Title: Maternal depression and child behaviours: sex-dependent mediation by glucocorticoid receptor gene methylation in a longitudinal study from pregnancy to age 5 years

Authors: Jonathan Hill[a], Andrew Pickles[b], Nicola Wright[c], John P Quinn[d], Chris Murgatroyd[e], Helen Sharp[c]

[a] School of Psychology and Clinical Language Sciences, University of Reading, Earley Gate, Reading RG6 6AL, UK, j.hill@reading.ac.uk; [b] Institute of Psychiatry, Psychology and Neuroscience, De Crespigny Park, London SE5 8AF, UK, andrew.pickles@kcl.ac.uk; [c] Department of Psychological Science, Institute of Psychology, Health and Society, Whelan Building, University of Liverpool, Brownlow Hill, Liverpool L69 3GB, UK, N.J.Wright@liverpool.ac.uk; hmsharp@liverpool.ac.uk; [d] Department of Molecular and Clinical Pharmacology, Institute of Translational Medicine, University of Liverpool, Brownlow Hill, Liverpool L69 3GB, UK, Jquinn@liverpool.ac.uk; [e] School of Healthcare Science, Manchester Metropolitan University, John Dalton Building, Chester Street, Manchester M1 5GD, UK, c.murgatroyd@mmu.ac.uk

Corresponding author: Jonathan Hill, School for Psychology and Clinical Language Sciences, University of Reading, Earley Gate, Reading, RG6 6AL, UK.

Email: j.hill@reading.ac.uk; Phone: 44 118 3785823

ABSTRACT

Background

Evolutionary hypotheses predict that male fetuses are more vulnerable to poor maternal conditions than females (Sex-biased Maternal Investment), but that the adaptive female fetus, with a more responsive hypothalamic-pituitary-adrenal (HPA) axis, is put at later risk of glucocorticoid mediated disorders where there is a mismatch between fetal and postnatal environments (Predictive Adaptive Response). Self-report measures of prenatal and postnatal depression and maternal report of child anxious depressed symptoms at 2.5, 3.5 and 5.0 years were obtained from an 'extensive' sample of first time mothers recruited from the general population (N = 794). Salivary *NR3C1* 1-F promoter methylation was assayed at 14 months in an 'intensive' subsample (N = 176) stratified during pregnancy by psychosocial risk. Generalised structural equation models (SEM) were fitted and estimated by maximum likelihood to allow inclusion of participants from both intensive and extensive samples.

Results

Postnatal depression was associated with *NR3C1* methylation and with anxious-depressed symptoms in the daughters of mothers lacking the hypothesised protective effect of high prenatal depression (prenatal-postnatal depression interaction for methylation, p =.00001; for child symptoms, p = .011). In girls, *NR3C1* methylation mediated the association between maternal depression and child anxious-depressed symptoms. The effects were greater in girls than boys, and the test of the sex differences in the effect of the prenatal-postnatal depression interaction on both outcomes gave $\chi^2(2) = 5.95$ (p=.051).

Conclusions

This is the first study to show in humans that, as a result of sex-biased reproductive investment and fetal adaptation, epigenetic and early behavioural outcomes may arise through different mechanisms in males and females. Epigenetic effects at the *NR3C1* promoter

47 mediated mismatch between prenatal and postnatal maternal conditions and child anxious-
48 depressed symptoms, specifically in females.
49
50 Key Words: maternal depression: *NR3C1* methylation; child anxiety-depression: sex
51 differences: parental reproductive investment: epidemiological sampling: mediation:
52 longitudinal design
53

54 **BACKGROUND**

55 The 'fetal origins' hypothesis was first proposed to account for associations between low
56 birth weight and obesity, cardiovascular disease, and Type II diabetes in middle and old age
57 [1]. According to this hypothesis, low birth weight reflects evolved adaptive mechanisms that
58 confer advantages later in life in food scarce environments, but create risk in the presence of
59 high calorie diets, common in industrialised societies. Far from being a mechanism specific
60 to nutrition in humans, adaptations prior to birth that anticipate later environments are found
61 across species, possibly reflecting a conserved 'Predictive Adaptive Response' (PAR)
62 mechanism [2, 3]. According to the PAR hypothesis matched prenatal and postnatal
63 conditions will be associated with good outcomes, while mismatching creates risks for later
64 offspring adaptation. In relation to effects on psychiatric disorders, many studies have
65 reported that anxiety, depression and behavioural symptoms in children are predicted by
66 prenatal stressors, maternal depression and anxiety, and by low birth weight [4 - 9] however
67 none has tested whether the PAR mechanism modifies these outcomes.

