, 2015). DNA methylation at CpG sites is carried out by a group of enzymes known as DNA methyltransferases (DNMTs). There are three main types of DNMTs. The DNMT group obtains the methyl group from S-adenosyl-l-methionine (SAM). The methyl group obtained is added to the fifth carbon of cytosines in CpG dinucleotides to form 5-methyl cytosine. Most methylated cytosines are observed mainly at CpG dinucleotides, while a few of them are observed at non-CpG sites (CpC, CpT, and CpA). The major functions of CpG and non-CpG methylation include gene activation or gene silencing depending on the CpG regions being methylated.

The hypermethylation of CpG sites in the promoter CpG island results in the silencing of genes (Jang et al., 2017). Little is known about the mechanism behind the hypermethylation of promoter CpG islands (Han et al., 2017). Nonetheless, this mechanism is believed to explain the carcinogenesis in human cancers (Zuberi et al., 2021), whilst levels of methylation at CpG sites are an indicator of the onset of major depressive disorder (MDD); suggesting that epigenome

variation, independent to genetic proximal variants may prospectively predict MDD onset (Humphreys et al., 2019). In a normal adult cell, most CpG sites are methylated except in promoter CpG islands. In other words, promoter CpG islands are typically unmethylated. However, promoter CpG regions contain regulatory elements that regulate the transcription of genes. Furthermore, *NR3C1* genetic variation has been evidenced in increased psychotic and cortisol symptoms, alongside depression pathophysiology, whilst DNA methylation at *NR3C1* has been associated with stress reactivity and stressful experiences in life (Palma-Gudiel, 2015). Attwood et al.'s (2011) study highlighted the impact of early life adversity (ELA) on *FKBP5* causing gene silencing, hypomethylation and dysregulation in promoters. When exposed to ELA or stress in early years this increases transcription factor binding sites in *FKBP5*. Dynamic ephrin type B receptor 1 (EphB2–NR1) interaction enhances N-Methyl-D-asoarate (NMDA) receptor current, which results in *Fkbp5* upregulation, as it is the *Fkbp5* gene that promotes anxiety development (Attwood et al., 2011). In addition, Zannas et al. (2016) indicated that a modulator of glucocorticoid signalling, the co-chaperone interacts with multiple steroid receptors. ELA that can lead *FKBP5* to hypomethylate; intron 7. The observation in childhood GR response element binding to GR causes demethylation of CpGs. The T allele has also shown dysregulation in GR resistance and transcription regulation (Zannas et al., 2015).

1.2.1 *OXTR*

OXTR is used to provide a connection between mother and child's development of behavioural and social skills during early years. Exon 1 of *OXTR* has been shown to be epigenetically regulated by DNA methylation (Baker et al., 2017). The environment has an impact on how the methylation develops, especially in infants who experienced adversity (Rama-Fernandez et al., 2021).

1.2.2. *FKBP5*

FKBP5 a co-chaperone affects the activity of the glucocorticoid receptor (GR) protein, which is involved in the regulation of the stress response in the Hypothalamic pituitary adrenal (HPA) axis . If there is an alteration in the methylation due to the environment, the stress response will be

affected, leading to the development of mood disorders or an adaptive response of robustness. When exposed to stressors *FKBP5* undergoes an epigenetic change in the chromatin, eventually changing . Changes in the chemical structures of DNA then force the DNA to reduce the production of methylation. The hormone cortisol needed for the stress response is impacted as a result. *FKBP5* intron 7 has been shown to involve GR response elements and a connection between the transcription start site and intrinsic are enhanced. Demethylation has been shown to be initiated by ELA (Geranton, 2019; Wiechmann, 2019).

1.2.3. *NR3C1*

NR3C1 1F is hypermethylated in those who experienced ELA. This suggests it is used in emotional regulation, especially through the HPA axis. CpG sites can dictate the outcomes of stress response, affecting the symptoms of depressive mood disorders (Makusic et al., 2020). The levels of methylation of *NR3C1* and maternal care affect the outcome of an infant's stress response. It affects how the cortisol reacts, which is used to cancel the HPA axis stress response (Conradt et al., 2019). Abuse inhibits the stability in the methylation in the gene, as it dysregulates the stress related causing hypermethylation (Cicchetti and Handley, 2017). A reduction in methylation has been shown to cause major depressive symptoms in life and a change in the epigenetics of the hippocampus causing social changes (Na et al., 2014).

1.3 The HPA axis

The HPA axis is at the heart of epigenetic mechanisms, and gene expression controls the HPA axis, which then controls the stress response (Tyrka, Ridout, and Parade, 2016). It has been found to play a central role in regulating stressors (Criado-Marrero et al., 2020). Consisting of the hypothalamus, pituitary gland, and adrenal cortex, the HPA axis integrates neuronal and endocrine feedback through a cascade of events to control a wide array of stress stimuli (Meloni, 2014). The physiological control, however, is mediated by the negative feedback obtained from

glucocorticoids or cortisol in the human body (Meloni, 2014). In other words, the stimulation of the HPA axis by external stimuli leads to the release of glucocorticoids or cortisol in the human body (Tyrka, Ridout, and Parade, 2016). GR (i.e., *NR3C1* and *FKBP5*), which are distributed throughout the brain and body, bind to cortisol to impede neuroendocrine responses. This inhibition helps neutralise prolonged stress effects in humans (Laryea et al., 2015).

Research reveals that stimulation of glucocorticoids (*NR3C1* and *FKBP5*) by stress regulatory genes can enable or inhibit the activation of the HPA axis (Doom, Cicchetti, and Rogosch, 2014). However, abnormal activation of the HPA axis may undermine a person's ability to respond to stressors effectively (Doom, Cicchetti, and Rogosch, 2014). Literature has shown, for instance, that HPA-axis dysfunction could result in a counter-regulatory response to chronic stress (Bosch et al., 2012). The dysfunction of the HPA axis has been linked to ELS or childhood adversity (Doom, Doyle and Gunnar, 2017). In some experiments, individuals who experienced moderate to severe ELS had an abnormal cortisol activity (Braquehais et al., 2012; Ruttle et al., 2011). In another study, children with a history of emotional abuse exhibited blunted cortisol responses (Tryka et al., 2012). These findings suggested that ELS posed a significant risk to HPA functioning.

OXTR is important for the mother-infant bond, however, if maternal depression is developed during pregnancy, the stress is passed from mother to foetus. *OXTR* is then dysregulated and the foetus HPA axis is primed for stress. This results in dysregulation of the mother-infant bond and the baby-HPA axis becomes further dysregulated (Pariante, 2014). Avoidance behavioural traits may develop in a young adult when a dysregulation in the *OXTR* levels occurs as a result of dysfunction of the HPA axis when exposed to a stressor (Ein-Dor et al., 2018). Prenatal adversities affect the maternal behaviour and childbirth, in addition to the wellbeing of the mother and infant, indicated by reduced methylation of OXTR (Unternaehrer et al., 2015).

1.4 Impacts of childhood adversity on epigenetic modification.

Childhood adversity or ELA has been linked to mood and mental disorders (Borghol et al., 2011). Research has shown that adverse childhood experiences and stress could enable epigenetic modifications through the activation of the HPA axis (Labonté et al., 2012). An exposure to ELS stimulates the release of CRF (corticotropin-releasing factor) and ACTH (adrenocorticotropic hormone) to finally produce glucocorticoids (Borghol et al., 2011). Depending on the level of ELS, glucocorticoids induce the expression of different genes including *OXTR, FKBP5, NR3C1, SLC6A4*, and *BDNF*. These protein-coding genes can alter the chromatin architecture by enabling or disabling methylation at CpG sites (Brenet et al., 2011; Moore, Le, and Fan, 2013). They have variable effects on cellular control, gene silencing, and genomic imprinting (Raftopoulos et al., 2015). Evidence suggests that low levels of DNA methylation enhance gene transcription, while high levels of DNA methylations are associated with epigenetic silencing (Hernando-Herraez et al., 2015). Below, we examine the impact of these genes on childhood adversity.

1.4.1 Methylation of *NR3C1* gene and childhood adversity

The *NR3C1* 1F gene has been linked to childhood adversity. Located on chromosome 5q31–32, this protein-coding gene has nine untranslated exons 1 variants and eight translated exons (Kosten and Nielsen, 2014). Exon 1 transcripts have the ability to adjust GR levels by differentially regulating RNA stabilities and translation efficiencies (Turner, et al., 2016). To understand the underlying mechanisms associated with *NR3C1* methylation and environmental exposures, researchers relied mainly on animal models. Low levels of maternal care (grooming and licking) in rodents were linked to a high level of *Nr3c1* methylation in the cerebellum and hippocampus (Kosten and Nielsen, 2014). Elevated methylation of *,Nr3c1* in rats, however, interferes with gene expression (Armstrong et al., 2014). When methylation of *Nr3c1* occurs at the CpG sites, there are associated reductions in GR-mediated negative feedback and GR levels, both of which have been linked to stress behaviours (Zhang et al., 2013).

In humans, there is a link between ELS and *NR3C1* methylation at alternate first exons (Braithwaite et al., 2015; Kertes et al., 2016). A survey of individuals with no history of psychopathology indicated ELS was associated with increased *NR3C1* methylation at exon 1F regions (Tyrka et al., 2012). Similar results have been found among children. In an experiment, impoverished pre-school-aged children showed greater DNA methylation levels of *NR3C1* at exon 1F regions than pre-schoolers exposed to little or no childhood adversity (Tyrka et al., 2015). However, in a study, this association was found only in male infants who received low maternal care (Braithwaite et al., 2015). In the vast majority of the studies published in the literature, a positive correlation was found between adversity exposure of adolescents and *NR3C1* methylation at exon 1F, 1B, 1H, and 1D (van der Knaap et al., 2014) shown in figure 1 below. Although the role of each of these exons in the stress response remains obscure, the vast majority of these studies indicated that *NR3C1* methylation at exon 1F regions could be induced by anxiety or depression.

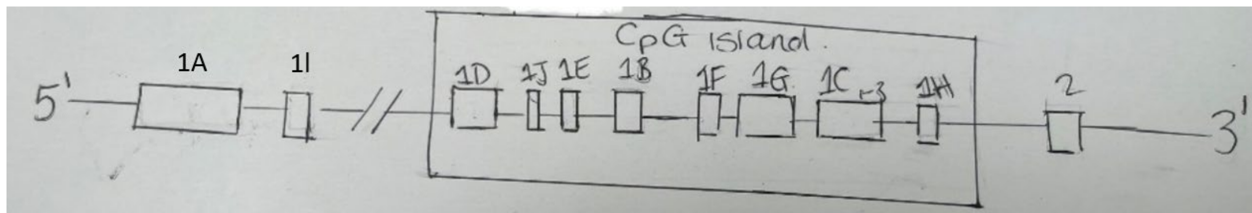


FIGURE 1 NR3C1 EXON AND CPG ISLAND

Furthermore, the literature suggests that *NR3C1* methylation could enhance the risk of maladaptive behavioural outcomes in children. Researchers have hypothesised that, in children, the mechanisms underlying the development of depression, anxiety, and mental disorders could be traced to *NR3C1* methylation (Paquette et al., 2015). Emerging work showed that elevations of *NR3C1* methylation were prevalent among new-borns and adolescents with personality disorders (van der Knaap et al., 2015). This observation is not peculiar to children or adults. Animal models also demonstrated that *NR3C1* methylation was associated with affective disorders in rodents (Pan et al., 2014). Overall, findings suggested that the *NR3C1* gene could be induced by ELS to regulate behavioural outcomes via the HPA axis.

It has been shown that an increase in DNA methylation of *NR3C1* 1F occurs in males across 10 CpG sites when the infants are exposed to maternal prenatal depression but no significance with females was observed (Braithwaite et al., 2015).

1.4.2. Methylation of *FKBP5* gene and childhood adversity

In addition to *NR3C1*, *FKBP5* has been found to regulate GR signalling and stress responses. This protein-coding gene is susceptible to a single nucleotide polymorphism (SNP), which improves GR's ability to bind to the CpG sites and introns 2, 5, and 7 (Zannas and Binder, 2014). By binding to the glucocorticoid response elements, *FKBP5* modulates and impairs the sensitivity of cortisol (Zannas and Binder, 2014). The methylation of the *FKBP5* gene at the intron 7 CpG site, however, alters the amount of GR imported into the nucleus (Klengel et al., 2013). This genetic alteration has been discovered to have a strong link to stress-related behaviours such as depression, PTSD, anxiety, and suicide (Zannas and Binder, 2014).

The relationship between ELS and *FKBP5* methylation in relation to children has been examined (Klengel et al., 2013; Keijser et al., 2021). However, results in the literature have been conflicting, at best. Researchers found that a negative association existed between childhood maltreatment and this gene's methylation (Tyrka et al., 2015). In a study of pre-schoolers with ELS, children with a history of ELS had lower levels of *FKBP5* intron 7 methylation in the DNA collected from saliva samples (Tyrka et al., 2015). This finding, however, was not in agreement with the research outcomes that showed that higher levels of ELS were associated with low methylation levels of *FKBP5* (Needham et al., 2015). It is possible that there are other factors that increase FKBP5 methylation apart from ELS. A study showed that *FKBP5* methylation was more pronounced in Holocaust survivors than in non-Holocaust survivors. However, the adult offspring of the Holocaust survivors showed low levels of *FKBP5* methylation compared to that of non-Holocaust survivors (Yehuda et al., 2016). This finding has also raised more doubts over the link between *FKBP5* methylation and childhood adversity (Tyrka, Ridout, and Parade, 2016).

More research efforts are needed to investigate how ELS influences *FKBP5* methylation, including the variants of the gene involved in the methylation. Methylation of *FKBP5*, nonetheless, has been linked to stress-related behaviours (Klengel et al., 2013; Zimmermann et al., 2011). People with maladaptive behavioural outcomes showed lower levels of *FKBP5* methylation compared to those with no history of childhood trauma (Klengel et al., 2013). For example, Individuals with bipolar disorder had elevated levels of cortisol and FKBP5 methylation compared to those without this mental health problem (Fries et al., 2015). These studies suggest that *FKBP5* methylation could be the underlying link between ELS and behavioural health outcomes.

1.4.3 Methylation of *OXTR* gene and childhood adversity

Oxytocin is a neuropeptide and peptide hormone produced in the hypothalamus and secreted by the pituitary gland. Current studies show that OXTR polymorphism not only influences HPA functioning but also controls social bonding, social recognition, sexual activity, maternal care, and relationship behaviours (Norman et al., 2012). In humans, oxytocin could minimise anxiety and enhance emotional control (Kubzansky et al., 2012). Studies reported that the greater the oxytocin levels in the body, the more depression could be reduced (Kubzansky et al., 2012). However, the artificial administering of oxytocin to depressed individuals did not result in prosocial behaviours (Olff et al., 2013). By understanding the molecular mechanism underlying *OXTR* methylation, patients with depression and anxiety could be treated more effectively (Tyrka, Ridout, and Parade, 2016).

Currently, studies that explored the relationship between childhood adversity and *OXTR* methylation are limited in literature (Cecil et al., 2014; Simons et al., 2017; Unternaehrer et al., 2015). There is a link between methylation of *OXTR* and childhood adversity. A study, for instance, found that children raised by non-biological parents had lower levels of *OXTR* methylation than those raised by their biological parents (Simons et al., 2017). In humans and rodents, maternal care had a positive association with *OXTR* methylation (Needham et al., 2015). A recent study of 309 African Americans provided more insights. The research findings revealed that childhood adversity was indirectly associated with *OXTR* methylation (Kogan et al., 2019).

However, research linking ELS with *OXTR* methylation has been inconsistent (Cecil et al., 2014; Gouin et al., 2017). While some studies reported that proximal forms of stress influenced methylation of *OXTR* (Simons et al., 2017), others documented those distal forms of stress affected this gene's epigenetic modification (Unternaehrer et al., 2012). Nevertheless, whether *OXTR* methylation can be induced by external stimuli, stress-related genes, or both remains unclear.

1.5 Methylation changes and parenting

Early life experience of parents has been shown to indicate transgenerational inheritance, especially through the maternal line from mothers to their children. For example, one study showed that offspring of Holocaust survivors showed different epigenetic regulation at the *FKBP5* intron 7 gene X environment (GXE) interaction that affects cortisol levels (Yehuda et al., 2016). In addition, it has been found that *NR3C1* and *FKBP5* genes can be epigenetically altered in a transgenerational way influenced by the mother's trauma (Van Aswegen et al., 2021). Childhood adversity affects the DNA methylation of the child and has been shown to be dependent on whether the child had been exposed to physical or sexual violence (Dunn et al., 2019). A study undertaken by Bryant et al. (2017) examined the effects upon children separated as a consequence of the Australian bush fires in 1983; this led to the development of attachment problems and symptoms associated with post-traumatic stress disorder (PTSD) later in adulthood (Bryant et al., 2017).

1.6 Aims and hypothesis

Therapies to reduce child's stress through targeting parental behaviour includes the Health Start Happy Start (HSHS) video feedback interventions study (O'Farrelly et al., 2021), which looks at how the intervention of parental behaviour can positively change the child's behaviour. The video feedbacks and interviews show how the parent/caregiver can change their behaviour by identifying the problematic behavioural traits of the caregiver that the child then develops. The HSHS study shows how early intervention reduces the problematic behavioural traits by changing parental interaction with their children (O'Farrlley et al.'s (2021). The video intervention study

has also presented that the mental health checker preschool prenatal accounts of childrens symptoms (PPACs) showed that the video intervention supports children's behaviour positively. The aim of the study were to determine if changes in epigenetic regulation of stress genes including *OXTR, NR3C1* and *FKBP5* was influenced by the video intervention to promote positive feedback (VIPP) treatment and if this correlated with changes in child behaviour child behaviour.

Specifically, our research questions and hypotheses were:

RQ1: Are levels of DNA methylation at the *OXTR, FKBP5* and *NR3C1* gene different for children who have received the VIPP intervention compared with those who have not?

Hypotheses: DNA methylation at the *OXTR, FKBP5* and *NR3C1* decrease in children exposed to VIPP intervention

RQ2: Does a degree of child behaviour change from TP 1 (pre-intervention) to TP 2 (2 year follow up) associated with percentage DNA methylation.

Hypotheses: Lower DNA methylation, as a result of intervention, correspond to improved child behaviour.

2 Methods: The HSHS study

This study was a 2-group, multisite randomised study conducted through six National Health Service trusts in England. Baseline and 5-month follow-up data were collected between July 30, 2015, and April 27, 2018. Of 818 eligible families, 227 declined to participate, and 300 were randomised into the trial. Target participants were caregivers of children who scored in the top 20% for behaviour problems on the Strengths and Difficulties Questionnaire. Participants were randomly allocated on a 1:1 basis to receive either VIPP-SD (VIPP- sensitive discipline) (n = 151) or usual care (n = 149), stratified by site and number of participating caregivers. Families allocated to VIPP-SD were offered six home-based video-feedback sessions of 1 to 2 hours' duration every 2 weeks.

Main measures included the PPACS, which is a structured interview of behaviour symptoms, designed to capture clinical phenotypes relating to hyperactivity, especially attention deficit hyperactivity disorder (AD/HD) and Hyperkinetic Disorder along with other related childhood psychiatric disorders (Chen and Taylor., 2006). Secondary outcomes included caregiver-reported total problems on the Child Behaviour Checklist (CBCL) and Strengths and Difficulties Questionnaire (SDQ) - both measure emotional and behavioural problems in children and adolescents (Mansolf et al., 2022). Child and parent demographics are provided in **Tables 1 and 2**.