68 Fetal adaptations may additionally vary by sex of the fetus. According to the Trivers-
69 Willard (T-W) hypothesis, if maternal health during pregnancy predicts later reproductive
70 fitness in the offspring, then a male predominance of births will be favoured when maternal

71 conditions are good, because healthy males compete successfully for females. By contrast,
72 when maternal conditions are poor, the sex ratio will be reversed, both to avoid bearing males
73 who compete less successfully for females, but also because, compared to females, health
74 outcomes for mothers following male births are poorer [10]. Although this hypothesis has
75 been subject to challenges and modifications [11], the central idea that reproductive strategies
76 associated with poor maternal conditions involve sacrifice of males and protection of females
77 has received substantial support. It is also consistent with well documented observations that
78 male fetuses are more vulnerable to threats such as preterm birth, and are more likely to
79 suffer neurodevelopmental consequences of fetal insults [12]. This hypothesis would appear
80 to predict better outcomes for females following poor maternal conditions. However, if this
81 protective effect in females arises from advantages conferred by fetal anticipation of matched
82 environments (PAR hypothesis), mismatches between maternal conditions during pregnancy
83 and the postnatal environment will create vulnerability. Combining the T-W and PAR
84 hypotheses leads to the prediction that the effects of prenatal risks will operate differently in
85 males and females. In females, vulnerability will be generated by particular combinations of
86 prenatal and postnatal risks, while in males poor outcomes will arise incrementally from
87 degree of prenatal risk. In the only human study we are aware of to have examined for the
88 combined effects predicted by the T-W and PAR hypotheses, matched environments indexed
89 by prenatal and postnatal depression (low-low and high-high) were associated with better
90 cognitive and motor outcomes over the first year of life than mismatched prenatal and
91 postnatal depression, and this effect was seen in females only [13]. However many studies
92 have reported sex differences in developmental outcomes in relation to prenatal risks, without
93 examining for the interplay with postnatal environments. Sex differences in fetal responses to
94 stress [14], and in later emotional and behavioural problems following maternal anxiety or
95 depression during pregnancy and low birth weight [4, 7, 8, 15, 16] have been identified.

In animal models, prenatal and postnatal stress cause long-term elevations in hypothalamic pituitary axis (HPA) reactivity and anxiety-like behaviours. This is mediated via reduced glucocorticoid receptor (GR) gene *NR3C1* expression, particularly in the hippocampus which impairs HPA axis feedback mechanisms [17]. The epigenetic process of DNA methylation involves the addition of methyl groups to CpG dinucleotides in gene regulatory regions that are associated with repressed gene expression. Animal findings of the epigenetic effects of early life stress have been translated to humans in a study reporting elevated *NR3C1* 1-F promoter methylation and reduced *NR3C1* expression in post-mortem hippocampal tissue of suicide completers who were abused during childhood, when compared to non-abused [18]. Other studies using peripheral DNA from blood or saliva of infants and adolescents have shown increased levels of *NR3C1* methylation associated with prenatal and childhood adversities [19, 20, 21]. Several clinical studies examining leukocytes have reported elevated methylation of the homologous human *NR3C1* 1-F promoter (homologous to the rat 1-7 promoter) at a specific CpG (CpG unit 22,23, Figure 1) associated with prenatal maternal depression [19, 22-24] and childhood stress [25]. Studies in humans also find associations between prenatal anxiety and postnatal depression in mothers, and adolescent depressive symptoms mediated via HPA axis dysregulation [26, 27], consistent with the role of HPA axis dysregulation in adolescent depression [28]. Higher *NR3C1* methylation levels, hypothesised to contribute to reduced *NR3C1* expression (18), have been associated with increased salivary cortisol stress responses in infants at 3 months [19] and a flattened cortisol recovery slope following stress in adolescents [29], suggesting methylation of *NR3C1* may impair negative feedback of the HPA axis.

In the first study to examine the interplay between prenatal and postnatal depression in relation to *NR3C1* gene methylation, we showed that the association between postnatal maternal depression and *NR3C1* 1-F promoter methylation in their children was stronger

where mothers had reported lower depression during pregnancy, in line with the PAR hypothesis [30]. However, we did not examine for sex differences. Sex differences in glucocorticoid mechanisms associated with prenatal stress have been shown in animal models. In rats many effects of prenatal stress on later development are seen only in females, and these are abolished by adrenalectomy [31]. The effects predicted by a combination of the T-W and PAR hypotheses, have been demonstrated in starlings where mismatched prehatch-posthatch conditions had a greater effect on corticosterone levels in female than male chicks, but prenatal risk increased mortality in male chicks [32, 33]. In humans, a sex difference in associations between prenatal depression and *NR3C1* 1-F promoter methylation has been reported [34], although the interplay with postnatal depression however was not analysed.