TABLE 1 CHILD DEMOGRAPHICS

		Total Sample				Intervention group				Control group			
	Variable	**N**	**%**	**Mean**	**Range**	**N**	**%**	**Mean**	**Range**	**N**	**%**	**Mean**	**Range**
Demographic variables	Age at T3	286		47.85	7.32	142		47.9	29.63	144		47.83	35.13
	Gender												
	Female	137	45.7			75	49.7			62	41.6		
	Male	163	54.3			76	50.4			87	58.4		
	Ethnicity												
	White	195	65			98	64.9			97	65.1		
	Mixed	58	19.3			33	21.9			25	16.8		
	Asian or Asian British	15	5			8	5.3			7	4.7		
	Black or Black British	13	4.3			3	2			10	6.7		
	Other	9	3			3	2			6	4		
Child Behaviour variables (rated by primary caregiver)	Behaviour - PACS T1	298		31.33	64	150		31.8	53.6	148		30.88	64
	Behaviour - PACS T2	286		27.93	58	140		27.2	49	146		28.67	58
	Behaviour - PACS T3	282		22.75	55.4	140		21.8	46	142		23.67	55.4
	Behaviour - CBCL T1	296		39.13	114	149		31.8	114	147		40.71	102
	Behaviour - CBCL T2	282		32.4	100	139		27.2	89	143		34.67	100
	Behaviour - CBCL T3	282		30.54	141	140		21.8	141	142		33.07	127
	Behaviour - SDQ T1	299		13.1	29	150		13	27	149		13.23	28
	Behaviour - SDQ T2	283		11.12	27	139		10.8	23	144		11.46	11.46
	Behaviour - SDQ T3	283		10.11	28	140		9.85	26	143		10.36	10.36

Abbreviation= T=timpoint, %= percentage, N= number of participants, PACS= preschool parental account of childrens symptoms, CBCL= child behaviour checklist, SDQ= strengths and difficulties questionnaire.

Table 2 Primary caregiver DemographicsT=Timepoint, %= percentage, N=number of

		Total Sample				Intervention group				Control group			
	Variable	**N**	**%**	**Mean**	**Range**	**N**	**%**	**Mean**	**Range**	**N**	**%**	**Mean**	**Range**
Demographic variables	Age	300		34.18	34	151		33.66	28	149		34.72	34
	Gender												
	Female	276	72			138	91.4			138	92.6		
	Male	13	4.3			8	5.3			5	3.4		
	Ethnicity												
	White	216	72			16	10.6			10	6.7		
	Mixed	22	7.3			10	6.6			10	6.7		
	Asian or Asian British	29	9.7			37	24.5			34	22.8		
	Black or Black British	14	4.7			32	21.2			39	26.2		
	Other	10	3.3			55	36.4			55	36.9		
	Highest Educational Qualification												
	Pre-GCSE	26	8.7			16	10.6			10	6.7		
	GCSE	20	6.7			10	6.6			10	6.7		
	College	71	23.7			37	24.5			34	22.8		
	Undergraduate degree	71	23.7			32	21.2			39	26.2		
	Postgraduate degree	110	36.7			55	36.4			55	36.9		
Mental health variables	Anxiety (GAD) T1	279		4.6	19	140		4.51	16	139		4.69	19
	Anxiety (GAD) T2	267		4.1	30	130		4.18	30	137		4.03	30
	Anxiety (GAD) T3	265		3.98	20	132		1.83	19	133		4.12	20
	Depression (PHQ) T1	292		4.48	26	146		4.58	26	146		4.39	25
	Depression (PHQ) T2	268		2.97	26	131		3.85	26	137		4.07	22
	Depression (PHQ) T3	267		4.12	47	132		4.14	47	135		4.1	38

participants, GAD= generalised anxiety disorder, PHQ= patient health questionnaire,

2.1 Ethics

Ethics were approved for the study at the Manchester Metropolitan University (MMU), “Nature via Nurture: Does parenting influence genetic regulation in young children?” (Ref; 10452) on 29th April 2019. Professor Paul Ramachanni provided the saliva samples from university of Cambridge.

2.2 Extraction of DNA from infant passive drool samples

Saliva samples were collected during the study and sent to MMU. These contained around ~ 200 µl volumes. These were extracted using the Qiagen DNA extraction kit using spin columns. The samples were transferred to an individual 1.5 ml tube to which was added protease K (20 µl) and AL buffer (200 µl). This was vortexed (15 sec) and incubated at 56 °C whilst being shaken (10 mins) to lyse the cells and release the nucleic acids. To enhance and influence the binding of nucleic acids to the silica spin columns, 200 µl of pure ethanol was added to the solution, which was then vortexed for 15 sec. The solution was then transferred to a DNA spin column and

centrifuged at 10,000 rpm for 1 min, taking care to ensure all the lysate had passed through. The liquid passed through the filter was then discarded and the DNA will be bound to the column. In order to remove residual cellular proteins and salt from the membrane a series of washing buffers and steps were performed; AW1 buffer (500 µl) was then added to the spin column and centrifuged at 10,000 rpm for 1 min, and the supernatant discarded; next AW2 buffer (500 µl) was added to the spin column and centrifuged (10,000 rpm for 1 min); this was followed by AW2 buffer (300 µl) again added to the spin column and centrifuged (10,000 rpm for 1 min). This empty spin column was then centrifuged again (13,000 rpm for 2 min) in order to dry the membrane and remove any residual wash buffer and ethanol. The top of the spin column was then placed on a fresh clean a 1.5 ml tube, and the DNA was eluted from the membrane to which was added 50 µl of AE buffer, incubating at room temperature for 1 min and centrifuging at 10,000 rpm for 1 min. The spin column sample solution was then re-eluted and centrifuged once again at a higher speed of 13,000 rpm for 2 min.

The eluted DNA was then measured for concentration and purity. This was done using a Nanodrop (Thermo, UK) and measuring wavelengths for 260 nm/280 nm and 260 nm/230 nm. DNA samples were then stored at -20°C.

2.3 Sodium bisulphite modification of DNA

The Epitect DNA bisulphite 96 well kit (Qiagen, UK) was used to sodium bisulphite treat DNA. 500ng of DNA was made up to 20 µl with RNase-free water accordingly (see **Appendix 1** for volume required for 500ng DNA) in a 96 well PCR plate. Sodium bisulphite solution (85 µl) was added to each of the wells followed by 35 µl of DNA buffer and mixed by pipetting. The 96 well plate was then sealed using polymerase chain reaction (PCR) film (Thermo, UK), briefly centrifuged and placed in an Eppendorf thermocycler with the following conditions in **Table 3**.

TABLE 3: PCR PROGRAMME FOR SODIUM BISULPHATE CONVERSION

Step	Time	Optimised temperature
Denaturation	5 minutes	95°C
Incubation	25 minutes	60°C
Denaturation	5 minutes	95°C

Incubation	85 minutes	60°C
Denaturation	5 minutes	95°C
Incubation	175 minutes	60°C
Hold	Indefinite	20°C

Following completion of the programme, the DNA was added to 560 µl of BL buffer and added to the 96 well Epitect plate. Aitpore tape was then used to seal the plate, which was then placed on a solute collection plate and centrifuged at 3250 g for 1 minute and eluate disposed. The spin column was washed with 500 µl of BW buffer that was then added and centrifuged at 3520 g for 1 min. Desulphonation was next performed by adding 250 µl BD buffer and incubating at room temperature for 15 min followed by centrifugation at 3250 xg for 1 minute. BW buffer (500 µl) was then added again and centrifuged at 3250 xg for 1 min and washed for final time with 250 µl for pure ethanol) (and centrifuged at 3250 xg for 1 min followed by a for 15 minute at 3250 g to remove residual ethanol. DNA was eluted from the plate by adding 50 µl EB buffer to the wells on the Epitect plate that was placed onto a new elution plate and centrifuged at 3250 g for 1 min. This bisulphite treated DNA was stored at -20 ^{o}C until used for PCR.

2.4 Bisulphite PCR

PCR was performed to amplify the DNA and label with biotin for pyrosequencing. A mastermix was prepared including for each reaction, 4 µL of 5x MyTaq reaction buffer (Bioline, UK) (0.5 µL of forward primer and 0.5 µL of reverse primer (see Table 4 for primer sequences) 0.2 µL MyTaq HS DNA polymerase (Bioline, UK), and 12.8 µL water to make a total solution volume of 18 µL. This solution was vortexed and, 18 µL aliquots were added to each well of a 96 well PCR plate.Table 4: Primers used for FKBP5, OXTR and NR3C1, location and sequence analysed

Gene	Primers. Forward (f), reverse (r),	Location	PCR size (bp)
NR3C1	F-(Biotin)AATTTTTTAGGAAAAAGGGTGG R-AACCCCTTTCCAAATAACACACTT	hg19; chr5:142,783,610-142,783,671	343

FKBP5	F-GGATTTGTTGGGATAATAATTTTGGG R-(Biotin)TCTTACCTCCAACACTACTACTAAA	Chr6: 35,558,486– 35,558,567	324
OXTR	F- GGGGGGAGTTAATTTTAGGTT R-(Biotin)CTCAATCCCCAAAAATCTTTACAATCT	hg19:Chr:3:8,810,807- 8,810,808	330

Then outside of the PCR hood (on the bench), DNA (2 µl) was added to each of the 96 well plates, then placed into an Eppendorf thermocycler. The PCR cycle shown in **Table 5** for the different primers.

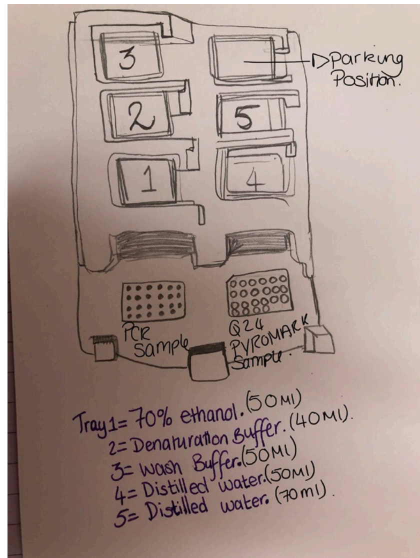
TABLE 5: THE TEMPERATURE AND TIMINGS OF PCR CYCLES FOR *NR3C1*, *FKBP5* AND *OXTR* GENES

Gene	Cycle
NR3C1	94 °C, 1 minuate; 60 °C, 1 minuate; 72°C, 1minute. 50 cycles
FKBP5	94 °C, 1 minute; 60 °C, 1 minute; 72°C1 minute, 50 cycles
OXTR	94 °C, 1 minute; 57 °C, 1 minute; 72°C1 minute, 50 cycles

Electrophoresis of the PCR products was performed to confirm whether the PCR reaction had worked regarding whether there is the correct band size, amount of product and whether there are primer dimers and specific single products. The procedure for producing a 2% agarose gel involved mixing 2 g agarose powder to 100 ml of 1x TBE buffer and microwaving for approximately 1 min until melted. 3 µl of SYBR™ Safe DNA Gel Stain (Thermo Fisher Scientific, UK) was then added and the agarose mixture was poured into a gel chamber with a comb.

2.5 Bisulphite Pyrosequencing

The Pyromark Q24 pyrosequencer (Qiagen, UK) was used to pyrosequence the bisulphite PCR products of the *NR3C1, FKBP5* and *OXTR* genes from the samples. The pyrosequecner was turned



on and the required solutions were then placed into the workstation. **Figure 2** shows the set-up of the PCR plate to PyroMark Q24 plate.

FIGURE 2: SCHEMATIC OF THE WASH STEPS FOR THE PYROMARK Q24

Pyromark q24 advanced software was opened and a new run file was created and the assay file identifying the sequence was loaded.

A master mix was prepared as seen in **Table 6,** 60 µl of which was then pipetted into each of the 24 well plates. 20 µl of PCR sample was then added to each well and covered with a sealing film. The plate was then placed on the shaker (taped on) and shaken at 1400 rpm for 10 min to allow the biotin labelled DNA to bind to the Sepharose beads.

TABLE 6: MASTER MIX PREPARATION

	One reaction	24 reactions plus 10% for error
Sepharose beads	1 µl	26.4 µl
Binding buffer	40 µl	1056 µl
PCR grade water	21.25 µl	510 µl

THE VACUUM PUMP WAS THEN TURNED ON APPLIED TO THE PCR SAMPLES TO ALLOW THEM TO ADHERE TO THE PUMP FILTERS THROUGH THE SEPHAROSE BEADS. THESE WERE THEN ADDED TO 70% ETHANOL TO WASH OFF UNBOUND DNA AND PRIMERS. THEN THIS WAS ADDED TO DENATURATION BUFFER TO RENDER THE DNA SINGLE STRANDED AND FINALLY ADDED TO WASH BUFFER TO REMOVE THE DENATURATION BUFFER. THE VACUUM WAS THEN SWITCHED OFF AND THE FILTERS PLACED IN A NEW PLATE CONTAINING ANNEALING BUFFER AND A SPECIFIC SEQUENCING PRIMER TO THE GENE OF INTEREST (TABLE 7). THIS WAS THEN TRANSFERRED TO A 85 °C HEATED PLATE AND LEFT THERE FOR 2 MIN TO DENATURE THE DNA AND REMOVE THE BINDING TO THE SEPHAROSE AND ALLOW EFFICIENT BINDING OF THE SEQUENCING PRIMERS. THE REQUIRED SEQUENCING AGENTS INCLUDING THE 4 DEOXYNUCLEOTIDE TRIPHOSPHATE (DNTPS) THE ENZYME AND SUBSTRATE MIXES WERE THEN LOADED INTO THE SEQUENCING CARTRIDGE AND PLACED TOGETHER WITH THE REACTION PLATE, CONTAINING THE DNA AND PRIMERS, INTO THE PYROSEQUENCERS AND THE RUN STARTED.

TABLE 7 SEQUENCING PRIMERS AND SEQUENCES TO BE ANALYSED

Gene	Sequencing primers.	Sequence to be analysed (CpGs numbered) 5'–3'
NR3C1	AACTCCCCAATAAATCTAAAAC	CR(CpG1)CR(CpG2)AAACTAAACR(CpG3)AAAACR(CpG4) AAAAAAAAATAAC
FKBP5	GGAGTTATAGTGTAGGTTT	TTTY(CpG1)GTGATTTTTGTGAAGGGTATAATTY(CpG2) GTTTAGTTTTGAAAAG
OXTR	ATTTATTTGTTAAGGTTTTGGATAA	TTTTGTTTTTGGAGGAG

2.6 Statistical analysis

Statistical analyses were conducted using SPSS version 26. For each analysis, $p < 0.05$ was regarded as statistically significant and graphs were produced using Microsoft Excel and SPSS. A statistical analysis plan was drawn up:

To address Research Question 1 t-test were used where the dependent variable is the percentage methylation, and the independent variable is the group (VIPP/control). The four t-tests independent variables were *NR3C1/ FKBP5* mean percentage methylation of CpG1 and CpG2, *OXTR* CpG1 and *OXTR* CpG2.

Research Question 2 was addressed by showing the correlations between TP one to TP 3 the percentage methylation with PACS, SDQ and CBCL through graphs. TP1 was when the child was

five to 24 months, TP2 was five month follow up and TP3 was a 24 month follow up. For the calculation of the change in score by TP2 orTP 3 minus TP 1.

Four linear regressions following independent (or outcome) variables: - *NR3C1* mean percentage methylation of CpG1 and CpG2, *FKBP5* mean percentage methylation CpG1 and CpG2, *OXTR* CpG 1 and *OXTR* CpG2. Confounders would be entered in block one of the model: - primary caregiver age, primary caregiver ethnicity, primary caregiver gender, primary caregiver education, primary caregiver depression (PHQ) at timepoint three, child age at TP three, and child gender. Block two then entered SDQ and compared.

2.6.1 methods used on SPSS IBM version 26

To analyse the statistics the following SPSS analysis was used; descriptive statistics, skewness and kurtosis, T-test, one way ANOVA, and stepwise regression.

Slips file was used to compare the VIPP and control.
Descriptive statistics methods used were: - select analyse, then descriptive statistics, then descriptive. A box opens which then transfers the necessary variables across to the description area. Once that was done, for more information to be added, options were selected and selected the necessary options and selected ok. Once completed pressed ok.
For skewness and kurtosis; analyse was selected, then the descriptive statistics and descriptive. Then transfered variables over and press options and selected skewness and kurtosis. Pressed selects again.
For the analysis of T-test, analyse seleceted, then compare means, then independent t-test. Selected define group and then continue.
For stepwise regression, analyse the regression and then linear, and transfer the independent and dependent variables across and pressed ok.
To create a histogram; graph selected then legacy dialogs and Histogram. Then moved variables to the variables. Select “displays normal curve” and clicked OK.
Square root transformation; transformed then compute variable. When then the table comes up the data was selected; “ALL” to function group box; scrolled down to function and specials

variables box andclicked “lg10”. Transfered the data across to the numeric expression. Once completed pressed ok and the new data will be added as a new column.

Finally for split file, started by going to date, split file and then selected ‘organise output by groups’. Moved to randomised groups into the groups based on box and selected ok. In this case the data separated to control and VIPP; 0 being control and 1 being VIPP.

3. RESULTS

3.1 Testing and establishing methods for saliva DNA extraction

I used personal saliva samples to establish and standardise the method for DNA extraction. Around 500 µl of passive drool was centrifuged to collect cells and DNA extraction was performed as described earlier (*see DNA extraction method*). This resulted in the following DNA concentrations (shown in **Table 8** below). Samples tested included 1. 1ml of saliva, 2. 0.5ml of saliva, sample 3 – 6 0.2ml of saliva.. this was to determine the variability of the yield.

TABLE 8 RESULTD OF TESTING SALIVA EXTRACTION PROTOCOLS

Sample		Concentration ng/µl	Total amount (µg/ 50ml)	A260/ 280	A260/230
1	1 ml of saliva	91.8	4.6	1.84	0.89
2	0.5 ml of saliva	41.8	2.05	1.86	0.76
3	0.2 ml of saliva	27.3	1.36	1.86	0.69
4	0.2 ml of saliva	9.4	0.47	1.96	0.35
5	0.2 ml of saliva	14.9	0.75	1.95	0.56
6	0.2 ml of saliva	18.6	0.93	1.75	0.5

3.2 DNA extraction from the Happy Start child passive drool samples

Saliva samples from 212 of the 300 mothers in the study who consented to DNA analysis were shipped to the MMU from Imperial College London on ice. These showed some variations in volumes, colours, and viscosities (**Figure 3**).

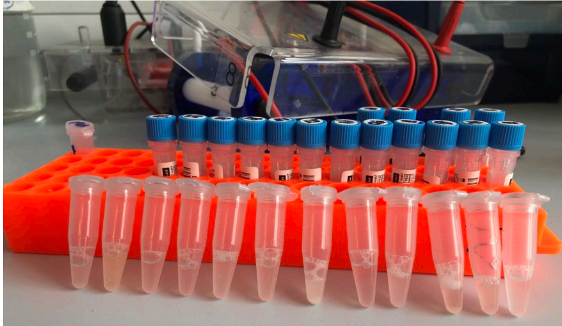


FIGURE 3 AN EXAMPLE OF CHILD PASSIVE DROOL SALIVA SAMPLES FROM THE HAPPY START COLLECTION DEMONSTRATING SOME VARIATION ON VOLUMES AND COLOURS.

As the method allowed reproducible extractions of DNA, the 212 samples from the study were extracted. This was performed in batches of 24 samples at a time per day. Overall, this resulted in an average of 2.308 µg of DNA with a 260/280 value of 1.869 (**Table 9**). Only one of the 212 samples failed to give any reasonable quantity of DNA. See **Appendix 1** for individual sample concentrations.

TABLE 9 AVERAGE RESULTS OF EXTRACTION DNA FROM SALIVA SAMPLES

	DNA Concentration (ng/ µl)	A260/280	A260/230	Total (µg)
Average	46.1195	1.8695	0.9609	2.308
Stand. Dev.	20.0966	0.0754	0.2648	1.007

3.3 establishment of DNA methylation analyses using bisulphite sequencing

3.3.1 DNA methylation of *FKBP5* in child DNA

Two CpG sites in a region of the *FKBP5* promoter previously linked to child maltreatment and behaviour (Klinger-König et al., 2019)) were tested for methylation. **Figure 4** shows the position

of the region in the *FKBP5* gene upstream of the coding region and the position of the two CpG sites.