In this study we examined predictions based on the T-W sex-biased parental investment and PAR hypotheses. In females, where individual and species vulnerability are reduced by matching environments but increased by mismatching, the presence of good prenatal conditions followed by adverse rearing experiences, and *vice versa*, will create vulnerability to child anxiety and depression. Based on the animal models, we predicted this effect in females will involve altered HPA axis reactivity arising from epigenetic modifications of the GR gene. In males, where individuals are sacrificed for species advantage, the presence of prenatal stress will create vulnerability, unmodified by later environment quality. The animal models suggest that glucocorticoid mechanisms are implicated in excess male deaths under unfavourable maternal conditions, but they may not contribute to effects of prenatal stress on functioning after birth.

These predictions were tested in a longitudinal study using measures of prenatal and postnatal depression, of *NR3C1* 1-F promoter region methylation at 14 months of age, and anxious depressed symptoms in children across the preschool period. We predicted that in girls but not boys, low prenatal depression followed by high postnatal maternal depression,

and high prenatal depression followed by low postnatal depression will be associated with elevated anxious depressed symptoms and elevated *NR3C1* methylation. In boys, prenatal and postnatal depression will be independent risks for elevated anxious-depressed symptoms, without the interaction between them predicted for females.

METHODS

Design

The participants were members of the Wirral Child Health and Development Study, a prospective epidemiological longitudinal cohort of first-time mothers recruited in pregnancy to study prenatal and infancy origins of emotional and behavioural disorders. The full cohort of 1233 mothers with live singleton births have participated in several waves of assessment with a stratified random sub-sample of 316 identified for additional, more intensive assessment (intensive sample). Strata were defined on the basis of low, medium and high psychosocial risk (scores of <=2, 3 or >3 on an inter-partner psychological abuse scale provided on entry to the study at 20 weeks of pregnancy), with higher selection probabilities for those at higher risk. Appropriately analysed, the design allows estimates of means and coefficients for the whole general population cohort to be derived even for measures available only in the intensive sample [35].

Approval for the procedures was obtained from the Cheshire North and West Research Ethics Committee (UK) (reference no. 05/Q1506/107). The extensive sample was identified from consecutive first time mothers who booked for antenatal care at 12 weeks gestation between 12/02/2007 and 29/10/2008. The booking clinic was administered by the Wirral University Teaching Hospital which was the sole provider of universal prenatal care on the Wirral Peninsula. Socioeconomic conditions on the Wirral range between the deprived inner city and affluent suburbs, but with few from ethnic minorities. The study was introduced to

171 the women by clinic midwives who asked for their agreement to be approached by study
172 research midwives when they attended for ultrasound scanning at 20 weeks gestation. After
173 complete description of the study to the women, written informed consent was obtained by
174 the study midwives, who then administered questionnaires and an interview in the clinic.

175 **Participants**

176 Of those approached by study midwives, 68.4% gave consent and completed the measures,
177 yielding an extensive sample of 1233 mothers with surviving singleton babies. The sampling
178 flow chart has been published previously [35]. The mean age at recruitment of extensive
179 sample participants was 26.8 years (s.d.5.8, range 18-51). Using the UK Index of Multiple
180 Deprivation (IMD) [36] based on data collected from the UK Census in 2001, 36.6 % of the
181 extensive sample reported socioeconomic profiles found in the most deprived UK quintile,
182 consistent with the high levels of deprivation in some parts of the Wirral. Forty eight women
183 (3.9%) described themselves as other than White British.

184 In addition to assessments of the mothers at 20 weeks gestation, mothers and infants
185 provided data at birth and postnatally at 5, 9, and 29 weeks, and at 14.19, s.d. 1.71 months
186 (‘14 months’), 30.86, s.d. 2.31 months (‘2.5 years’), 41.90 s.d. 2.48 months (‘3.5 years’) and
187 58.64 s.d. 3.74 months (‘5 years’). Two hundred and sixty eight mothers and infants came
188 into the lab at 14 months for detailed observational, interview and physiological measures.
189 This was the first occasion at which saliva for DNA was collected. Seven parents declined
190 consent for DNA collection, 3 samples were spoilt, and 25 assessments were curtailed before
191 saliva collection because of time constraints. Sufficient DNA for methylation analyses was
192 obtained from 181 infants. Maternal reports of child anxious-depressed symptoms were
193 available on 253 of the intensive sub-cohort at 2.5 years, on 825 of the whole cohort at 3.5
194 years and on 768 of the whole cohort at 4.5 years.

Measures

Maternal depression

Maternal symptoms of depression were assessed at 20 weeks gestation and at every follow up point using the Edinburgh Postnatal Depression Scale (EPDS), which has been used extensively to assess prenatal and postnatal depression [37].

DNA methylation

Methylation status in the *NR3C1* 1-F promoter was examined at the same CpGs (CpG unit 22 and 23, shown in Figure 1) identified in previous studies (24). DNA collected from Oragene® saliva samples, was extracted, bisulphite treated, amplified (Forward, GACCTGGTCTCTCTGGGG; Reverse, TGCAACCCCGTAGCCCCTTTC) and run on a Sequenom EpiTYPER system (Sequenom Inc., San Diego, US), providing an average for methylation across the two CpG units. Data was transformed to percentage of methylation at CpG unit 22 and 23 to allow for comparison with previous analysis of differential methylation at this locus.

Child anxious-depressed symptoms

Child symptoms were assessed by maternal report at 2.5, 3.5 and 5.0 years using the preschool Child Behavior Checklist (CBCL) [38]. It has 99 items each scored 0 (not true), 1 (somewhat or sometimes true), and 2 (very true or often true), which are summed to create seven syndrome scales. Only the anxious/depressed scale was analysed for this report, and as recommended in the CBCL manual, raw scores were used [39].

Stratification variable and confounders

Partner psychological abuse was assessed using a 20 item questionnaire covering humiliating, demeaning or threatening utterances in the partner relationship during pregnancy over the

previous year [40]. Maternal age (at this first pregnancy), marital status at 20 weeks gestation, and socioeconomic status were included as covariates because of their established associations with adult depression. Socioeconomic status was determined using the revised English Index of Multiple Deprivation (IMD) [36] based on neighborhood deprivation. All mothers were given IMD ranks according to the postcode of the area where they lived and assigned to a quintile, based on the UK distribution of deprivation. Mother's years of education at enrolment in the study was recorded. Information about smoking was obtained at 20 and 32 weeks gestation and was included because of published associations with altered DNA methylation [41]. Birth records provided sex of infant, one-minute Apgar score, and birth weight and gestational age, from which a measure of fetal growth was obtained. Low fetal growth is associated with elevated fetal glucocorticoid exposure and so might be associated with elevated *NR3C1* gene methylation. Obstetric risk was rated using a weighted severity scale developed by a collaboration of American and Danish obstetricians and paediatric neurologists [42].

Statistical Analysis

All analyses were undertaken in Stata 14 (StataCorp, 2015). Generalised structural equation models (SEM) were fitted using the `sem` procedure and estimated by maximum likelihood to allow inclusion of participants from both intensive and extensive strata. The anxiety-depression scores at 2.5, 3.5 and 5.0 years and *NR3C1* percent methylation at 14 months were highly skewed so scores were log-transformed and Winsorized at 2.5 standard deviations to reduce their skew. For further robustness, we report standard errors and p-values based on the heteroscedastic consistent estimator of the parameter covariance matrix. The main analyses included the stratification variable and confounds except for perinatal confounds as they may lie on a mediational pathway from prenatal depression, however the effect of adding those variables was examined. Model estimates and tests allowed for

243 differential missingness associated with any of the covariates and observed responses
244 included in the model, accounting for the stratified study design.

245 The pre-post environment mismatch predictions on both methylation and child
246 symptoms were examined first by testing for two-way interactions between prenatal and
247 postnatal depression in models estimated separately in females and males. We then tested for
248 the sex difference by examining the three-way, sex by prenatal depression by postnatal
249 depression interactions in a model that included both genders. The effects of combinations of
250 prenatal and postnatal depression giving rise to these interaction effects are shown in the
251 figures. The prediction of additive effects of prenatal and postnatal depression in boys was
252 examined in models without interaction terms.

253 In the fitted models methylation was specified as a factor, measured without error by
254 the observed methylation, a device that implicitly imputes rates of methylation where these
255 have not been observed, but doing so in a manner which recognises our uncertainty in these
256 unobserved values. This enables participants with partial data that would be informative for
257 some parts of the model to be included.

258

259 **RESULTS**

260 *Descriptive Statistics*

261 Table 1 gives summary statistics for males and females separately for the measures included
262 in the analysis, and shows the sample size at each data collection point. As described in the
263 statistical analysis section, differences in the available sample for different measures were
264 accounted for by use of weighted, maximum likelihood or covariate adjusted estimators.
265 Figure 2 shows the structure of the SEM model in which maternal history of depression
266 predicts *NR3C1* methylation (solid red arrows) and maternal report of child anxious-

267 depressed symptoms (solid black arrows). These analyses included the 412 girls and 382
268 boys on whom there were measures of maternal depression and maternal report of child
269 anxious-depressed symptoms at a minimum of one follow up point as well as all confounders.