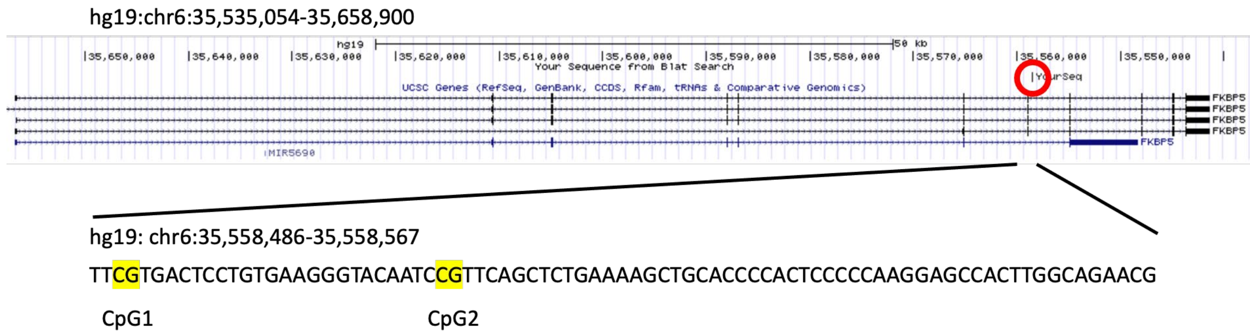


Figure 42 Region of the Human *FKBP5* promoter tested at CpG1 and CpG2

3.3.2 Establishment of *FKBP5* bisulphite sequencing

PCR was performed using 2 µl of bisulphite DNA and the protocol described in the methods. This produced clear bands of a size of 328bp (**Figure 5**).

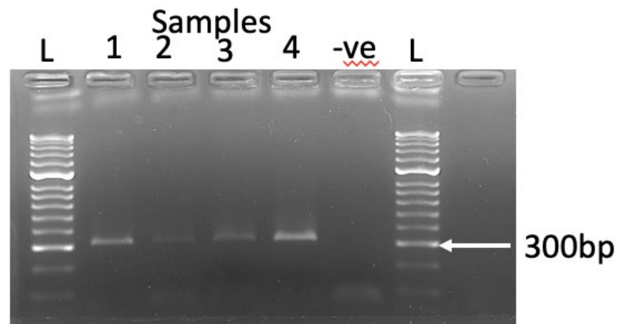


Figure 5 PCR of the region of the *FKBP5* promoter harbouring CpG1 and CpG2 representative agarose gel of four samples and a negative (-ve) and a 50bp DNA ladder (L).3.3.3 *FKBP5* methylation levels across the samples.

Results of the DNA methylation across all the samples showed very high levels of methylation for CpG1 (97.47%) with lower levels of methylation for CpG2 (78.68%). The average methylation across the two CpG sites in each sample was 88.07% (**Figure 6**).

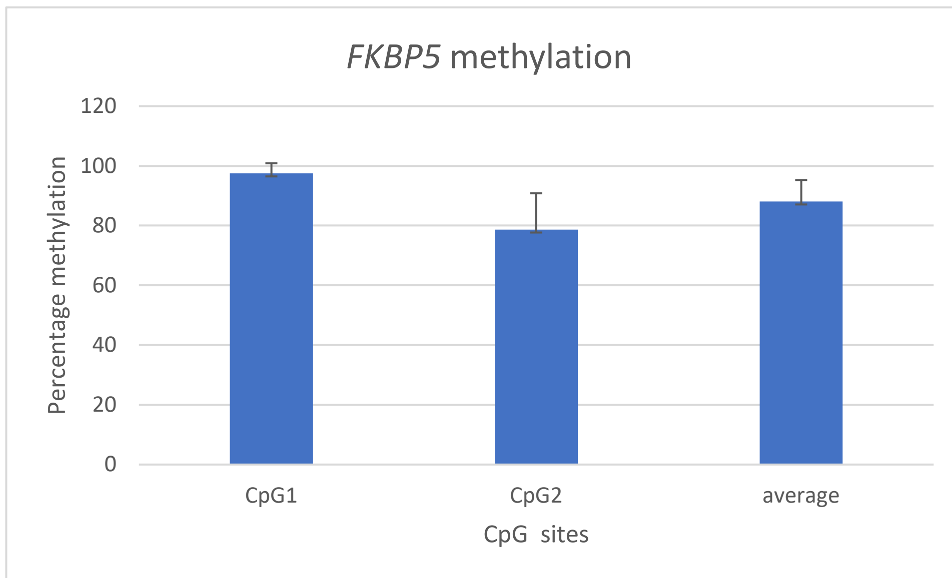


FIGURE 6 A BAR CHART SHOWING LEVELS OF DNA METHYLATION AT CPG1 AND CPG2 AT THE *FKBP5* PROMOTER AND THE AVERAGE METHYLATION OF BOTH CPG SITE (N=211)

3.3.4 DNA methylation at *NR3C1* in child DNA

Four CpG sites in a region of the *NR3C1* promoter previously linked to early childhood adversity and child behaviour (Parade et al., 2016) were tested for methylation. **Figure 7** shows the position of the region in the *NR3C1* gene with alternative untranslated exons, the coding region and the position of the four CpG sites in the promoter region of the alternative untranslated 1st exon 7.

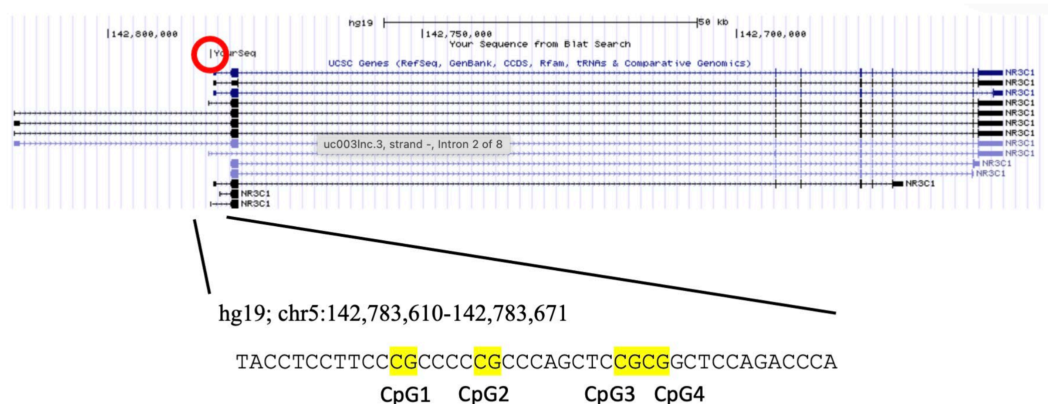


FIGURE 7 REGION OF THE HUMAN *NR3C1* PROMOTER TESTED FOR DNA METHYLATION AT CPG1, CPG2, CPG3 AND CPG4

3.3.5 Establishment of *NR3C1* bisulphite sequencing

PCR was performed using 2 µl of bisulphite DNA and the protocol described in the methods. This produced clear bands of a size of 356bp (**Figure 8**).

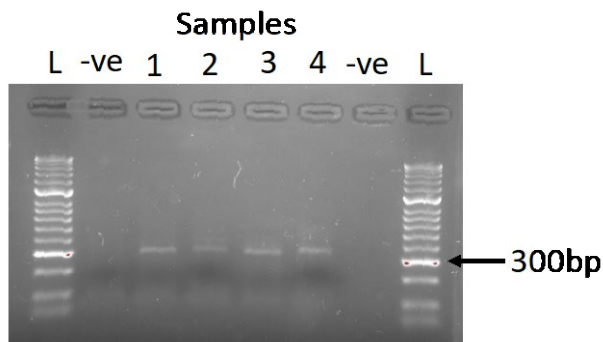


FIGURE 8 PCR OF THE REGION OF THE HUMAN *NR3C1* PROMOTER HARBOURING CPG1 AND CPG2. REPRESENTATIVE AGAROSE GEL OF FOUR SAMPLES AND A NEGATIVE (-VE) AND A 50BP DNA LADDER (L).

3.3.6 *NR3C1* methylation levels across the samples

Results of the DNA methylation across all the samples showed relatively lower levels of methylation than *NR3C1* for CpG1 (8.89%), CpG2 (4.5%), CpG3 (5.6%) and CpG4 (47.2%) (**Figure 9**). CpG sites one and two were focussed on as these have been previously described in relation to child behaviour and stress and to increase statistical power. Not all the samples were able to produce clean PCR bands and reliable results and only 139 of the samples produced values that could be used in the study.

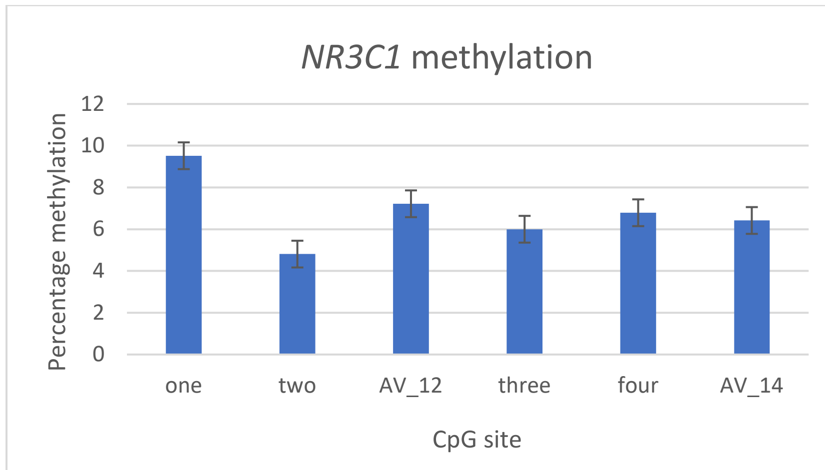


Figure 9 A bar chart showing levels of DNA methylation of CpG1 and CpG2 at the *FKBP5* promoter and the average of both CpG sites (n=139). AV_12 is the average between CpG 1 and CpG2. AV_14 is the avergae between the four CpG sites.

3.3.7 DNA methylation of *OXTR* in child DNA

DNA methylation one CpG site (-934) in a region of the *OXTR* promoter has previously been linked to child conduct disorders (Klinger-konig et al., 2019) were tested for methylation. **Figure 10** shows the position of the region in the *OXTR* untranslated second intron gene upstream of the coding region and the position of the two CpG sites (CpG1 is the -934 site).

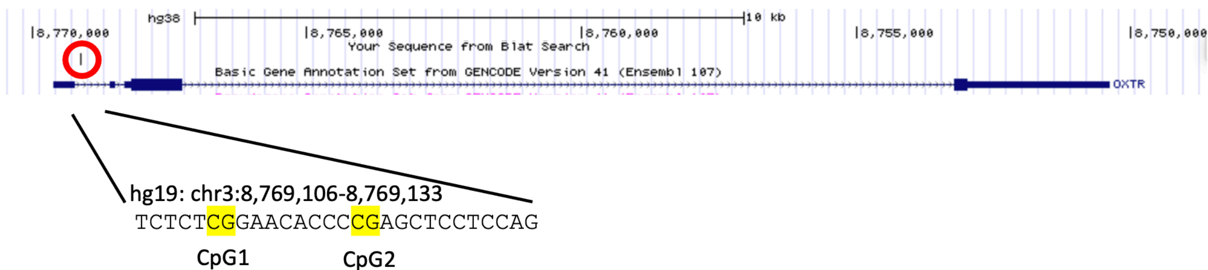


Figure 10 Regions of the Human *OXTR* promoter tested for DNA methylation at CpG1 and CpG2

3.2.8 Establishment of *OXTR* bisulphite sequencing

PCR was performed using 2 µl of bisulphite DNA and the protocol described in the methods. This produced clear bands of a size of 328bp (**Figure 11**).

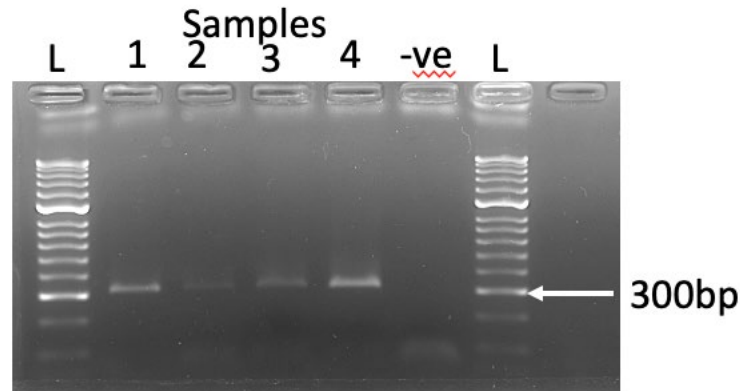


Figure 11 PCR of the region of the Human *OXTR* promoter harbouring CpG1 and CpG2. representative agarose gel of four and a negative (-ve) and a 50bp DNA ladder (L).

3.3.9 *OXTR* methylation levels across the samples

Results of the DNA methylation across all the samples showed levels of methylation at *OXTR* of 47.57% methylation for CpG1 and 69.05% methylation for CpG2 (**Figure 11**).

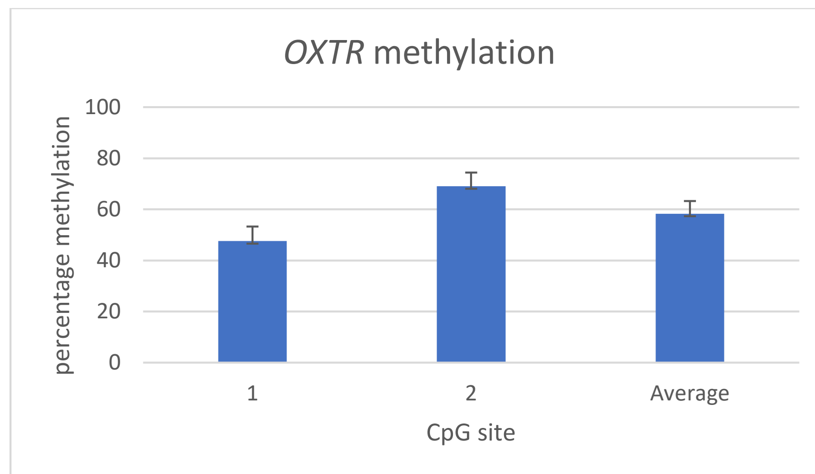


Figure 12 A bar chart showing levels of DNA methylation at CpG1 and CpG2 at the *OXTR* promoter and the average methylation of both CpG sites (n=211).

3.4. Inspecting and testing distribution of DNA methylation data

The PCRs above were run on the Pyromark sequencer as described in the methods to determine levels of DNA methylation at the CpG sites, given as a percentage. The DNA methylation levels for each of the CpG sites at each of the three gene regions across the samples were then checked for skewness and kurtosis. As these are the dependent variables they should be normally distributed for parametric analysis. This revealed that the methylation data are not normally distributed and are all skewed and display kurtosis (**Table 10**).

TABLE 10 DESCRIPTIVE STATISTICS OF METHYLATED CPGS AT *OXTR, NR3C1*, AND *FKBP5* INCLUDING MEAN, STANDARD DEVIATION, SKEWNESS AND KURTOSIS

Descriptive Statistics

	N	Minimum	Maximum	Mean	Std. Deviation	Skewness		Kurtosis	
	Statistic	Statistic	Statistic	Statistic	Statistic	Statistic	Std. Error	Statistic	Std. Error
OXTR CpG1	223	22.215000000000000	66.333333333333330	47.570029895366220	5.437619228759963	-.099	.163	4.486	.324
OXTR CpG2	223	35.345000000000000	83.500000000000000	69.050089686098720	5.350013627341616	-2.094	.163	12.205	.324
FKBP5 CpG1	220	71.000000000000000	100.00000000000000	97.468181818181790	3.711329990255925	-3.202	.164	15.704	.327
FKPB5 CpG2	220	33.000000000000000	98.000000000000000	78.681060606060580	12.126915102362696	-1.371	.164	1.730	.327
NR3C1 CpG1	139	1	95	8.86	12.469	3.365	.206	16.783	.408
NR3C1 CpG2	139	1	31	4.50	4.719	3.177	.206	12.344	.408
NR3C1 CpG3	139	2	56	5.59	7.223	4.715	.206	25.407	.408
NR3C1 CpG4	139	3	83	7.22	7.637	7.611	.206	71.474	.408
Valid N (listwise)	136								

The methylation data were therefore log transformed to normalise the data. However, this revealed that some of the methylation data were still very kurtosed (**Table 11**).

.

TABLE 11 DESCRIPTIVE STATISTICS FOR LOG TRANSFORMED METHYLATION DATA FOR *OXTR, NR3C1* AND *FKBP5* INCLUDING MEAN, STANDARD DEVIATION, SKEWNESS AND KURTOSIS, YELLOW HIGHLIGHTED DATA SHOW KURTOSED VALUES.

Descriptive Statistics

	N	Minimum	Maximum	Mean	Std. Deviation	Skewness		Kurtosis	
	Statistic	Statistic	Statistic	Statistic	Statistic	Statistic	Std. Error	Statistic	Std. Error
OXTRCpG1_Log_EB	223	1.35	1.82	1.6743	.05294	-1.705	.163	12.241	.324
OXTRCpG2_Log_EB	223	1.55	1.92	1.8376	.03834	-3.673	.163	25.553	.324
FKBP5CpG1_log_EB	220	1.85	2.00	1.9885	.01774	-3.701	.164	20.837	.327
FKBP5CpG2_log_EB	220	1.52	1.99	1.8896	.07846	-1.982	.164	4.685	.327
NR3C1CpG1_log_EB	139	.00	1.98	.6629	.47932	.515	.206	-.633	.408
NR3C1CpG2_log_EB	139	.00	1.49	.5156	.32055	.727	.206	.380	.408
Valid N (listwise)	136								

The data were therefore square root transformed instead and again tested (**Table 12**). This showed that the data were slightly better, and so we therefore used this square root transformed data in the further analyses.

TABLE 12 DESCRIPTIVE STATISTICS FOR SQUARE ROOT TRANSFORMED METHYLATION DATA FOR *OXTR*, *NR3C1* AND *FKBP5* INCLUDING MEAN, STANDARD DEVIATION, SKEWNESS AND KURTOSIS. YELLOW HIGHLIGHTED DATA SHOWN KURTOSED VALUES.

Descriptive Statistics

	N	Minimum	Maximum	Mean	Std. Deviation	Skewness		Kurtosis	
	Statistic	Statistic	Statistic	Statistic	Statistic	Statistic	Std. Error	Statistic	Std. Error
OXTRCpG1_sqrt_EB	223	4.71	8.14	6.8854	.40274	-.800	.163	7.175	.324
OXTRCpG2_sqrt_EB	223	5.95	9.14	8.3027	.34087	-2.822	.163	17.923	.324
FKBP5CpG1_sqrt_EB	220	8.43	10.00	9.8707	.19445	-3.441	.164	18.098	.327
FKBP5CpG2_sqrt_EB	220	5.74	9.90	8.8399	.73427	-1.649	.164	2.946	.327
NR3C1CpG1_sqrt_EB	139	1.00	9.75	2.5221	1.58754	1.570	.206	2.724	.408
NR3C1CpG2_sqrt_EB	139	1.00	5.57	1.9489	.84292	1.843	.206	4.156	.408
Valid N (listwise)	136								

Histograms of the data were then checked to see the spread of the data and identify any possible outliers (**Figure 12**). This revealed a few low outliers for OXTR CpGs 1 and 2, and a high extreme

value for *NR3C1* CpG1. An extreme values table was developed to allow to find which cases needed to be removed (**Table 13**).