270 Table 2 shows for girls and boys separately the estimated path coefficients from the
271 standardised prenatal depression, postnatal depression and their interaction (product) of
272 primary interest accounting for the stratification, attrition and confounders. We first tested the
273 prediction that there would be an interaction between prenatal and postnatal depression in
274 girls but not in boys. In girls there was a significant effect of the interaction between prenatal
275 and postnatal depression on both child anxiety-depression (p=.011) and *NR3C1* 1-F promoter
276 methylation (p =.00001). For boys, by contrast, anxious-depressed symptoms were not
277 predicted by the prenatal and postnatal depression interaction term (p=.920), and the effect on
278 *NR3C1* methylation was smaller than for girls, though still significant (p=.003). Adding the
279 three additional potential confounders that were assessed after the prenatal exposure
280 (obstetric risk index, 1-minute Apgar score and birthweight/gestational age) made no material
281 difference to these associations. Fitting this model to boys and girls together, but allowing
282 the effects of prenatal and postnatal depression exposure on the two correlated outcomes to
283 differ by sex (in addition to a gender main effect), a Wald test of the sex differences in the
284 effect of the prenatal-postnatal depression interaction on both outcomes (a difference of 0.20
285 for anxiety-depression and 0.18 for methylation) gave $\chi^2(2)$ of 5.95 (p=.051), with the two
286 individual interactions contributing equally (anxiety-depression p=.088, methylation p=.069).

287 We then tested the prediction that in boys there would be independent and additive
288 effects of prenatal and postnatal depression, by estimating the model (not shown in the Table)
289 for boys without the interaction term. This showed a significant effect on child anxiety-
290 depression of postnatal depression (standardised coefficient 0.17, CI 0.04 to 0.30, p = .011)
291 and an effect of similar magnitude, that was non-significant, of prenatal depression (0.15, CI -

292 0.02 to 0.33, p=.080). Independent effects on methylation were not seen (prenatal 0.05, CI -
293 0.17 to 0.27, p=.640; postnatal 0.13, CI -.09 to 0.36, p=.241).

294 Figure 3 displays how the interactions between prenatal and postnatal depression in the
295 prediction of anxious-depressed symptoms differed between girls and boys. It can be seen
296 that, in girls, at a low level of prenatal depression (1 standard deviation below the mean),
297 increasing postnatal depression was strongly associated with increasing child anxious-
298 depressed symptoms, while at a high level there was no association. With prenatal depression
299 at the mean, the association was intermediate between the low and high prenatal levels. In
300 boys, by contrast, as evidenced in parallel regression lines, there was no interplay between
301 prenatal and postnatal maternal depression.

302 As shown in Figure 4, the effects of prenatal-postnatal mismatch on methylation were
303 again strongly evident in girls, with the greatest association between postnatal depression and
304 methylation in the presence of low prenatal depression, and progressively weaker
305 associations at higher levels of prenatal depression. The progressive effect of prenatal
306 depression was also evident in boys but it was less strong.

307 In girls, replacing the correlation between the methylation and anxiety-depression
308 factors by a causal effect, higher *NR3C1* methylation at 14 months was associated with
309 higher anxiety-depressed symptoms (standardised coefficient 0.36 CI 0.05 to 0.67, p=.025) .
310 The residual direct effect of the prenatal-postnatal interaction on child anxiety-depression
311 symptoms was substantially reduced, from -0.19 (shown in Table 2) to -0.06 (CI -0.26 to
312 0.15), becoming wholly nonsignificant (p=.600). For boys there was no evidence of an effect
313 of methylation on symptoms (standardised coefficient -0.03, CI -0.31 to 0.24, p=.820).

314

315 **DISCUSSION**

316 Many, although not all, of our predictions based on the evolutionary T-W and PAR
317 hypotheses for sex-biased parental investment and fetal programming were supported in this
318 longitudinal study, from 20 weeks of pregnancy and over the first 5 years of children's lives.
319 Mismatching between prenatal and postnatal maternal depression was associated with greater
320 anxious-depressed symptoms and *NR3C1* methylation in girls. Both effects were most
321 evident in girls exposed to high levels of postnatal depression. Their symptoms and *NR3C1*
322 methylation were higher where their mothers had reported low levels of depression during
323 pregnancy, in line with the idea that they had not been prepared by the fetal environment for
324 postnatal exposure to maternal depression. In girls only, elevated *NR3C1* was associated with
325 higher anxious-depressed symptoms, and mediated the association between maternal
326 depression and child symptoms. In boys there was no evidence of effects of prenatal –
327 postnatal mismatch on anxious depressed symptoms. However, and contrary to our
328 prediction, the prenatal-postnatal mismatch effect on *NR3C1* methylation was seen in boys as
329 well as in girls, although the size of the effect was smaller.

330 The strengths of the investigation include prospective study with a general population
331 sample, accounting for a number of plausible confounds and factors associated with attrition.
332 Also, by using SEM to create a latent variable from measurement at 3 time points over 2.5
333 years we reduced the risks arising from multiple testing for each time point, and we were able
334 to examine the predictions in relation to persistently elevated symptoms likely to confer risk
335 for an elevated trajectory for anxious-depressed symptoms over childhood [43]. The method
336 adopted for missing methylation data exploited the properties of maximum likelihood for
337 accounting for data assumed missing at random. Most missingness was by design because of
338 the systematic stratification of the intensive sample, thus meeting this assumption, and
339 inclusion of multiple covariates allowed us account for unplanned attrition. It is nevertheless

possible that not all the necessary confounds to deal with non-random missingness were identified.

There were four principal limitations in relation to the measurement of *NR3C1* methylation. First, peripheral cell samples, both from blood and saliva, are heterogeneous, which may account for some of the variability in methylation. This can introduce a confound where other variables are associated with cellular heterogeneity [44]. Second, while studies combining peripheral cell and CNS post mortem estimations suggest that they are often substantially correlated [45], it cannot be assumed that DNA methylation in peripheral tissues reflects methylation in relevant CNS regions. This is particularly a concern because of substantial variations in epigenetic effects across brain regions and cell types. Third there are many combinations of CpG sites, even on a relatively circumscribed region such as the *NR3C1* 1-F promoter that could be examined, leading to the risk of multiple analyses and 'significant' findings occurring by chance. Fourth, although we accounted for a number of plausible confounds, environmental variables other than those included in analyses may better account for the findings.

No one study can establish the validity of estimates of peripheral cell methylation as indices of CNS methylation, however a finding of the same pattern of associations for peripheral cell methylation and for behaviours that undoubtedly reflect CNS function, and for mediation of the association between maternal depression and symptoms by *NR3C1* methylation is relevant to the issue. As is evident from the SEM models, and as seen in Figures 3 and 4, there were striking similarities between the patterns of associations involving interactions between prenatal and postnatal depression and sex differences, not only for child anxious-depressed symptom but also for *NR3C1* methylation. Furthermore, in this study we reduced risks arising from multiple analyses of many potential methylation sites by

364 examining only one site that had been identified from a meta-analysis of previous studies
365 [24].

366

367 **CONCLUSIONS**

368 Our findings are important in five major ways. First they provide pointers to study
369 designs that could be introduced into animal models where mechanisms can be examined
370 using experimentally controlled risks. These would for example examine the interplay
371 between prenatal and postnatal risks in relation to the role of the placenta in regulating
372 passage of maternal glucocorticoids to the foetus, which in turn can be controlled by further
373 epigenetic modifications of specific placental genes [46]. Second they illustrate how
374 evolutionary hypotheses regarding parental investment in offspring can be used to generate
375 novel, and in some ways surprising, predictions regarding parenting and early development in
376 humans. Third, testing in this way can generate further productive questions. In this study,
377 while there was good evidence for mismatch effects in females on *NR3C1* methylation and
378 child symptoms, and for a sex difference in relation to child symptoms, the prenatal-postnatal
379 depression mismatch was also associated with *NR3C1* methylation in males, which was
380 contrary to the predictions. Further study is needed into the conditions under which fetal
381 programming effects are seen in males as well as females, and under what conditions there
382 are sex differences in the behavioural implications of *NR3C1* methylation. Fourth they show
383 that, even though human development is subject to many complex social and psychological
384 influences, biological mechanisms conserved across many non-human species, can be highly
385 influential. Fifth they suggest that some prenatal effects on epigenetic and behavioural
386 outcomes in early childhood, differ radically in males and females, and so further study of

sex specific mechanisms is needed. This will have implications for our understanding of the biology of psychiatric disorders arising in childhood.

Abbreviations

HPA - Hypothalamic-Pituitary-Adrenal Axis

PAR - Predictive Adaptive Response

NR3C1 - Nuclear Receptor Subfamily 3 Group C Member 1 Gene

SEM – Structural Equation Modelling

T-W - Trivers-Willard Hypothesis,

GR - Glucocorticoid Receptor

DNA - Deoxyribonucleic Acid.