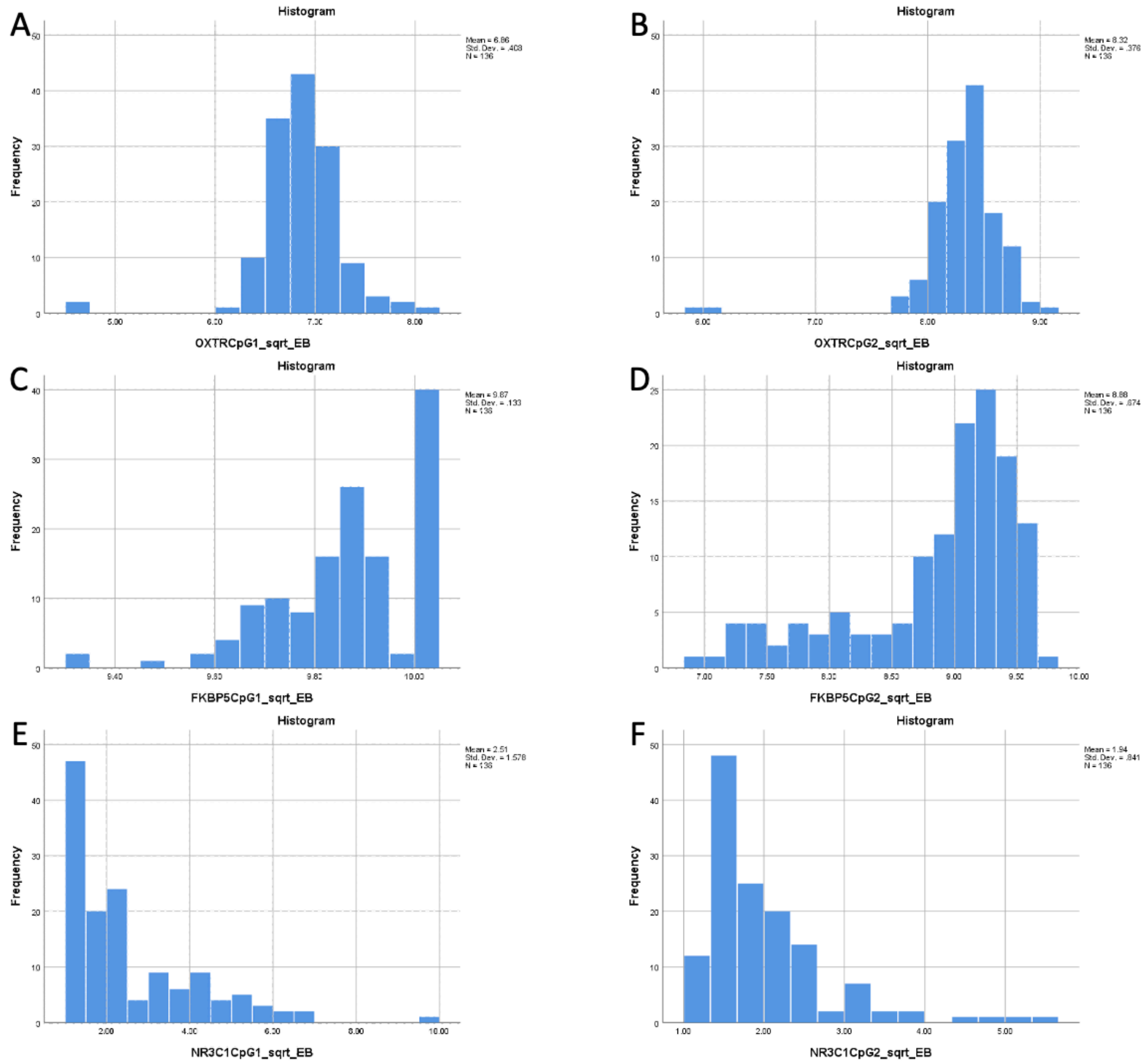


FIGURE 13 HISTOGRAM OF SQUARE ROOT TRANSFORMED DATA *OXTR* CPG1 (A) AND CPG (C) AND CPG2 (D), AND *NR3C1* CPG1 (E) AND CPG2 (F) AND *FKBP5* CPG1 (C) AND CPG2 (D).

Therefore, the two outliers were removed from *OXTR* CpG1, two outliers removed from OXTR CpG2 and one outlier removed from *NR3C1* CPG1 (in green, Table **11**).

TABLE 13 EXTREME VALUES FOR DATA POINTS FOR CPG VALUES FROM *OXTR, FKBP5* AND *NR3C1* EXHIBITING THOSE CASES WITH EXTREME VALUES THAT NEEDED TO BE REMOVED (GREEN)

Extreme Values

			Case Number	Value
OXTR CpG1_sqrt_EB	Highest	1	78	8.14
		2	135	7.81
		3	163	7.78
		4	299	7.62
		5	39	7.55[a]
	Lowest	1	5	4.71
		2	6	4.72
		3	188	6.16
		4	19	6.32
		5	288	6.40[b]
OXTR CpG2_sqrt_EB	Highest	1	163	9.14
		2	176	8.92
		3	57	8.89
		4	56	8.83
		5	234	8.83
	Lowest	1	6	5.95
		2	5	6.03
		3	288	7.75
		4	123	7.75
		5	37	7.78
FKBP5CpG1_sqrt_EB	Highest	1	6	10.00
		2	9	10.00
		3	12	10.00

		4	15	10.00
		5	19	10.00[c]
	Lowest	1	24	9.33
		2	20	9.33
		3	101	9.49
		4	164	9.59
		5	54	9.59
FKBP5CpG2_sqrt_EB	Highest	1	95	9.75
		2	23	9.64
		3	121	9.64
		4	19	9.59
		5	28	9.59[d]
	Lowest	1	101	6.93
		2	27	7.00
		3	108	7.21
		4	158	7.28
		5	61	7.28[e]
NR3C1 CpG1_sqrt_EB	Highest	1	155	9.75
		2	17	6.93
		3	18	6.78
		4	22	6.40
		5	14	6.00
	Lowest	1	300	1.00
		2	299	1.00
		3	297	1.00
		4	243	1.00
		5	190	1.00[f]

NR3C1 CpG2_sqrt_EB	Highest	1	18	5.57
		2	17	5.20
		3	174	4.90
		4	86	4.58
		5	15	3.74[g]
	Lowest	1	300	1.00
		2	299	1.00
		3	243	1.00
		4	190	1.00
		5	165	1.00[f]

a. Only a partial list of cases with the value 7.55 are shown in the table of upper extremes.

b. Only a partial list of cases with the value 6.40 are shown in the table of lower extremes.

c. Only a partial list of cases with the value 10.00 are shown in the table of upper extremes.

d. Only a partial list of cases with the value 9.59 are shown in the table of upper extremes.

e. Only a partial list of cases with the value 7.28 are shown in the table of lower extremes.

f. Only a partial list of cases with the value 1.00 are shown in the table of .lower extremes.

g. Only a partial list of cases with the value 3.74 are shown in the table of upper extremes.

The descriptive statistics were checked again for distribution (**Table 14**) and this revealed that just *FKBP5* CpG1 looks slightly skewed and kurtosed, whilst all other data now looks normally distributed.

TABLE 14 DESCRIPTIVE STATISTICS FOR SQUARE ROOT TRANSFORMED METHYLATION FOR *OXTR, NR3C1* AND *FKBP5* FOLLOWING REMOVAL OF OUTLIERS. GREEN HIGHLIGHTED DATA SHOW SKEWED AND KURTOSED DATA.

Descriptive Statistics									
	N	Minimum	Maximum	Mean	Std. Deviation	Skewness		Kurtosis	
	Statistic	Statistic	Statistic	Statistic	Statistic	Statistic	Std. Error	Statistic	Std. Error
OXTRCpG1_sqrt_EB	221	6.16	8.14	6.9050	.34704	.813	.164	1.145	.326
OXTRCpG2_sqrt_EB	221	7.42	9.14	8.3236	.26078	-.166	.164	.458	.326
FKBP5CpG1_sqrt_EB	220	8.43	10.00	9.8707	.19445	-3.441	.164	18.098	.327
FKBP5CpG2_sqrt_EB	220	5.74	9.90	8.8399	.73427	-1.649	.164	2.946	.327
NR3C1CpG1_sqrt_EB	138	1.00	6.93	2.4698	1.46797	1.226	.206	.612	.410
NR3C1CpG2_sqrt_EB	139	1.00	5.57	1.9489	.84292	1.843	.206	4.156	.408
Valid N (listwise)	133								

3.5 Primary caregiver and child demographics

The study included 300 families in total with 151 in the intervention group and 149 in the control groups. There were no significant differences in age of the mothers or fathers between the intervention and control groups, gender of the primary caregivers, ethnicity, or level of education of the mother or father (**Table 15**).

	Total Sample				Intervention group				Control group			
	N	%	Mean	Range	N	%	Mean	Range	N	%	Mean	Range
Age	300		34.18	34	151		33.66	28	149		34.72	34
Gender												
Female	276	72			138	91.4			138	92.6		
Male	13	4.3			8	5.3			5	3.4		
Ethnicity												
White	216	72			16	10.6			10	6.7		
Mixed	22	7.3			10	6.6			10	6.7		
Asian or Asian British	29	9.7			37	24.5			34	22.8		
Black or Black British	14	4.7			32	21.2			39	26.2		
Other	10	3.3			55	36.4			55	36.9		
Highest Educational Qualification												
Pre-GCSE	26	8.7			16	10.6			10	6.7		
GCSE	20	6.7			10	6.6			10	6.7		
College	71	23.7			37	24.5			34	22.8		
Undergraduate degree	71	23.7			32	21.2			39	26.2		
Postgraduate degree	110	36.7			55	36.4			55	36.9		

TABLE 15 PRIMARY CAREGIVER DEMOGRAPHICS

Abbreviations: %= percentage, N= number of participants.

Within the child demographics there were no significant differences in age of the children between the intervention and control groups. There were more males in the control group than the intervention. Ethnicity overall did not differ (**Table 16**).

TABLE 16 CHILD DEMOGRAPHICS

	Total Sample				Intervention group				Control group			
	N	**%**	**Mean**	**Range**	**N**	**%**	**Mean**	**Range**	**N**	**%**	**Mean**	**Range**
Age at T3	286		47.85	7.32	142		47.86	29.63	144		47.83	35.13
Gender												
Female	137	45.7			75	49.7			62	41.6		
Male	163	54.3			76	50.4			87	58.4		
Ethnicity												
White	195	65			98	64.9			97	65.1		
Mixed	58	19.3			33	21.9			25	16.8		
Asian or Asian British	15	5			8	5.3			7	4.7		
Black or Black British	13	4.3			3	2			10	6.7		
Other	9	3			3	2			6	4		
Behaviour - PACS T1	298		31.33	64	150		31.77	53.6	148		30.88	64
Behaviour - PACS T2	286		27.93	58	140		27.15	49	146		28.67	58
Behaviour - PACS T3	282		22.75	55.4	140		21.81	46	142		23.67	55.4
Behaviour - CBCL T1	296		39.13	114	149		31.77	114	147		40.71	102
Behaviour - CBCL T2	282		32.4	100	139		27.15	89	143		34.67	100
Behaviour - CBCL T3	282		30.54	141	140		21.81	141	142		33.07	127
Behaviour - SDQ T1	299		13.1	29	150		12.97	27	149		13.23	28
Behaviour - SDQ T2	283		11.12	27	139		10.77	23	144		11.46	11.46
Behaviour - SDQ T3	283		10.11	28	140		9.85	26	143		10.36	10.36

Abbreviations: T3: Timepoint 3. PACS: CBCL: child behaviour checklist; SDQ: strength and difficulties questionnaire.

3.6 Testing for differences in DNA methylation between children who have received the VIPP intervention compared with those who have not

To answer whether methylation differs between the groups of children who received the VIPP intervention and the controls, independent samples t-tests were conducted where the

dependent variable is the percentage methylation, and the independent variable is group (VIPP or control). To increase statistical power, we took the average methylation across *NR3C1* CpG one and two as previously described and *FKBP5* CpG one and two as previously described. CpGs one and two for OXTR were tested independently (**Table 17**).

TABLE 17 DESCRIPTIVE STATISTICS FOR AVERAGE DNA METHYLATION FOR ***NR3C1*** AND ***FKBP5*** AND ***OXTR*** CPGS1 AND 2

		N	Mean	Std. Deviation	Std. Error Mean
NR3C1 Mean CpG1 and 2 SQRT	Control	72	2.2544	1.14749	.13523
	VIPP	65	2.1167	1.05643	.13103
FKBP5 Mean CpG1 and 2 SQRT	Control	105	9.3396	.46160	.04304
	VIPP	103	9.3678	.38794	.03823
OXTR CpG1_sqrt_EB	Control	115	6.9199	.37095	.03459
	VIPP	104	6.8902	.32284	.03166
OXTR CpG2_sqrt_EB	Control	115	8.3038	.26671	.02487
	VIPP	104	8.3454	.25582	.02509

The t-tests revealed there were no difference between VIPP and control groups for average methylation at *NR3C1, FKBP5* and *OXTR* CpGs 1 and 2 (**Table 18**). This is supported by bar graphs also showing no differences between VIPP and control groups for average methylation at *NR3C1* (**Figure 14**), FKBP5 (**Figure 15**) and *OXTR* CpGs 1 and 2 (**Figure 16**).

TABLE 18 INDEPENDENT SAMPLES T-TESTED FOR DIFFERENCES IN AVERAGE DNA METHYLATION FOR ***NR3C1*** AND ***FKBP5*** AND ***OXTR*** CPGS 1 AND 2 BETWEEN VIPP AND CONTROL GROUPS

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
NR3V1 Mean CpG1 and 2 SQRT	Equal variances assumed	.023	.880	.728	135	.468	.13772	.18910	-.23627	.51171
	Equal variances not assumed			.731	134.944	.466	.13772	.18830	-.23468	.51013
FKBP5 Mean CpG1 and 2 SQRT	Equal variances assumed	1.999	.159	-.486	216	.628	-.02822	.05812	-.14277	.08633
	Equal variances not assumed			-.490	215.154	.624	-.02822	.05757	-.14169	.08524
OXTRCpG1_sqrt_EB	Equal variances assumed	2.715	.101	.629	217	.530	.02969	.04722	-.06338	.12276
	Equal variances not assumed			.633	216.691	.527	.02969	.04689	-.06273	.12211
OXTRCpG2_sqrt_EB	Equal variances assumed	.010	.919	-1.175	217	.241	-.04159	.03540	-.11136	.02818
	Equal variances not assumed			-1.177	216.238	.240	-.04159	.03532	-.11122	.02803

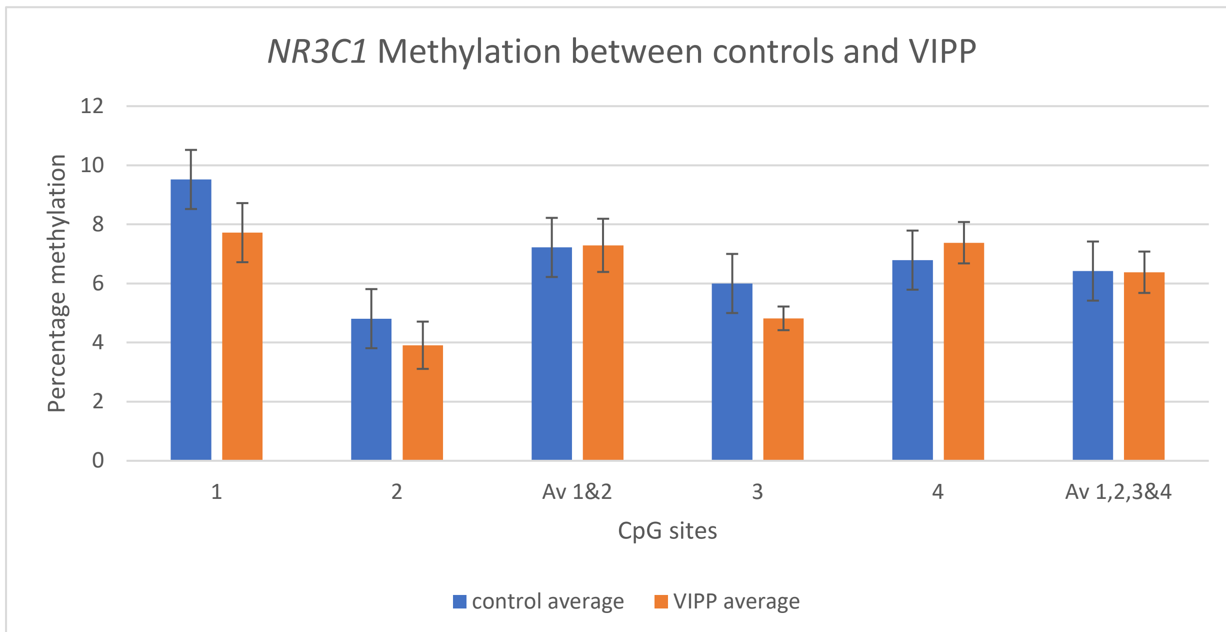


Figure 14 A bar chart showing levels of DNA methylation across all CpG sites at the *NR3C1* promoter between VIPP (n=6.38) and control (n=6.42) groups at average across all sites.

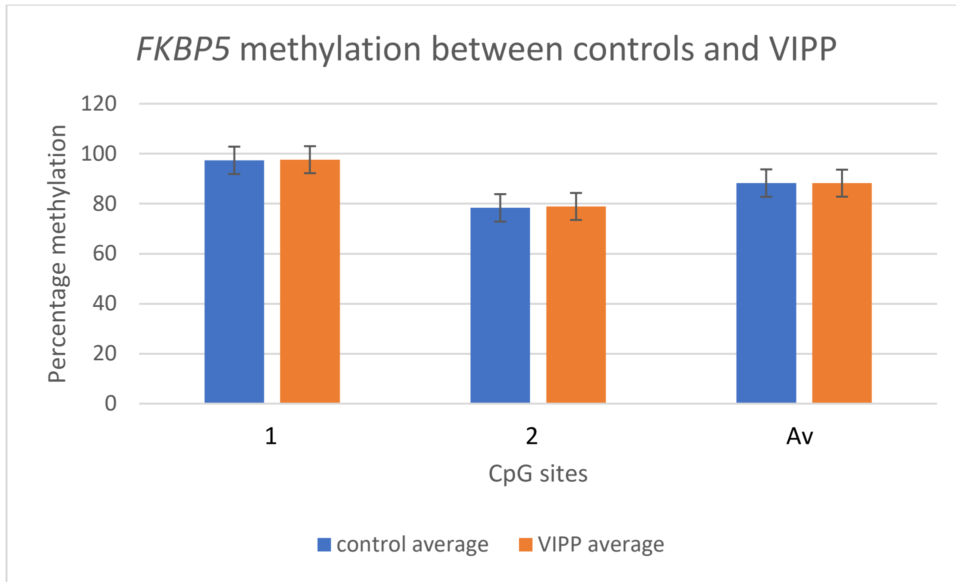


Figure 15 A bar chart showing levels of DNA methylation at CpG1 and CpG2 at the *FKBP5* promoter between VIPP (N=88.22) and control (N= 88.25) groups

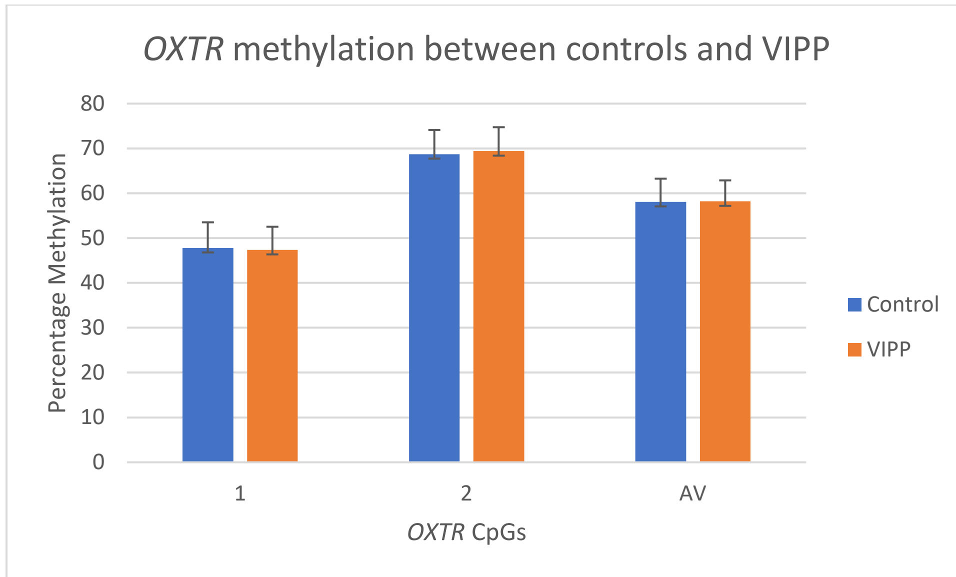


Figure 16 A bar chart showing levels of DNA methylation of CpG1 (control N=47.8; VIPP= 47.37) and CpG2 (control N=68.73; VIPP= 69.39) and the averages (control N=58.07; VIPP N=58.19)

3.7 Testing whether the degree of child behaviour change from pre-intervention (TP1) to 2-year follow up (TP2) associated with levels of DNA methylation

To address the question of whether DNA methylation at the three genes is associated with changes in in child behaviour following the intervention we firstly performed Pearson's correlations to show any relationships between two variables, namely between the DNA methylation variables, and behaviour change scores from TP1 to TP3 on the behaviour. For simplicity SDQ score was focussed on only and calculated the change score by subtracting TP3-TP1. Again, to maintain statistical power we took the average methylation across *NR3C1* CpG 1 and 2 and *FKBP5* CpG 1 and 2 and tested CpGs 1 and 2 for *OXTR*. For the regression the confounders we included the primary caregiver age, gender, ethnicity, and highest qualification, and depression (PHQ) and the infant gender and age at TP 3 in months (see below) (**Table 19**).