CpG – Cytosine Phosphate Guanine

IMD – Index of Multiple Deprivation

EPDS - Edinburgh Postnatal Depression Scale

CBCL - Child Behavior Checklist

CNS – Central Nervous System

DECLARATIONS

Ethics approval and consent to participate

Ethical approval for the study was granted by the Cheshire North and West Research Ethics Committee, UK, on the 27th June 2006. The letter confirming ethical agreement for the study (reference number 05/Q1506/107) stated, 'On behalf of the Committee, I am pleased to confirm a favourable ethical approval for the above research on the basis described in the application form, protocol and supporting document as revised.' At recruitment at 20 weeks pregnancy, after complete description of the study to the women, written informed consent was obtained by the study midwives, who then administered questionnaires and an interview in the clinic.

Consent for publication

Not applicable

Availability of data and material

There is not open access to the data, because that is not permitted by our ethical approval. However requests for access to the data can be considered via contact with the first author.

Competing interests

None of the authors has a conflict of interest.

Funding

This study was funded by grants from the UK Medical Research Council (G0400577 and G0900654).

Authors' contributions

JH, HS, AP designed the study, CM, JQ conducted the methylation estimations, JH, HS, NW supervised data collection, JH, AP, NW analysed the data, JH, AP, HS, NW wrote the paper, and all authors read and approved the final manuscript.

Acknowledgements

We are very grateful to all participating families and to the research staff who contributed to this work. We also thank Wirral University Teaching Hospital NHS Foundation Trust who supported initial recruitment and Cheshire and Wirral Partnership NHS Foundation Trust and Wirral Community NHS Trust for their current support and the NIHR Biomedical Research Centre for Mental Health at the South London and Maudsley NHS Foundation Trust and King's College London. We would also like to extend our thanks to all the staff involved in the Wirral Child Health and Development Study.

REFERENCES

1. Barker DJ: **The origins of the developmental origins theory**. *J Intern Med* 2007, **261**(5):412-417.
2. Bateson P, Gluckman P, Hanson M: **The biology of developmental plasticity and the Predictive Adaptive Response hypothesis.** *The Journal of Physiology* 2014, **592**(Pt 11):2357-2368.
3. Wells JC: **Environmental quality, developmental plasticity and the thrifty phenotype: a review of evolutionary models.** *Evol Bioinform Online* 2007, **3**:109-120.
4 Costello EJ, Worthman C, Erkanli A, Angold A: **Prediction from low birth weight to female adolescent depression: a test of competing hypotheses.** *Arch Gen Psychiatry* 2007, **64**(3):338-344.
5. Barker ED, Jaffee SR, Uher R, Maughan B: **The contribution of prenatal and postnatal maternal anxiety and depression to child maladjustment.** *Depress Anxiety* 2011, **28**(8):696-702.
6. O'Connor TG, Heron J, Golding J, Glover V: **Maternal antenatal anxiety and behavioural/emotional problems in children: a test of a programming hypothesis.** *J Child Psychol Psychiatry* 2003, **44**(7):1025-1036.
7. Quarini C, Pearson RM, Stein A, Ramchandani PG, Lewis G, Evans J: **Are female children more vulnerable to the long-term effects of maternal depression during pregnancy?** *J Affect Disord* 2016, **189**:329-335.
8. Sharp H, Hill J, Hellier J, Pickles A: **Maternal antenatal anxiety, postnatal stroking and emotional problems in children: outcomes predicted from pre- and postnatal programming hypotheses.** *Psychol Med* 2015, **45**(2):269-283.
9. MacKinnon N, Kingsbury M, Mahedy L, Evans J, Colman I: **The Association Between Prenatal Stress And Externalizing Symptoms In Childhood: Evidence**

From The Avon Longitudinal Study Of Parents And Children. *Biol Psychiatry*, in press.