Table 19 Pearson's correlation for DNA methylation and change in SDQ scores from TP 1 and 3

Correlations

		NR3V1 Mean CpG1 and 2 SQRT	FKBP5 Mean CpG1 and 2 SQRT	OXTRCpG1_sqrt_EB	OXTRCpG2_sqrt_EB	SDQ Total score change from T1 to T3	Primary caregiver age	primary caregiver ethnicity - 5 categories	primary caregiver gender	Primary caregiver highest qualification	PHQ total caregiver 1 timpoint 3	Infant gender	Infant age at time point 3 in months
NR3C1 Mean CpG1 and 2 SQRT	Pearson Correlation	1	.228**	-.116	-.044	-.149	.063	-.054	.132	-.027	-.083	.192*	.084
	Sig. (2-tailed)		.008	.182	.612	.084	.463	.535	.124	.758	.350	.025	.330
	N	138	135	135	135	136	137	134	137	137	128	137	137
FKBP5 Mean CpG1 and 2 SQRT	Pearson Correlation	.228**	1	-.263**	-.153*	-.037	.024	.038	.042	-.035	-.099	.011	-.018
	Sig. (2-tailed)	.008		.000	.024	.590	.722	.583	.538	.608	.154	.868	.796
	N	135	220	218	218	216	218	215	218	218	208	218	218
OXTRCpG1_sqrt_EB	Pearson Correlation	-.116	-.263**	1	.576**	.087	.004	-.063	.042	-.067	.040	-.074	-.022
	Sig. (2-tailed)	.182	.000		.000	.202	.958	.356	.532	.326	.561	.273	.741
	N	135	218	221	221	217	219	216	219	219	209	219	219
OXTRCpG2_sqrt_EB	Pearson Correlation	-.044	-.153*	.576**	1	.071	.027	-.087	-.053	-.152*	.018	-.067	.041
	Sig. (2-tailed)	.612	.024	.000		.297	.688	.205	.436	.024	.796	.324	.550
	N	135	218	221	221	217	219	216	219	219	209	219	219
SDQ Total score change from T1 to T3	Pearson Correlation	-.149	-.037	.087	.071	1	-.085	-.015	.061	-.120*	.236**	.083	-.031
	Sig. (2-tailed)	.084	.590	.202	.297		.152	.804	.308	.045	.000	.166	.606
	N	136	216	217	217	282	282	278	282	282	264	282	282
Primary caregiver age	Pearson Correlation	.063	.024	.004	.027	-.085	1	-.051	.084	.258**	-.069	.062	.048
	Sig. (2-tailed)	.463	.722	.958	.688	.152		.385	.148	.000	.260	.285	.417
	N	137	218	219	219	282	300	296	300	300	267	300	286
primary caregiver ethnicity - 5 categories	Pearson Correlation	-.054	.038	-.063	-.087	-.015	-.051	1	-.045	.079	-.056	.013	.002
	Sig. (2-tailed)	.535	.583	.356	.205	.804	.385		.436	.174	.362	.819	.976
	N	134	215	216	216	278	296	296	296	296	265	296	282
primary caregiver gender	Pearson Correlation	.132	.042	.042	-.053	.061	.084	-.045	1	-.079	.043	.054	.057
	Sig. (2-tailed)	.124	.538	.532	.436	.308	.148	.436		.172	.482	.353	.337
	N	137	218	219	219	282	300	296	300	300	267	300	286
Primary caregiver highest qualification	Pearson Correlation	-.027	-.035	-.067	-.152*	-.120*	.258**	.079	-.079	1	-.336**	-.019	-.172**
	Sig. (2-tailed)	.758	.608	.326	.024	.045	.000	.174	.172		.000	.739	.004
	N	137	218	219	219	282	300	296	300	300	267	300	286
PHQ total caregiver 1 timpoint 3	Pearson Correlation	-.083	-.099	.040	.018	.236**	-.069	-.056	.043	-.336**	1	-.005	.072
	Sig. (2-tailed)	.350	.154	.561	.796	.000	.260	.362	.482	.000		.932	.238
	N	128	208	209	209	264	267	265	267	267	267	267	267
Infant gender	Pearson Correlation	.192*	.011	-.074	-.067	.083	.062	.013	.054	-.019	-.005	1	-.014
	Sig. (2-tailed)	.025	.868	.273	.324	.166	.285	.819	.353	.739	.932		.819
	N	137	218	219	219	282	300	296	300	300	267	300	286
Infant age at time point 3 in months	Pearson Correlation	.084	-.018	-.022	.041	-.031	.048	.002	.057	-.172**	.072	-.014	1
	Sig. (2-tailed)	.330	.796	.741	.550	.606	.417	.976	.337	.004	.238	.819	
	N	137	218	219	219	282	286	282	286	286	267	286	286

**. Correlation is significant at the 0.01 level (2-tailed).

*. Correlation is significant at the 0.05 level (2-tailed).

No correlations were found between methylation at any of the genes and change in behaviour (SDQ) following the VIPP intervention. Interestingly, there was a negative correlation between DNA methylation at *OXTR* CpG2 and primary caregiver highest qualification.

When looking at correlations in DNA methylation between the genes we found that average *FKBP5* DNA methylation correlated with average *NR3C1* methylation and *OXTR* CpG1 and CpG2 methylation.

While correlation shows the relationship between the two variables, regression allows us to see how one affects the other, i.e., when one changes, so does the other, and not always in the same direction allowing for the establishment of possible cause and effect relationships. The type of regression we performed was stepwise which allows the inclusion of multiple variables of interest to identify a set of predictors.

Using DNA methylation from the different genes as the independent (or outcome variable) and included the following confounders that were entered into block 1 of the model: 1. Primary caregiver age, 2. Primary caregiver ethnicity, 3. Primary caregiver gender, 4. Primary caregiver education, 5. Primary caregiver depression (PHQ) at TP3, 6. Child age at TP3, 7. Child gender. In block 2 SDQ change score was entered.

When performing the stepwise regression for the mean methylation of *NR3C1* (**Table 20**) we found a significant association with SDQ change score and infant gender suggesting increased methylation with increased SDQ score change dependent on infant gender.

TABLE 20 STEPWISE REGRESSION FOR AVERAGE ***NR3C1*** DNA METHYLATION AND SDQ CHANGE SCORE

Model		Unstandardized Coefficients		Standardized Coefficients	t	Sig.
		B	Std. Error	Beta		
1	(Constant)	.468	1.110		.422	.674
	Primary caregiver age	-.002	.020	-.008	-.082	.935
	primary caregiver ethnicity - 5 categories	-.053	.084	-.057	-.628	.531
	primary caregiver gender	.101	.581	.016	.174	.862
	Primary caregiver highest qualification	.025	.101	.024	.251	.802
	GAD total caregiver 1 timpoint 3	-.021	.025	-.079	-.848	.398
	Infant age at time point 3 in months	.022	.014	.140	1.543	.126
	Infant gender	.439	.202	.198	2.175	.032
2	(Constant)	.356	1.098		.325	.746
	Primary caregiver age	-.001	.019	-.005	-.051	.959
	primary caregiver ethnicity - 5 categories	-.053	.083	-.057	-.636	.526
	primary caregiver gender	.176	.575	.028	.307	.760
	Primary caregiver highest qualification	.011	.100	.010	.111	.912
	GAD total caregiver 1 timpoint 3	-.014	.025	-.050	-.538	.592
	Infant age at time point 3 in months	.018	.014	.116	1.286	.201
	Infant gender	.499	.202	.225	2.474	.015
	SDQ Total score change from T1 to T3	-.037	.019	-.181	-1.988	.049

Coefficients[a]

a. Dependent Variable: NR3V1 Mean CpG1 and 2 SQRT

When performing the stepwise regression for the mean methylation of *FKBP5* (**Table 21**) no significant association was found with SDQ change score methylation at this gene, thus does not associate with SDQ score changes. However, there was an association with primary caregiver gender.

TABLE 21 STEPWISE REGRESSION FOR AVERAGE FKBP5 DNA METHYLATION AND SDQ CHANGE SCORE

Model		Unstandardized Coefficients B	Unstandardized Coefficients Std. Error	Standardized Coefficients Beta	t	Sig.
1	(Constant)	9.708	.338		28.726	.000
	Primary caregiver age	.006	.006	.079	1.065	.288
	primary caregiver ethnicity - 5 categories	.028	.027	.072	1.017	.310
	primary caregiver gender	-.396	.170	-.167	-2.325	.021
	Primary caregiver highest qualification	-.030	.029	-.075	-1.023	.308
	GAD total caregiver 1 timpoint 3	-.009	.007	-.088	-1.220	.224
	Infant age at time point 3 in months	-.001	.004	-.011	-.160	.873
	Infant gender	-.010	.061	-.011	-.163	.871
2	(Constant)	9.700	.340		28.500	.000
	Primary caregiver age	.006	.006	.078	1.054	.293
	primary caregiver ethnicity - 5 categories	.028	.028	.072	1.012	.313
	primary caregiver gender	-.392	.172	-.165	-2.284	.023
	Primary caregiver highest qualification	-.030	.029	-.075	-1.026	.306
	GAD total caregiver 1 timpoint 3	-.008	.007	-.086	-1.175	.242
	Infant age at time point 3 in months	-.001	.004	-.012	-.173	.863
	Infant gender	-.008	.062	-.009	-.131	.896
	SDQ Total score change from T1 to T3	-.001	.006	-.017	-.232	.817

Coefficients[a]

a. Dependent Variable: FKBP5 Mean CpG1 and 2 SQRT

WHEN PERFORMING THE STEPWISE REGRESSION FOR THE MEAN METHYLATION OF *OXTR* CPGS 1 AND 2 (TABLES 22 AND 23) WE FOUND NO SIGNIFICANT ASSOCIATIONS WITH SDQ CHANGE SCORE. HOWEVER, THERE WAS AGAIN A NEGATIVE ASSOCIATION BETWEEN *OXTR* CPG2 AND PRIMARY CAREGIVER HIGHEST QUALIFICATION, SUGGESTING A HIGHER LEVEL OF EDUCATIONAL QUALIFICATION IS ASSOCIATED WITH REDUCED LEVELS OF *OXTR* METHYLATION. TABLE 22 STEPWISE REGRESSION FOR AVERAGE *OXTR* CPG 1 DNA METHYLSTION SDQ CHANGE SCORE.

Coefficients[a]							
Model		Unstandardized Coefficients		Standardized Coefficients	t	Sig.
		B	Std. Error	Beta		
1	(Constant)	7.105	.277		25.634	.000
	Primary caregiver age	.001	.005	.008	.108	.914
	primary caregiver ethnicity - 5 categories	-.019	.023	-.060	-.834	.405
	primary caregiver gender	-.005	.139	-.002	-.034	.973
	Primary caregiver highest qualification	-.014	.024	-.042	-.574	.566
	GAD total caregiver 1 timpoint 3	.006	.006	.070	.962	.337
	Infant age at time point 3 in months	-.002	.004	-.037	-.515	.607
	Infant gender	-.045	.050	-.065	-.911	.364
2	(Constant)	7.130	.278		25.644	.000
	Primary caregiver age	.001	.005	.011	.153	.878
	primary caregiver ethnicity - 5 categories	-.019	.023	-.060	-.833	.406
	primary caregiver gender	-.019	.140	-.010	-.138	.890
	Primary caregiver highest qualification	-.013	.024	-.042	-.564	.573
	GAD total caregiver 1 timpoint 3	.005	.006	.060	.815	.416
	Infant age at time point 3 in months	-.002	.004	-.032	-.440	.660
	Infant gender	-.051	.050	-.073	-1.028	.305
	SDQ Total score change from T1 to T3	.005	.005	.077	1.070	.286

a. Dependent Variable: OXTRCpG1_sqrt_EB

TABLE 23 STEPWISE REGRESSION FOR AVERAGE *OXTR* CPG2 DNA METHYLATION AND SDQ CHANGES SCORE

Coefficients[a]

Model		Unstandardized Coefficients		Standardized Coefficients	t	Sig.
		B	Std. Error	Beta		
1	(Constant)	8.552	.206		41.424	.000
	Primary caregiver age	.003	.004	.065	.893	.373
	primary caregiver ethnicity - 5 categories	-.019	.017	-.079	-1.124	.262
	primary caregiver gender	-.064	.104	-.044	-.617	.538
	Primary caregiver highest qualification	-.046	.018	-.189	-2.593	.010
	GAD total caregiver 1 timpoint 3	-.001	.004	-.017	-.245	.807
	Infant age at time point 3 in months	1.677E-6	.003	.000	.001	.999
	Infant gender	-.036	.037	-.069	-.984	.326
2	(Constant)	8.571	.207		41.408	.000
	Primary caregiver age	.003	.004	.069	.942	.347
	primary caregiver ethnicity - 5 categories	-.019	.017	-.079	-1.123	.263
	primary caregiver gender	-.076	.104	-.052	-.726	.469
	Primary caregiver highest qualification	-.046	.018	-.188	-2.584	.010
	GAD total caregiver 1 timpoint 3	-.002	.004	-.028	-.391	.696
	Infant age at time point 3 in months	.000	.003	.006	.079	.937
	Infant gender	-.041	.037	-.078	-1.110	.269
	SDQ Total score change from T1 to T3	.004	.003	.081	1.143	.255

a. Dependent Variable: OXTRCpG2_sqrt_EB

4. Discussion

This thesis aimed to test whether changes in epigenetic regulation of stress genes including *OXTR, NR3C1* and *FKBP5* in infants are influenced by changes in parenting through a video intervention treatment and if this correlated with changes in child behaviour. Specifically, we found that levels of DNA methylation at the *OXTR, FKBP5* and *NR3C1* gene did not significantly differ between children who have received the VIPP intervention compared with those who did not. We also found no significant correlations between DNA methylation at the three genes and the degree of child behaviour change from pre-intervention and 2 years follow up. Interestingly, we did find significant correlations in DNA methylation with behavioural changes in association with infant's gender for *NR3C1*, with primary caregiver gender for *FKBP5* and levels of educational attainment shown to have an influence on *OXTR*. Shown in child demographics table one and 16 showing the control and intervention groups.

Behaviour problems are one of the most common mental health disorders in childhood and these are associated with a wide range of adverse outcomes in adulthood. Therapies to reduce these long-term effects could have important implications and central to this would be the importance in understanding mechanisms and having the ability to predict who would respond better with markers to predict response. Here we investigated the outcomes of the HSHS video feedback interventions study that has been shown to be effective in reducing children's problematic behavioural traits through targeting parental behaviour (O'Farrelly et al., 2021). Here we studied the epigenetic regulation of the stress genes *OXTR*, *NR3C1* and *FKBP5* to see if these associate with child behaviour and if epigenetic patterns change following the intervention.

When comparing the group children who received the VIPP intervention with the group who did not, we found no differences in DNA methylation in *NR3C1*, *FKBP5* or *OXTR*. We also found no correlations with methylation at any of the genes with changes in behaviour (SDQ) following the intervention. Interestingly, we did find a significant association with *NR3C1* methylation and SDQ change score and infant gender suggesting increased methylation with increased SDQ score change dependent on gender. We also found an interesting negative association between

OXTR CpG2 and primary caregiver highest qualification suggesting a higher level of educational qualification is associated with reduced levels of *OXTR* methylation.

Reduced SDQ scores from TP 1 to TP 2, two years later, reflects improvements in behaviour, associated with reduced *NR3C1* methylation supporting the hypothesis that child behavioural problems and stress-related disorders are associated with higher levels of methylation of *NR3C1*. This further aligns with the theory and importance of maternal depression and parenting behaviour playing an important role in offspring development and that part of these mechanisms could be linked to epigenetic changes in stress—related genes. There is a wide and varied literature, including animal studies, that have investigated the effects of maternal care and maternal stress with epigenetic mechanisms demonstrating long-lasting effects until adulthood (Harris, A, R. and Santos, P, H., 2020). Most of these studies suggest that early-life stress significantly modifies epigenetic marks in several HPA axis genes in multiple brain regions (Kosten and Nielson, 2015). Several human studies have replicated similar results in both brain and peripheral samples; for example, McGowan et al. (2011) found epigenetic changes in *NR3C1* in post-mortem brain tissues from individuals who suffered childhood trauma (McGowan et al., 2011) while other studies have found changes in blood (e.g. Sheilds et al., 2016) and saliva (e.g. Schechter et al., 2015). A recent systematic review of childhood maltreatment and DNA methylation found that most studies in children demonstrated increased *NR3C1* methylation with maltreatment. Furthermore, in adults, several studies documented greater *NR3C1* methylation in those exposed to childhood maltreatment (Cecil, Chang, and Nolte, 2020). Deficiencies in early-life nurturing have been associated with increased methylation of *NR3C1* promoter in leukocytes (Tykra et al., 2012) and epigenetic alterations in this gene have also been linked to even broader parenting behaviours, such as harsh parenting practices (Lewis et al., 2021). Our results therefore support the notion that improving parental care can epigenetically reduce DNA methylation at *NR3C1* that might further link to improvements in child behaviour.

The methylation levels for the *NR3C1* gene promoter were around 9% for CpG1 and around 5% for CpG2. Though these values are similar to previously reported levels in other studies (e.g.

Folger et al., 2019; Lester et al., 2015; Na et al., 2014), these values are quite low, with limited ability to reliably detect group differences between the VIPP and control groups. However, given that we find correlations with improvements in behaviour this would support the role of epigenetic regulation at this gene. A previous study has shown that increased *NR3C1* promoter methylation correlated with reduced GR gene expression (Mcgowan et al., 2009). Furthermore, as GR is known to regulate negative feedback of the HPA axis, then the hypothesis here would be that lower methylation and higher GR gene expression might be expected in conditions with lower cortisol - there is some evidence for this (Fischer et al., 2021). Hence, the idea that the VIPP treatment might be reducing HPA activity and cortisol levels and the *NR3C1* methylation may reflect this.

In relation to the role of *NR3C1* and HPA axis, we also found a positive correlation between average *FKBP5* DNA methylation with average *NR3C1* methylation. As *FKBP5* modulates the sensitivity of the GR (Criado-Marriado et al., 2020; Tykra et al., 2015; Hartmann et al., 2021), this might further support the role of this mechanism in the behavioural changes seen following VIPP in this study. To provide further evidence of this, we would need to measure cortisol levels longitudinally across the two timepoints to understand if there are correlations between the methylation and the levels of this hormone, and perhaps also test cortisol responses to stress. For example, one study found that people with depression, compared to controls, had blunted cortisol reactivity to a stressor and further showed increased *NR3C1* methylation (Bakusic et al., 2014).

A further part of these findings are the effects of gender, i.e., the association of *NR3C1* methylation with changes in behaviour was dependent on infant gender. Some studies have found gender differences in *NR3C1* methylation in response to stress either prenatally (Braithwaite et al., 2015) or postnatally (Hill et al., 2019). For example, in the study by Hill et al., (2019) *NR3C1* promoter methylation mediated the association between maternal depression and child anxious-depressed symptoms in girls and not in boys, suggesting that epigenetic and early behavioural outcomes may arise through different mechanisms in males and females. This

further highlights the importance of sex-differences in the epigenetic regulation of behaviour as shown in Braithwaite et al. (2015).

In this study when examining *OXTR* methylation we found lower levels of CpG2 methylation correlated with primary caregiver highest qualification. This would suggest that those parents with a higher level of educational qualification led to reduced levels of *OXTR* methylation in the infants during this study. Studies have shown that children of parents with low education have a 2 to 3-fold increased risk of psychiatric disorders such as attention deficit hyperactivity disorder (ADHD) and depression (Brown et al., 2020; Braquhais et al., 2012; Hiller et al., 2020), compared to children of parents with high education. A recent meta-analysis has found that increases in *OXTR* DNA methylation are associated with callous-unemotional traits in youth, social cognitive deficits in Autistic Spectrum Disorder (ASD), rigid thinking in anorexia nervosa, affect regulation problems, and problems with facial and emotional recognition (Maud et al., 2018). Therefore, the hypothesis could be that higher parental educational attainment might associate with reduced risk of behavioural problems in the children that would further associate with reduced *OXTR* methylation.

This again supports parenting in the regulation and development of child behaviour and the role of oxytocin. That we did not find direct differences in *OXTR* methylation between the VIPP and control groups, this might reflect that the differences were not large enough to be detected within the size of this study. Measuring levels of serum oxytocin might help support the hypothesis that the oxytocinergic system could be important in mediating the behavioural responses to the treatment. Also, since *OXTR* methylation did not directly correlate with behavioural changes, while parental education did, this might reflect the complexities of the mechanisms involved in the response to VIPP treatment.

Interestingly, *OXTR* methylation at both CpG1 and CpG2 negatively correlated with *FKBP5* methylation possibly supporting its role in modulating reactivity to stressors and increases in social sensitivity via the HPA axis. Oxytocin is known to respond to cortisol (Carter et al., 2020; Florea et al., 2022) and may be co-released following a range of both positive and negative challenges (Kuchenbecker., 2021). Therefore, this might suggest that the epigenetic regulation

of *FKBP5*, important in regulating the HPA axis, might link to the epigenetic regulation of *OXTR* as part of the mechanism important in regulating responses to this VIPP intervention. Methylation at *FKBP5* did not differ in response to VIPP treatment nor did it correlate with behaviour or behavioural changes following treatment. The levels of methylation we found in this study were very similar to previously reported levels in other studies, however, these levels are relatively high, i.e., over 95% at CpG1 and around 85% at CpG2. The spread of values was also quite narrow, and this might have impacted the statistical power needed to find significant effects. However, this is associated with methylation, which would support a theory that a level of coordination epigenetic regulation exists between *FKBP5* and both *NR3C1* and *OXTR,* as their role in controlling behaviour and the behavioural responses following this VIPP intervention.

5 Limitations

The potential issues and limitations throughout this research include having had limited access to the laboratory due to COVID-19 restrictions in addition to laboratory renovations. This led to a delay in accessing the laboratory, whilst due to renovations equipment and samples had to be moved and some reordered. Sample may have degraded leading to errors in results. Equipment upon moving malfunctioned and troubleshooting had to be undertaken, which led to a further delay of approximately two weeks. Furthermore, issues also arose regarding accessing further DNA samples due to limited running of the Liverpool University biobank (despite these having been ordered to be in place some months before access to the laboratory).

Two samples were removed from this research study as there was insufficient DNA concentration in one sample and the other sample had not received full consent. Not all *NR3C1* samples were completed as not all the samples were able to work in the PCR cycler. The issue was unknown as troubleshooting had shown no known cause of why the results didn't come out.

There are a number of limitations in relation to the general measurement of DNA methylation in this study and the technique used: 1, Cells within the saliva were not heterogeneous, i.e., may come from different types such as blood cells and epithelial. This may therefore account for some of the variability in methylation observed and may possibly relate to other variables associated with cellular heterogeneity that may affect the analyses, i.e., if some children might have had a mild illness or partaken in exercise etc. that could have affected changes in the numbers of different blood cells. (Borgol et al., 2012; Bustamante et al 2018). 2, Although we consider that the methylation here in the saliva might correlate with brain in regulating behaviour – as some studies have suggested (Bearer and Mulligan., 2018) – this cannot be taken for certain. Some studies show variations in epigenetic effects across brain regions and cell types and therefore it cannot be assumed that variations in the *NR3C1* promoter in saliva reflect variations in GR synthesis in the hippocampal regions involved in HPA axis regulation. (Chincehetti and handley., 2017). 3, DNA methylation is only one of several different epigenetic processes that can regulate the activity of a gene – therefore it might not reflect a direct measure of the activity of the gene. (Conradt et al., 2015; Dunn et al., 2019). 4, There are many combinations of CpG sites, even on a

relatively concise and specific gene region such as those examined in the *FKBP5*, *OXTR* and *NR3C1* promoters that could also be - perhaps even more – important. However, we focussed on only a few key target CpG sites to avoid the risk of multiple analyses and 'significant' findings occurring by chance. (Essex et al., 2013; Ein-dor et al., 2017; Dun et al., 2019; Hartmann et al., 2021).

Finally, two samples were removed from this research study as there was insufficient DNA concentration in one sample and the other sample had not received full consent of the caregiver.

6. Further research

Further research could include more longitudinal measures to determine if there are any dynamic changes in these epigenetic marks. We did not find clear group differences in methylation at the genes between the VIPP and control groups after two years, however, there might have been earlier changes such as during the VIPP intervention, perhaps less than a year. Furthermore, the associations we found in this study might be maintained following the two years here, perhaps into later adolescence and perhaps even adulthood, which may further associate with possible long term behavioural changes from this study – though this has yet to be examined.

Though we tested the epigenetic marks at genes important for regulating the hormones oxytocin and cortisol, we did not measure these actual hormones. In doing so, this could allow us to test what role these epigenetic marks have in controlling these hormones following the intervention and whether these further associate with the behavioural changes.

Though this study had clear significant positive behavioural outcomes the number of participants in the study might not have been large enough for the epigenetic analyses in this study.

Further research could also include the impact that COVID-19 has had on early life stress and associated financial distress. Studies (Araújo et al., 2021; Shevlin et al., 2020; Wu et al., 2020) are emerging about the impact of the COVID-19 pandemic on mental health, indicating a profound effect on the health of children and adolescents. COVID-19 has impacted everyone since the first lockdown due to various factors from home schooling to social isolation, in addition to many experiencing deaths of loved ones and domestic violence.

7. Conclusion

In conclusion, though we found no clear differences in methylation at *OXTR, NR3C1* and *FKBP5* between VIPP and control groups or correlations in methylation at any of the genes with changes in behaviour following the intervention, we did find a significant association with NR3C1 methylation and sex-specific behavioural changes and a negative association between *OXTR* methylation and primary caregiver educational attainment.

NR3C1 is significantly associated with change in SDQ score and infant gender. Higher methylation has been shown to be associated with increased SDQ score change and association with infant gender. *OXTR* was not associated with CpG2 and the primary caregiver's highest qualification, but higher levels of educational qualification were associated with reduced levels of methylation. *FKBP5* high levels of methylation at CpG1 and low levels at CpG2 were not associated with SDQ score but with primary caregivers' genders.

There are multiple factors to consider such as childhood traumatic events, general psychopathology, social support, and the fears and personal fears relating to COVID-19. During the lockdown both healthy people and people with pre-existing mental health conditions showed a decline at baseline level (Seitz, Bertsch, and Herpertz, 2021).

8. References

Araújo, L., Veloso, C., Souza, M., Azevedo, J. and Tarro, G. (2021) 'The potential impact of the COVID-19 pandemic on child growth and development: a systematic review' *Jornal de Pediatria*, 97(4), pp.369-377.

Armstrong, D. A., Lesseur, C., Conradt, E., Lester, B. M. and Marsit, C. J. (2014) 'Global and gene-specific DNA methylation across multiple tissues in early infancy: Implications for children's health research' *FASEB Journal*, 28, pp. 2088–2097.

Attwood, B., Bourgognon, J., Patel, S., Mucha, M., Schiavon, E., Skrzypiec, A., Young, K., Shiosaka, S., Korostynski, M., Piechota, M., Przewlocki, R. and Pawlak, R. (2011) 'Neuropsin cleaves EphB2 in the amygdala to control anxiety.' *Nature*, 473(7347), pp.372-375.

Alonzo T. Folger,T, A., Ding, L., Ji, H., Yolton, K., Robert T. Ammerman, T, R., Ginkel, J and Bowers, K. (2019) 'Neonatal NR3C1 methylation and social-emotional development at 6 and 18 months of age.' *Front Behav Neurosci* 13, 14.

Baker, M., Lindell, S, G., Driscol, c, a., Zhou, Z., Yuan, Q., Schwandt, M, L., Miller-Crews, I., Simpson, E, A., Annika, p., Farrari, P, F., Sindhu, R, K ., Razagyar, M., Sommer, W, H., Lopez, J, F., Tompson, R, C., Goldman, D, Heiling, M., Higley, J, D., Suomi, S, J., and Bar, C, S. (2017) 'Early rearing history influences oxytocin receptor epigenetic regulation in rhesus macaques' *proc natl acad sci*, 114 (44), 11769-11774.

Bearer, L. E., and Mulligan, S. B. (2018) 'Epigenetic Changes Associated with Early Life Experiences: Saliva, A Biospecimen for DNA Methylation Signatures' *Current Epigenetics*, 19(8), pp.676-698.

Bakusic, J., Vrieze, E., Ghosh, M., Bekaert, B., Claes, S. and Godderis, L. (2020) 'Increased methylation of NR3C1 and SLC6A4 is associated with blunted cortisol reactivity to stress in major depression.' *Neurobiology of Stress*, 13, p.100272.

Braithwaite, E. C., Kundakovic, M., Ramchandani, P. G., Murphy, S. E., and Champagne, F. A. (2015) 'Maternal prenatal depressive symptoms predict infant NR3C1 1F and BDNF IV DNA methylation.' *Epigenetics*, 10, pp. 408–417.

Braithwaite, E., Pickles, A., Wright, N., Sharp, H. and Hill, J. (2020) 'Sex differences in foetal origins of child emotional symptoms: a test of evolutionary hypotheses in a large, general population cohort.' *Journal of Child Psychology and Psychiatry*, 61(11), pp.1194-1202.

Braquehais, M. D., Picouto, M. D., Casas, M., and Sher, L. (2012) 'Hypothalamic–pituitary–adrenal axis dysfunction as a neurobiological correlate of emotion dysregulation in adolescent suicide' *World Journal of Pediatrics*, 8, pp.197–206.

Brenet, F., Moh, M., Funk, P., Feierstein, E., Viale, A. J. Socci, N. D. (2011) 'DNA methylation of the first exon is tightly linked to transcriptional silencing.' *PLOS ON*E, 6, e14524.

Borghol, N., Suderman, M., McArdle, W., Racine, A., Hallett, M., Pembrey, M., Hertzman, C., and Power, C., M. Szyf. (2011) 'Associations with Early-life Socio-economic Position in Adult DNA Methylation' *International Journal of Epidemiology*, 41(1), pp.62-74.

Bosch, M., Reise, H., Reijneveld S, A., Bakker, M, P., Verhulst., Ormel , J., Oldeinkel, A,J. (2012) 'Timing matters: long term effects of adversities from prenatal period up to adolescence on adolescents' cortisol stress response, the TRIALS study'. *Psychoneuroendochtinology*, 37 (9) pp. 1439-1447

Bustamante, A., Aiello, A., Guffanti, G., Galea, S., Wildman, D. and Uddin, M. (2018) 'FKBP5 DNA methylation does not mediate the association between childhood maltreatment and depression symptom severity in the Detroit Neighbourhood Health Study' *Journal of Psychiatric Research*, 96, pp.39-48.

Bryant, R., Creamer, M., O'Donnell, M., Forbes, D., Felmingham, K., Silove, D., Malhi, G., van Hoof, M., McFarlane, A. and Nickerson, A. (2017) 'Separation from parents during childhood

trauma predicts adult attachment security and post-traumatic stress disorder.' *Psychological Medicine*, 47(11), pp.2028-2035.

Carter. S. C., Kenkel. W. M., MacLean. E. L., Wilson. S. R., Perkeybile. A. M., Yee. J, R., Ferris. C R., Nazarloo. H. P., Porges. S. W., Davis. J. M., Connelly. J. J., Kingsbury. M A., and Dantzer. R. (2020) 'Is oxytocin "Nature's medicine"?' *Pharmacol rev*, 74 (4) pp.829-861.

Cecil, C. A. M., Lysenko, L. J., Jaffee, S. R., Pingault, J.-B., Smith, R. G., Relton, C. L. and Barker, E. D. (2014) 'Environmental risk, oxytocin receptor gene (OXTR) methylation and youth callous-unemotional traits: A 13-year longitudinal study.' *Molecular Psychiatry*, 19, p.1071–1077.

Cecil, Zhang and Nolte, (2020). 'Childhood maltreatment and DNA methylation: a systematic review'. *Neuroscince Biobehavior Review* 112, pp.392-409.

Chen, W., and Taylor, E. (2006) 'Parental Account of Children's Symptoms (PACS), ADHD Phenotypes and its Application to Molecular Genetic Studies. In R. D. Oades *(Ed.),* Attention-deficit/hyperactivity disorder (AD/HD) and the hyperkinetic syndrome (HKS): Current ideas and ways forward*.' Nova Science Publishers,* pp.3-20.

Cicchetti, D. and Handley, E. (2017) 'Methylation of the glucocorticoid receptor gene, nuclear receptor subfamily 3, group C, member 1 (NR3C1), in maltreated and non-maltreated children: Associations with behavioral undercontrol, emotional lability/negativity, and externalizing and internalizing symptoms.' *Development and Psychopathology*, 29(5), pp.1795-1806.

Conradt, E., Ostlund, B., Guerin, D., Armstrong, D., Marsit, C., Tronick, E., LaGasse, L. and Lester, B. (2019) 'DNA methylation of NR3c1 in infancy: Associations between maternal caregiving and infant sex.' *Infant Mental Health Journal*, 40(4), pp.513-522.

Conradt, E., Fei, M., LaGasse, L., Tronick, E., Guerin, D., Gorman, D., Marsit, C. and Lester, B. (2015) ‘Prenatal predictors of infant self-regulation: the contributions of placental DNA methylation of NR3C1 and neuroendocrine activity.’ *Frontiers in Behavioral Neuroscience*, 9.

Criado-Marrero, M., Smith, T.M., Gould, L.A., Kim, S., Penny, H.J., Sun, Z., Gulick, D., Dickey, C.A. and Blair, L.J. (2020) ‘FKBP5 and early life stress affect the hippocampus by an age-dependent mechanism.’ *Brain, Behavior, & Immunity-Health*, *9*, p.100143.

Deidda, M., Boyd, K., Minnis, H., Donaldson, J., Brown, K., Boyer, N. and McIntosh, E. (2018) ‘Protocol for the economic evaluation of a complex intervention to improve the mental health of maltreated infants and children in foster care in the UK (The BeST? services trial).’ *BMJ Open*, 8(3), p.e020066.

Doom, J. R., Cicchetti, D., and Rogosch, F. A. (2014) ‘Longitudinal patterns of cortisol regulation differ in maltreated and no maltreated children.’ *Journal of the American Academy of Child & Adolescent Psychiatry*, 53, pp.1206–1215.

Doom, J. R., Doyle, C. M., and Gunnar, M. R. (2017) ‘Social Stress Buffering by Friends in Childhood and Adolescence: Effects on HPA and Oxytocin Activity.’ *Soc neurosci*, 12(1): pp.8-12.

Dunn, E., Soare, T., Zhu, Y., Simpkin, A., Suderman, M., Klengel, T., Smith, A., Ressler, K. and Relton, C. (2019) ‘Sensitive Periods for the Effect of Childhood Adversity on DNA Methylation: Results from a Prospective, Longitudinal Study.’ *Biological Psychiatry*, 85(10), pp.838-849.

Ein-Dor, T., Verbeke, W., Mokry, M. and Vrtička, P. (2018) ‘Epigenetic modification of the oxytocin and glucocorticoid receptor genes is linked to attachment avoidance in young adults.’ *Attachment Human Development*, 20(4), pp.439-454.

Essex, M., Thomas Boyce, W., Hertzman, C., Lam, L., Armstrong, J., Neumann, S. and Kobor, M. (2011) 'Epigenetic Vestiges of Early Developmental Adversity: Childhood Stress Exposure and DNA Methylation in Adolescence.' *Child Development*, 84(1), pp.58-75.
Folger, A.T, Ding, L., Hong, J., Yolton, K., Ammerton, R, T., Van Ginkel, J,b., and Bowers, K, (2019) 'Neonatal NR3C1 methylation and social-emotional development at 6 and 18 months of age', *Frontiers in Behavioural Neuroscience*, 13.

Festante, F., Rayson, H., Paukner, A., Kaburu, S,K S., Toschi, G., Fox, N, A., and Ferrari, P, F. (2021) 'Oxytocin prootes prosocial behaviour and related neural responses in infant macaques at-risk for compromised social development.' Dev cogn neurosci

Florea, T., Matei Palimariciuc, M., Ana Caterina Cristofor, C. A., Dobrin, I., Chiriță, R., Magdalena Bîrsan, M., Dobrin, R. P., and Pădurariu, M. (2022) 'Oxytocin: Narrative expert review of current perspectives on the relationship with other neurotransmitters and the impact on the main psychiatric disorders.' *Medicina (Kaunus)* 58 (7), 923.

Fries, G. R., Vasconcelos-Moreno, M. P., Gubert, C., dos Santos, B. T., Sartori, J., Eisele, B., Ferrari, P., Fijtman, A., Ruegg, J., Gassen, N., Kapczinski, F., Rein, T., Kauer-sant'Anna, M. (2015) 'Hypothalamic–pituitary–adrenal axis dysfunction and illness progression in bipolar disorder'. *International Journal of Neuropsychopharmacology*, 18(1)

Fischer, S., Schumacher, T., Knaevelsrud, C., Ehlert, U., and Sarah Schumacher, S (2009) 'Genes and hormones of the hypothalamic–pituitary–adrenal axis in post-traumatic stress disorder. What is their role in symptom expression and treatment response?' *J Neural Transm (Vienna),* 126 (9) pp.1279-1286.

Géranton, S. (2019) ‘Does epigenetic ‘memory’ of early-life stress predispose to chronic pain in later life? A potential role for the stress regulator FKBP5.’ *Philosophical Transactions of the Royal Society B: Biological Sciences*, 374(1785), p.20190283.

Gouin, J. P., Zhou, Q. Q., Booij, L., Boivin, M., Côté, S. M., Hébert, M., Vitaro, F. (2017) ‘Associations among oxytocin receptor gene (OXTR) DNA methylation in adulthood, exposure to early life adversity, and childhood trajectories of anxiousness.’ *Scientific Reports, 7*(7446).

Gottschalk, M, G and Domschke, K (2016) ‘Novel developments in genetics and epigenetic mechanism of anxiety’ *current opinion psychiatry* 29 (1): pp 32-38.

Gotlib, I., Borchers, L., Chahal, R., Gifuni, A. and Ho, T. (2020) ‘Early Life Stress Predicts Depressive Symptoms in Adolescents During the COVID-19 Pandemic: The Mediating Role of Perceived Stress.’ *frontiers in phycology*, 11: 603748.

Goldman, D., Heilig, M., Higley, J., Suomi, S. and Barr, C. (2017) ‘Early rearing history influences oxytocin receptor epigenetic regulation in rhesus macaques.’ *Proceedings of the National Academy of Sciences*, 114(44), pp.11769-11774.

Hammerton, G., Murray, J., Maughan, B., Barros, F., Gonçalves, H., Menezes, A., Wehrmeister, F., Hickman, M. and Heron, J. (2019) ‘Childhood Behavioural Problems and Adverse Outcomes in Early Adulthood: A Comparison of Brazilian and British Birth Cohorts.’ *Journal of Developmental and Life-Course Criminology*, 5(4), pp.517-535.

Hartmann, J., Bajaj, T., Klengel, C., Chatzinakos, C., Ebert, T., Dedic., N., McCullough, K, M., Lardenoije, R., Joels, M., Meijer, O, C., MaCann, K, E., Dudek, S, M., Sarabdjitsingh, R, A., Klengel, T., Gassen, N, C., Schidt, M, V., and Ressler, K, J, (2021) ‘Mineralocorticoid receptors

dampen glucocortricoid receptor sensitivity to stress via regulation of FKBP5' Cell reports, 35 (9), 109185.

Han, K., Won, E., Sim, Y., Kang, J., Han, C., Kim, Y., Kim, S., Joe, S., Lee, M., Tae, W. and Ham, B. (2017) 'Influence of FKBP5 polymorphism and DNA methylation on structural changes of the brain in major depressive disorder.' *Scientific Reports*, 7(1).

Harris, A, R., and Santos, P, H. (2020) 'Maternal depression in Latinas and child socioemotional development: A systematic review.' 5 (3), e0230256.

Han, H., Cortez, C.C., Yang, X., Nichols, P.W., Jones, P.A. and Liang, G. (2011) 'DNA methylation directly silences genes with non-CpG island promoters and establishes a nucleosome occupied promoter.' *Human molecular genetics*, *20*(22), pp.4299-4310.

Hernando-Herraez, I., Garcia-Perez, R., Sharp, A. J., and Marques-Bonet, T. (2015) 'DNA methylation: Insights into human evolution' *PLOS Genetics*, 11, e1005661.

Hill, J., Pickles, A., Wright, N., Quinn, J., Murgatroyd, C. and Sharp, H. (2019) 'Mismatched Prenatal and Postnatal Maternal Depressive Symptoms and Child Behaviours: A Sex-Dependent Role for NR3C1 DNA Methylation in the Wirral Child Health and Development Study.' *Cells*, 8(9), p.943.

Hiller, R., Halligan, S., Meiser-Stedman, R., Elliott, E. and Rutter-Eley, E. (2020). 'Supporting the emotional needs of young people in care: a qualitative study of foster carer perspectives.' *BMJ Open*, 10(3), p.e033317.

Hohne, N., Poidinger, M., Merz, F., Pfister, H., Bruckl, T., Zimmermann, P., Uhr, M., Holsboer, F. and Ising, M. (2014). 'FKBP5 Genotype-Dependent DNA Methylation and mRNA Regulation

After Psychosocial Stress in Remitted Depression and Healthy Controls.' *International Journal of Neuropsychopharmacology*, 18(4),.

Holmes, L., Shutman, E., Chinaka, C., Deepika, K., Pelaez, L. and Dabney, K. (2019) 'Aberrant Epigenomic Modulation of Glucocorticoid Receptor Gene (NR3C1) in Early Life Stress and Major Depressive Disorder Correlation: Systematic Review and Quantitative Evidence Synthesis' *International Journal of Environmental Research and Public Health*, 16(21), p.4280.

Hoeijmakers, L., Harbich, D., Schmid, B., Lucassen, P., Wagner, K., Schmidt, M. and Hartmann, J. (2014) 'Depletion of FKBP51 in Female Mice Shapes HPA Axis Activity.' *PLoS ONE*, 9(4), p.e95796.

Humphreys, K.L., Moore, S.R., Davis, E.G., Maclsaac, J.L., Lin, D.S., Kober, M.S., and Gotlib, I.H. (2019) 'DNA methylation of HPA-axis genes and the onset of major depressive disorder in adolescent girls: a prospective analysis.' *Translational Psychiatry* 9, 245.

Jang, H.S., Shin, W.J., Lee, J.E. and Do, J.T. (2017) 'CpG and non-CpG methylation in epigenetic gene regulation and brain function.' *Genes*, *8*(6), p.148.

Keijser, R., Olofsdotter, S., Nilsson, K.W. and Åslund, C. (2021) 'Three-way interaction effects of early life stress, positive parenting and FKBP5 in the development of depressive symptoms in a general population.' *Journal of Neural Transmission*, *128*(9), pp.1409-1424.

Kertes, D., Kamin, H., Hughes, D., Rodney, N., Bhatt, S. and Mulligan, C. (2016) 'Prenatal Maternal Stress Predicts Methylation of Genes Regulating the Hypothalamic-Pituitary-Adrenocortical System in Mothers and Newborns in the Democratic Republic of Congo.' *Child Development*, 87(1), pp.61-72.

Kim, J.K., Samaranayake, M. and Pradhan, S. (2009). ‘Epigenetic mechanisms in mammals.’ *Cellular and Molecular Life Sciences*, *66*(4), pp.596-612.

Klengel, T., Mehta, D., Anacker, C., Rex-Haffner, M., Pruessner, J., Pariante, C., Pace, T., Mercer, K., Mayberg, H., Bradley, B., Nemeroff, C., Holsboer, F., Heim, C., Ressler, K., Rein, T. and Binder, E. (2013) ‘Allele-specific FKBP5 DNA demethylation mediates gene–childhood trauma interactions’ N*ature Neuroscience*, 16(1), pp.33-41.

Kuchenbecker, Y. S., Pressman, S. D., Jared Celniker, J., Grewen, K. M., Sumida, M. D., Naveen Jonathan, N., Brendan Everett, B., George M Slavich, M. G. (2021) ‘Oxytocin, cortisol, and cognitive control during acute and naturalistic stress.’ 24 (4), pp.370-383.

Klinger-König, J., Hertel, J., Van der Auwera, S., Frenzel, S., Pfeiffer, L., Waldenberger, M., Golchert, J., Teumer, A., Nauck, M., Homuth, G., Völzke, H. and Grabe, H. (2019) ‘Methylation of the FKBP5 gene in association with FKBP5 genotypes, childhood maltreatment and depression.’ *Neuropsychopharmacology*, 44(5), pp.930-938.

Kogan, S.M., Bae, D., Cho, J., Smith, A.K. and Nishitani, S. (2019) ‘Childhood adversity, socioeconomic instability, oxytocin-receptor-gene methylation, and romantic-relationship support among young African American men.’ *Psychological Science*, *30*(8), pp.1234-1244.

Kosten, T. A., and Nielsen, D. A. (2014) ‘Litter and sex effects on maternal behaviour and DNA methylation of the Nr3c1 exon 17 promoter gene in hippocampus and cerebellum.’ *International Journal of Developmental Neuroscience*, 36, pp.5–12.

Kubzansky, L.D., Mendes, W.B., Appleton, A.A., Block, J. and Adler, G.K. (2012) ‘A heartfelt response: oxytocin effects on response to social stress in men and women’ *Biological Psychology*, *90*(1), pp.1-9.

.Kulski, J. K. (2016) ‘Next-Generation Sequencing — An Overview of the History, Tools, and “Omic” Applications, Next Generation Sequencing - Advances, Applications and Challenges. London: Intech Open.’ pp.3–60.

Lewis, R, C., Breitenstein, S, R., Henderson, A., Hayley A Sowards, A, H., Piras, S, I., Huentelman, J, M., Doane, D, L., Lemery-Chalfant, K., (2021). ‘Harsh Parenting Predicts Novel HPA Receptor Gene Methylation and NR3C1 Methylation Predicts Cortisol Daily Slope in Middle Childhood.’ Cell Molecular Neurobiology, (4), pp.783-793.

Labonté, B., Suderman, M., Maussion, G., Navaro, L., Yerko, V., Mahar, I., Bureau, A., Mechawar, N., Szyf, M., Meaney, M.J. and Turecki, G. (2012) ‘Genome-wide epigenetic regulation by early-life trauma’ *Archives of General Psychiatry*, *69*(7), pp.722-731.

Laryea, G., Muglia, L., Arnett, M., and Muglia, L. J. (2015) ‘Dissection of glucocorticoid receptor-mediated inhibition of the hypothalamic–pituitary– adrenal axis by gene targeting in mice.’ *Frontiers in Neuroendocrinology*, 36, pp.150–164.

Lieberman, R., Kranzler, H., Levine, E. and Covault, J. (2017) ‘Examining FKBP5 mRNA expression in human iPSC-derived neural cells.’ *Psychiatry Research,* 247, pp.172-181.

Lester, M, B., Marsit, J, C., Giarraputo, J., Hawes, K., LaGasse, L., L and Padburuy, F, J., (2015). ‘Neurobehaviour related to epigenetic differences in preterm infants.’ *Epigenomics* 7(7), pp.1123-1136

Martín-Blanco, A., Soler, J., Villalta, L., Feliu-Soler, A., Elices, M., Pérez, V., Arranz, M., Ferraz, L., Álvarez, E. and Pascual, J. (2014) ‘Exploring the interaction between childhood maltreatment

and temperamental traits on the severity of borderline personality disorder.' *Comprehensive Psychiatry*, 55(2), pp.311-318.

Maud, C., Ryam, J., Mclntosh., J., and Olsson, C., (2018) 'The role of oxytocin receptor (OXTR) DNA methylation (DNAm) in trauma social and emotional functioning: A systematic narrative review'. *BMC Psychiatry*, 18: 154.

Mak, M., Yin, L., Li, M., Cheung, R. and Oon, P. (2020). 'The Relation between Parenting Stress and Child Behavior Problems: Negative Parenting Styles as Mediator.' *Journal of Child and Family Studies*, 29(11), p.2993-3003.

Mansolf, M., Blackwell, C., Cummings, P., Choi, S. and Cella, D. (2022) 'Linking the Child Behavior Checklist to the Strengths and Difficulties Questionnaire.' *Psychological Assessment*, 34(3), pp.233-246.

Mcleigh, J., Tunnell, K. and Lazcano, C. (2021) 'Developmental Status of Young Children in Foster Care.' *J Dev Behav Pediatr*, 42(5), pp.389- 400.

McGowan, P., Sasaki, A., D'Alessio, A., Dymov, S., Labonté, B., Szyf, M., Turecki, G. and Meaney, M. (2009) 'Epigenetic regulation of the glucocorticoid receptor in human brain associates with childhood abuse.' *Nature Neuroscience*, 12(3), pp.342-348.

Mcgowan O, P., Suderman, M., Sasaki, A., Huang, T., Hallott, M., Meaney, J, M., and Szyf, M. (2011) 'Broad epigenetic signature of maternal care in the brain of adult rats.' *PLoS ONE*, 6(2) e14739.

McLeigh, J., Tunnell, K. and Lazcano, C. (2021) 'Developmental Status of Young Children in Foster Care.' *Journal of Developmental & amp Behavioral Pediatrics*, 42(5), pp.389-400.

Meloni, M. (2014) 'The social brain meets the reactive genome: Neuroscience, epigenetics and the new social biology.' *Frontiers in Human Neuroscience*, 8, (309).

Menke, A., Klengel, T., Rubel, J., Brückl, T., Pfister, H., Lucae, S., Uhr, M., Holsboer, F. and Binder, E. (2013) 'Genetic variation in FKBP5 associated with the extent of stress hormone dysregulation in major depression.' *Genes, Brain and Behavior*, 12(3), pp.289-296.

Mulder, R., Rijlaarsdam, J., Luijk, M., Verhulst, F., Felix, J., Tiemeier, H., Bakermans-Kranenburg, M. and Van Ijzendoorn, M. (2017) 'Methylation matters: FK506 binding protein 51 (FKBP5) methylation moderates the associations of FKBP5 genotype and resistant attachment with stress regulation.' *Development and Psychopathology*, 29(2), pp.491-503.

Mulligan, C., D'Errico, N., Stees, J. and Hughes, D. (2012) 'Methylation changes at NR3C1 in newborns associate with maternal prenatal stress exposure and newborn birth weight.' *Epigenetics*, 7(8), pp.853-857.

Murgatroyd, C. and Spengler, D. (2011) 'Epigenetics of Early Child Development.' *Frontiers in Psychiatry*, 2(16).

Na, K., Chang, H., Won, E., Han, K., Choi, S., Tae, W., Yoon, H., Kim, Y., Joe, S., Jung, I., Lee, M. and Ham, B. (2014) 'Association between Glucocorticoid Receptor Methylation and Hippocampal Subfields in Major Depressive Disorder.' *PLoS ONE*, 9(1), p.e85425.

Needham, B.L., Smith, J.A., Zhao, W., Wang, X., Mukherjee, B., Kardia, S.L., Shively, C.A., Seeman, T.E., Liu, Y. and Diez Roux, A.V. (2015) 'Life course socioeconomic status and DNA methylation in genes related to stress reactivity and inflammation: The multi-ethnic study of atherosclerosis.' *Epigenetics*, *10*(10), pp.958-969.

Norman, G.J., Hawkley, L., Luhmann, M., Ball, A.B., Cole, S.W., Berntson, G.G. and Cacioppo, J.T. (2012) ‘Variation in the oxytocin receptor gene influences neurocardiac reactivity to social stress and HPA function: a population-based study.’ *Hormones and Behavior*, *61*(1), pp.134-139.

Nugent, N.R., Tyrka, A.R., Carpenter, L.L. and Price, L.H. (2011). ‘Gene–environment interactions: early life stress and risk for depressive and anxiety disorders.’ *Psychopharmacology*, *214*(1), pp.175-196.

Oberlander, T., Weinberg, J., Papsdorf, M., Grunau, R., Misri, S. and Devlin, A (2008). ‘Prenatal exposure to maternal depression, neonatal methylation of human glucocorticoid receptor gene (NR3C1) and infant cortisol stress responses.’ *Epigenetics*, 3(2), pp.97-106.

O’Farrelly, C., Barker, Beth., Watt, H., Babalis, D., Bakermans- Kranenburg, M., Sarah Byford, S., Poushali Ganguli, P., Grimås, E., Iles, J., Mattock, H., McGinley, J., Phillips, C., Ryan, R., Scott, S., Jessica Smith, Stein, A., Stevens, E., van IJzendoorn, M., Warwick, J., and Ramchandani, P (2021). ‘A video feedback parenting intervention to prevent enduting behaviour problems in at risk children aged 12-36 months: the Health Start Happy Start.’ *health technol assess* 25 (29) pp.1-84.

Olff, M., Frijling, J.L., Kubzansky, L.D., Bradley, B., Ellenbogen, M.A., Cardoso, C., Bartz, J.A., Yee, J.R. and Van Zuiden, M. (2013) ‘The role of oxytocin in social bonding, stress regulation and mental health: an update on the moderating effects of context and interindividual differences.’ *Psychoneuroendocrinology*, *38*(9), pp.1883-1894.

Pan, P., Fleming, A. S., Lawson, D., Jenkins, J. M., and McGowan, P. O. (2014) ‘Within- and between-litter maternal care alter behavior and gene regulation in female offspring.’ *Behavioral Neuroscience*, 128, pp.736–748.

Paquette, A. G., Lester, B. M., Lesseur, C., Armstrong, D. A., Guerin, D. J., Appleton, A. A., Marsit, C. (2015) ‘Placental epigenetic patterning of glucocorticoid response genes is associated with infant neurodevelopment.’ *Epigenomics*, 7, p.767–779

Parade, S., Parent, J., Rabemananjara, K., Seifer, R., Marsit, C., Yang, B., Zhang, H. and Tyrka, A. (2017) ‘Change in FK506 binding protein 5 (FKBP5) methylation over time among preschoolers with adversity.’ *Development and Psychopathology*, 29(5), pp.1627-1634.

Parade, S., Ridout, K., Seifer, R., Armstrong, D., Marsit, C., McWilliams, M. and Tyrka, A. (2016) ‘Methylation of the Glucocorticoid Receptor Gene Promoter in Preschoolers: Links with Internalizing Behavior Problems’. *Child Development*, 87(1), pp.86-97.

Pariante, C. (2014) ‘Depression during pregnancy: molecular regulations of mothers’ and children's behaviour.’ *Biochemical Society Transactions*, 42(2), pp.582-586.

Palma-Gudiel, H., Córdova-Palomera, A., Leza, J. C. and Fañanás, L. (2015) ‘Glucocorticoid receptor gene (NR3C1) methylation processes as mediators of early adversity in stress-related disorders causality: a critical review.’ *Neuroscience Biobehavioral Review* 55, pp.520–535.

Perroud, N., Paoloni-Giacobino, A., Prada, P., Olié, E., Salzmann, A., Nicastro, R., Guillaume, S., Mouthon, D., Stouder, C., Dieben, K., Huguelet, P., Courtet, P. and Malafosse, A. (2011) ‘Increased methylation of glucocorticoid receptor gene (NR3C1) in adults with a history of childhood maltreatment: a link with the severity and type of trauma.’ *Translational Psychiatry*, 1(12), p.e59

Perroud, N., Salzmann, A., Prada, P., Nicastro, R., Hoeppli, M., Furrer, S., Ardu, S., Krejci, I., Karege, F. and Malafosse, A. (2013) ‘Response to psychotherapy in borderline personality

disorder and methylation status of the BDNF gene.' *Translational Psychiatry*, 3(1), pp.e207-e207.

Fries, G., Gassen, N, C., Rein, T. (2015). 'The FKBP51-Glucocorticoid Receptor Balance in Stress-Related Mental Disorders' *Current Molecular Pharmacology*, 9(2), pp.126-140.

Ramo-Fernández, L., Gumpp, A., Boeck, C., Krause, S., Bach, A., Waller, C., Kolassa, I. and Karabatsiakis, A. (2021) 'Associations between childhood maltreatment and DNA methylation of the oxytocin receptor gene in immune cells of mother–newborn dyads.' *Translational Psychiatry*, 11(1).

Raftopoulos, L., Katsi, V., Makris, T., Tousoulis, D., Stefanadis, C. and Kallikazaros, I. (2015) 'Epigenetics, the missing link in hypertension.' *Life sciences*, 129, pp.22-26.

Ruttle, P. L., Shirtcliff, E. A., Serbin, L. A., Fisher, D. B., Stack, D. M., and Schwartzman, A. E. (2011) 'Disentangling psychobiological mechanisms underlying internalizing and externalizing behaviors in youth: Longitudinal and concurrent associations with cortisol.' *Hormones and Behaviour*, 59, pp.123–132.

Santo, H., Nephew, B., Bhattacharya, A., Tan., X., Smith, L., Alyamani, R., Martin, E., Perreira K., Fry, R., and Murgatroyd, C. (2018) 'Discrimination exposure and DNA methylation of stress-related genes in Latina mothers.' *Psychoneuroendocrinology*, 98, pp.131-138.

Schechter, S, D., Moser, A, D., Paoloni-Giacobino, A., Stenz, L, Gex-Fabry, M., Tatjana Aue, T., Adouan, W., Cordero, I, M., Suardi, F., Aurelia Manini, A.,Ana Sancho Rossignol,S., Merminod, G., Ansermet, F., Alexandre G Dayer, G, A., Sandra Rusconi Serpa, R,S. (2015) 'Methylation of NR3C1 is related to maternal PTSD parenting stress and maternal medial prefrontal cortical activity.' *front psychol* 6:690.

Shevlin, M., McBride, O., Murphy, J., Miller, J., Hartman, T., Levita, L., Mason, L., Martinez, A., McKay, R., Stocks, T., Bennett, K., Hyland, P., Karatzias, T. and Bentall, R. (2020) 'Anxiety, depression, traumatic stress and COVID-19-related anxiety in the UK general population during the COVID-19 pandemic.' *BJPsych Open*, 6(6).

Shields, A.E., Wise, L. A., Ruiz-Narvaez, A, E., Seddigh, B., Byun, H., Cozier, Y, C., Rosenburg, L., Palmer, J, R., and Baccarelli, A, A, (2016) 'Childhood abuse, promoter methylation of Leukocyte *nr3c1* and the potential modifying effect of emotional support', *Epigenomics*, 8(11), pp. 1507–1517.

Sumner, J., Gambazza, S., Gao, X., Baccarelli, A., Uddin, M. and McLaughlin, K. (2022) 'Epigenetics of early-life adversity in youth: cross-sectional and longitudinal associations.' *Clinical Epigenetics*, 14(1).

Simons, R.L., Lei, M.K., Beach, S.R., Cutrona, C.E. and Philibert, R.A. (2017) 'Methylation of the oxytocin receptor gene mediates the effect of adversity on negative schemas and depression.' *Development and Psychopathology*, *29*(3), pp.725-736.

.

Smart, C., Strathdee, G., Watson, S., Murgatroyd, C. and McAllister-Williams, R. (2015) 'Early life trauma, depression and the glucocorticoid receptor gene – an epigenetic perspective.' *Psychological Medicine*, 45(16), pp.3393-3410.

Turner R,J., Thomas C, S., Brown T, H., (2016) Childhood adversity and adult health: Evaluating intervening mechanisms. *Soc Sci Med* ; 156:114-24.

Tyrka, A. R., Lee, J. K., Graber, J. A., Clement, A. M., Kelly, M. M., DeRose, L., Warren., M, Brooks-Gunn, J. (2012). 'Neuroendocrine predictors of emotional and behavioural adjustment in boys: Longitudinal follow-up of a community sample.' *Psychoneuroendocrinology*, 37, pp.2042–2046.

Tyrka, A. R., Parade, S. H., Eslinger, N. M., Marsit, C. J., Lesseur, C., Armstrong, D. A., Phillip, N., Josefson, N., Seifer, R. (2015). 'Methylation of exons 1D, 1F, and 1H of the glucocorticoid receptor gene promoter and exposure to adversity in preschool-aged children.' *Development and Psychopathology*, 27, pp.577–585.

Tyrka, A., Price, L., Marsit, C., Walters, O. and Carpenter, L. (2012) 'Childhood Adversity and Epigenetic Modulation of the Leukocyte Glucocorticoid Receptor: Preliminary Findings in Healthy Adults.' PLoS ONE, 7(1), p.e30148.

Tyrka, A., Ridout, K., Parade, S., Paquette, A., Marsit, C. and Seifer, R. (2015). 'Childhood maltreatment and methylation of FK506 binding protein 5 gene (FKBP5).' *Development and Psychopathology*, 27(4pt2), pp.1637-1645.

Tyrka, A.R., Ridout, K.K. and Parade, S.H. (2016). 'Childhood adversity and epigenetic regulation of glucocorticoid signaling genes: Associations in children and adults.' *Development and Psychopathology*, *28*(4pt2), pp.1319-1331.

Unternaehrer, E., Meyer, A. H., Burkhardt, S. C. A., Dempster, E., Staehli, S., Theill, N., Meinlschmidt, G. (2015) 'Childhood maternal care is associated with DNA methylation of the genes for brain-derived neurotrophic factor (BDNF) and oxytocin receptor (OXTR) in peripheral blood cells in adult men and women.' *Stress*, 18, 451–461

Unternaehrer, E., Bolten, M., Nast, I., Staehli, S., Meyer, A., Dempster, E., Hellhammer, D., Lieb, R. and Meinlschmidt, G. (2016). ‘Maternal adversities during pregnancy and cord blood oxytocin receptor (OXTR) DNA methylation.’ *Social Cognitive and Affective Neuroscience*, 11(9), p.1460-1470.

Van Aswegen, T., Bosmans, G., Goossens, L., Van Leeuwen, K., Claes, S., Van Den Noortgate, W. and Hankin, B. (2021) ‘Epigenetics in Families: Covariance between Mother and Child Methylation Patterns.’ *Brain Sciences*, 11(2), p.190.

van der Knaap, L. J., Riese, H., Hudziak, J. J., Verbiest, M. M., Verhulst, F. C., Oldehinkel, A. J., Van Oort, F, V, A. (2014) ‘Glucocorticoid receptor gene (NR3C1) methylation following stressful events between birth and adolescence: The TRAILS study.’ *Translational Psychiatry*, 4, e381.

Weaver, I., Cervoni, N., Champagne, F., D’Alessio, A., Sharma, S., Seckl, J., Dymov, S., Szyf, M., and Meaney, M. (2007). ‘Epigenetic Programming by Maternal Behaviour.’ *Epigenetics*, 2(1), pp.22-28.

Wiechmann, T., Röh, S., Sauer, S., Czamara, D., Arloth, J., Ködel, M., Beintner, M., Knop, L., Menke, A., Binder, E. and Provençal, N. (2019). ‘Identification of dynamic glucocorticoid-induced methylation changes at the FKBP5 locus.’ *Clinical Epigenetics*, 11(1).

Wu, Y., Zhang, C., Liu, H., Duan, C., Li, C., Fan, J., Li, H., Chen, L., Xu, H., Li, X., Guo, Y., Wang, Y., Li, X., Li, J., Zhang, T., You, Y., Li, H., Yang, S., Tao, X., Xu, Y., Lao, H., Wen, M., Zhou, Y., Wang, J., Chen, Y., Meng, D., Zhai, J., Ye, Y., Zhong, Q., Yang, X., Zhang, D., Zhang, J., Wu, X., Chen, W., Dennis, C. and Huang, H. (2020) ‘Perinatal depressive and anxiety symptoms of pregnant women during the coronavirus disease 2019 outbreak in China.’ *American Journal of Obstetrics and Gynecology*, 223(2), p.240.e1-240.e9.

Yehuda, R., Daskalakis, N., Desarnaud, F., Makotkine, I., Lehrner, A., Koch, E., Flory, J., Buxbaum, J., Meaney, M. and Bierer, L. (2013) 'Epigenetic Biomarkers as Predictors and Correlates of Symptom Improvement Following Psychotherapy in Combat Veterans with PTSD.' *Frontiers in Psychiatry*, 4.

Wilkinson, M. F. (2015) 'Evidence that DNA methylation engenders dynamic gene regulation.' *Proc. Natl. Acad. Sci. U.S.A.* 112, p.E2116.

Yehuda, R., Daskalakis, N.P., Bierer, L.M., Bader, H.N., Klengel, T., Holsboer, F. and Binder, E.B. (2016) 'Holocaust exposure induced intergenerational effects on FKBP5 methylation. Biological psychiatry.' 80(5), pp.372-380.

Zannas AS, Wiechmann T, Gassen NC, Binder EB. (2016) 'Gene-Stress-Epigenetic Regulation of FKBP5: Clinical and Translational Implications.' *Neuropsychopharmacology* 41(1): pp.261-74.

Zuberi, M., Dholariya, S., Khan, I., Mir, R., Guru, S., Sumi, M. and Saxena, A. (2021) 'Epigenetic Silencing of DAPK1and p16INK4a Genes by CpG Island Hypermethylation in Epithelial Ovarian Cancer Patients.' *Indian Journal of Clinical Biochemistry*, *36*(2), pp.200-207.

Zannas, A. S., and Binder, E. B. (2014) 'Gene–environment interactions at the FKBP5 locus: Sensitive periods, mechanisms and pleiotropism.' *Genes, Brain and Behavior*, 13, pp.25–37.

Zhang, T. Y., Labonte, B., Wen, X. L., Turecki, G., and Meaney, M. J. (2013) 'Epigenetic mechanisms for the early environmental regulation of hippocampal glucocorticoid receptor gene expression in rodents and humans.' *Neuropsychopharmacology*, 38, pp.111–123.

Zimmermann, P., Brückl, T., Nocon, A., Pfister, H., Binder, E.B., Uhr, M., Lieb, R., Moffitt, T.E., Caspi, A., Holsboer, F. and Ising, M. (2011) 'Interaction of FKBP5 gene variants and adverse life events in predicting depression onset: results from a 10-year prospective community study.' *American Journal of Psychiatry*, *168*(10), pp.1107-1116.

9. Appendices

Appendix 1. Sodium bisulfite treatment (500 NG DNA)

Sample	Concentration	A260/280	A260/230	Volume	Amount
3	71.2	1.83	1.46	50	3560
4	55.4	2.07	1.12	50	2770
6	14.2	2.16	0.5	50	715
7	49.5	1.96	1.06	50	2470
8	9.3	2.15	0.37	50	465
9	22.4	2.05	0.67	50	1120
10	59.7	1.92	1.34	50	2985
11	18.6	2.12	0.54	50	930
12	36.1	1.95	1.01	50	1085
13	45.2	1.94	1.12	50	2260
14	24.5	1.99	0.94	50	1225
15	33.4	1.97	1.01	50	1670
16	24.2	1.94	0.55	50	1210
17	40.7	1.89	1.1	50	2035
18	43.9	1.94	1.06	50	2195
19	27.7	2.02	0.91	50	1360
20	34.2	1.94	0.94	50	1710

21	28.1	1.99	0.92	50	1405
22	20.1	1.96	0.66	50	1005
23	46	1.87	1.01	50	2300
24	23.2	1.85	0.7	50	1160
25	29.9	1.9	0.74	50	1495
26	26.9	1.98	0.9	50	1395
27	52.8	1.87	1.25	50	2640
28	23.5	1.94	0.74	50	2675
29	29.7	1.87	0.82	50	1485
30	24.2	2.02	0.79	50	1210
31	32.9	1.9	0.84	50	1645
32	53.9	1.89	1.3	50	2695
33	26.9	1.96	0.94	50	1345
34	33.9	1.91	0.85	50	1695
35	41.4	1.93	1.06	50	2070
36	43.5	1.9	0.92	50	2175
37	20.6	2.05	0.72	50	1030
38	25.1	2.05	0.77	50	1255
39	47	1.95	1	50	2350
40	17.1	2.03	0.47	50	855
41	57.3	1.86	1.15	50	2865
42	66.8	1.83	1.45	50	3340
43	80.7	1.81	1.29	50	4035
44	86.7	1.83	1.24	50	4335
45	76.2	1.83	1.52	50	2760
46	26.6	1.84	0.84	50	1330
47	32.1	1.86	1	50	1605
48	22.4	1.85	0.81	50	1120
49	53.2	1.84	1.19	50	2660
50	26.9	1.8	1.05	50	1345
51	33.5	1.82	0.93	50	1675
52	47.5	1.85	1.3	50	2375
53	33.3	1.87	1.04	50	1665
54	54.9	1.83	1.18	50	2745
55	37.5	1.79	0.87	50	1875
56	25.1	1.82	0.79	50	1255
57	32.1	1.83	0.85	50	1605
58	44.5	1.9	1.09	50	2225
59	38.3	1.83	1.09	50	1915
60	54	1.87	1.32	50	2700
61	24.8	1.79	0.86	50	1225

62	46.6	1.86	0.89	50	1635
63	24.5	1.86	0.71	50	285
64	32.7	1.92	1.06	50	1660
65	5.7	1.71	0.27	50	285
66	33.2	1.68	0.79	50	1660
67	25.7	1.91	1.82	50	1285
68	67.1	1.9	0.79	50	1855
69	19.1	1.99	0.65	50	955
70	38.8	1.84	0.85	50	1940
71	36.2	1.92	0.76	50	1810
72	30.2	1.86	0.8	50	1510
73	33.7	1.92	0.73	50	1685
74	27.1	1.99	0.71	50	1355
75	40.5	1.89	1	50	2025
76	36.3	1.83	0.84	50	1815
77	37.4	1.73	0.67	50	1870
78	23.2	1.83	0.53	50	1160
79	87.9	1.74	0.85	50	4395
80	55.1	1.84	0.9	50	2755
81	33.6	1.9	0.81	50	1680
82	76.4	1.88	1.23	50	3820
83	49.3	1.87	1.32	50	2470
84	25.4	1.94	0.69	50	1270
85	70	1.86	1.29	50	3500
86	58	1.85	1.37	50	2900
87	27.4	1.91	1.79	50	1370
88	23.9	1.83	0.66	50	1195
89	40.3	1.84	0.98	50	2015
90	33.8	1.85	0.94	50	1960
91	26.2	1.86	0.56	50	1310
92	23.6	1.8	0.77	50	1180
93	37	1.91	0.95	50	1850
94	60.9	1.8	1.31	50	3045
95	48.9	1.84	1.13	50	2445
96	18.4	1.84	0.61	50	920
97	38.9	1.88	0.64	50	1945
98	57	1.84	1.84	50	2850
99	28.1	1.67	0.67	50	1405
100	33.6	1.75	0.96	50	1680
101	33.2	1.81	0.86	50	1660
102	47.7	1.76	0.7	50	2385

103	34.2	1.75	0.78	50	1710
104	47.8	1.82	1.05	50	2390
105	101.4	1.82	1.23	50	5070
106	46.1	1.77	1	50	2305
107	25.7	1.9	0.79	50	1285
108	31.5	1.86	0.87	50	1575
109	36.4	1.81	0.75	50	1820
110	39	1.76	0.85	50	1950
111	36.5	1.86	0.82	50	1825
112	42.8	1.81	0.86	50	1640
113	40.6	1.78	0.76	50	2030
114	50.9	1.89	1.14	50	2545
115	34.1	1.8	0.87	50	1705
116	52.6	1.91	1.08	50	2630
117	38.3	1.87	0.92	50	1915
118	48.2	1.88	0.95	50	2410
119	52	1.89	1.15	50	2600
120	12.7	1.88	0.36	50	635
121	22.3	1.96	0.67	50	1115
122	49.8	1.9	0.83	50	2490
123	42.7	1.9	0.9	50	2135
124	43.9	1.86	0.78	50	2195
125	35.7	1.88	0.92	50	1785
126	31.5	1.83	0.77	50	1575
127	43.4	1.92	1.71	50	1575
128	24.1	1.92	0.64	50	1205
129	22.4	1.77	0.55	50	1120
130	37.5	1.88	0.99	50	1875
131	61.7	1.89	1.03	50	3085
132	27.2	1.97	0.63	50	1360
133	27.9	1.88	0.69	50	1395
134	26.9	1.93	0.64	50	1345
135	31.9	1.93	0.92	50	1595
136	28.7	1.98	0.76	50	1435
137	64.2	1.88	0.94	50	3210
138	62.1	1.89	1.14	50	3105
139	71.8	1.86	1.15	50	3590
140	75.6	1.91	1.27	50	3780
141	65.2	1.88	1.12	50	3260
142	45.6	1.93	0.96	50	2260
143	34.7	1.85	0.73	50	1735

144	44.9	1.88	0.99	50	2245
145	45.9	1.85	0.81	50	2295
146	52.9	1.89	0.99	50	2630
147	55.8	1.88	1.09	50	2790
148	32.2	1.9	0.82	50	1610
149	36.8	1.97	0.65	50	1840
150	42.6	1.86	0.92	50	2130
151	43.3	1.91	1.02	50	2165
152	32.2	1.96	0.81	50	1610
153	22.1	1.95	0.54	50	1105
154	32.2	1.9	0.85	50	1610
155	32.7	1.84	0.81	50	1635
156	34.8	1.88	0.88	50	1740
157	36.3	1.85	0.78	50	1815
158	60.4	1.83	1.13	50	3020
159	34.8	1.81	0.84	50	1740
160	61.9	1.83	1.11	50	3095
161	73.4	1.82	1.03	50	3670
162	63.1	1.67	0.58	50	3155
163	61.1	1.75	0.69	50	2055
164	45	1.84	0.76	50	2250
165	62.9	1.85	1.02	50	3145
166	56.8	1.86	0.87	50	2840
167	40.4	1.87	0.77	50	2020
168	40.8	1.87	0.7	50	2040
169	61.5	1.78	0.78	50	3075
170	57.5	1.82	0.81	50	2875
171	84.2	1.8	1.07	50	4210
172	75.3	1.82	1.02	50	3765
173	83.3	1.7	0.78	50	4165
174	41.6	1.83	0.83	50	2080
175	58.4	1.85	1.12	50	2920
176	27.7	1.86	0.67	50	1385
177	67	1.85	1.08	50	3350
178	95.6	1.85	1.33	50	4780
179	74.5	1.72	0.82	50	3725
180	76.5	1.88	1.26	50	3815
181	65.3	1.87	0.73	50	3265
182	71	1.81	1.02	50	83550
183	67.1	1.68	0.76	50	3355
184	63.5	1.82	1.02	50	3175

185	71	1.81	1.27	50	3550
186	62.4	1.82	1.22	50	3120
187	72.4	1.85	1.3	50	3620
188	91.1	1.83	1.29	50	4555
189	75.9	1.85	1.38	50	3795
190	35.6	1.88	0.85	50	1780
191	59.8	1.84	1.11	50	2990
192	51.9	1.89	1.06	50	2595
193	43.1	1.83	0.91	50	2155
194	87.8	1.83	1.38	50	4390
195	126.3	1.88	1.68	50	6315
196	67.4	1.83	1.24	50	3370
197	46.8	1.86	0.92	50	2340
198	74.5	1.87	1.39	50	3725
199	76.2	1.82	1.3	50	3810
200	48	1.85	1.02	50	2400
201	51	1.81	1.03	50	2550
202	44.6	1.87	1.05	50	2230
203	40.8	1.79	0.96	50	2040
204	54.9	1.81	1.16	50	3745
205	71.5	1.82	1.23	50	3575
206	81.8	1.84	1.45	50	4090
207	87.4	1.88	1.45	50	4370
208	50.4	1.82	1	50	2520
209	66.9	1.86	1.51	50	3345
210	58.4	1.83	1.05	50	2920
211	97	1.83	1.36	50	3850
212	72.7	1.86	1.4	50	3635
213	48.8	1.84	1.19	50	2440
214	56.5	1.85	1.36	50	2825
215	15.3	1.83	0.58	50	765
216	114.8	1.84	1.66	50	5715
217	45.4	1.86	0.97	50	2270
218	47.9	1.84	1.02	50	2395
219	46.5	1.92	0.91	50	2325
220	56.1	1.87	1.03	50	2805
221	96.8	1.85	1.22	50	4840
222	52.3	1.84	1.15	50	2615
223	61.4	1.83	1.23	50	3070
224	54.1	1.83	0.64	50	2705
225	97.1	1.86	1.56	50	4855

average	46.97027	1.867838	0.971532	50	2685.18
std dev	20.69892	0.074487	0.274501	0	5547.997