10. Trivers RL, Willard DE: **Natural selection of parental ability to vary the sex ratio of offspring.** *Science* 1973, **179**(4068):90-92.
11. Schindler S, Gaillard J-M, Grüning A, Neuhaus P, Traill LW, Tuljapurkar S, *et al.*: **Sex-specific demography and generalization of the Trivers-Willard theory.** *Nature* 2015, **526**(7572):249-252.
12. Aiken CE, Ozanne SE: **Sex differences in developmental programming models.** *Reproduction* 2013, **145**(1):R1-3.
13. Sandman CA, Glynn LM, Davis EP: **Is there a viability-vulnerability tradeoff? Sex differences in fetal programming.** *J Psychosom Res* 2013, **75**(4):327-335.
14. Doyle C, Werner E, Feng T, Lee S, Alternus M, Isler JR, *et al.*: **Pregnancy distress gets under fetal skin: Maternal ambulatory assessment & sex differences in prenatal development.** *Dev Psychobiol* 2015, **57**(5):607-625.
15. Glover V, Hill J: **Sex differences in the programming effects of prenatal stress on psychopathology and stress responses: An evolutionary perspective.** *Physiol Behav* 2012, **106**(5):736-740.
16. Van den Bergh BR, Van CB, Smits T, Van HS, Lagae L: **Antenatal maternal anxiety is related to HPA-axis dysregulation and self-reported depressive symptoms in adolescence: a prospective study on the fetal origins of depressed mood.** N*europsychopharmacology* 2008, **33**(3):536-545.
17. Meaney MJ, Szyf M, Seckl JR: **Epigenetic mechanisms of perinatal programming of hypothalamic-pituitary-adrenal function and health.** *Trends Mol Med* 2007, **13**(7):269-277.

18. McGowan PO, Sasaki A, D'Alessio AC, Dymov S, Labonte B, Szyf M, *et al.*: **Epigenetic regulation of the glucocorticoid receptor in human brain associates with childhood abuse.** *Nature Neuroscience* 2009, **12**: 342-348.

19. Oberlander TF, Weinberg J, Papsdorf M, Grunau R, Misri S, Devlin AM: **Prenatal exposure to maternal depression, neonatal methylation of human glucocorticoid receptor gene (NR3C1) and infant cortisol stress responses.** *Epigenetics* 2008, **3**: 97–106.

20. Tyrka AR, Price LH, Marsit C, Walters OC, Carpenter LL (2012): **Childhood adversity and epigenetic modulation of the leukocyte glucocorticoid receptor: preliminary findings in healthy adults**. *PLoS One* 2012, **7**:e30148.

21. Conradt E, Hawes K, Guerin D, Armstrong DA, Marsit CJ, Tronick E, *et al.*: **The contributions of maternal sensitivity and maternal depressive symptoms to epigenetic processes and neuroendocrine functioning.** *Child Development* 2016, **87**(1):73-85.

22. Conradt E, Lester BM, Appleton AA, Armstrong DA, Marsit CJ: **The roles of DNA methylation of NR3C1 and 11beta-HSD2 and exposure to maternal mood disorder in utero on newborn neurobehavior.** *Epigenetics : Official Journal of the DNA Methylation Society* 2013, **8**(12):1321-1329.

23. Hompes T, Izzi B, Gellens E, Morreels M, Fieuws S, Pexsters A, *et al.*: **Investigating the influence of maternal cortisol and emotional state during pregnancy on the DNA methylation status of the glucocorticoid receptor gene (NR3C1) promoter region in cord blood.** *J Psychiatr Res* 2013, **47**(7):880-891.

24. Palma-Gudiel H, Cordova-Palomera A, Eixarch E, Deuschle M, Fananas L: **Maternal psychosocial stress during pregnancy alters the epigenetic signature of the**

glucocorticoid receptor gene promoter in their offspring: a meta-analysis. *Epigenetics : Official Journal of the DNA Methylation Society* 2015, **10**(10):893-902.

25. Melas PA, Wei Y, Wong CCY, Sjöholm LK, Äberg E, Mill J, *et al.*: **Genetic and epigenetic associations of MAOA and NR3C1 with depression and childhood adversities.** *Int J Neuropsychopharmacol* 2013, **1**:1-16.
26. Halligan SL, Herbert J, Goodyer I, Murray L: **Disturbances in morning cortisol secretion in association with maternal postnatal depression predict subsequent depressive symptomatology in adolescents.** *Biol Psychiatry* 2007, **62**(1):40-46.
27. Van den Bergh BR, Mulder EJ, Mennes M, Glover V: **Antenatal maternal anxiety and stress and the neurobehavioural development of the fetus and child: links and possible mechanisms. A review.** *Neurosci Biobehav Rev* 2005, **29**(2):237-258.
28. Goodyer IM, Herbert J, Tamplin A: **Psychoendocrine antecedents of persistent first-episode major depression in adolescents: a community-based longitudinal enquiry.** *Psychol Med* 2003, **33**(4):601-610.
29. Van der Knaap LJ, Oldehinkel AJ, Verhulst FC, Van Oort FV, Riese H: **Glucocorticoid receptor gene methylation and HPA-axis regulation in adolescents. The TRAILS study.** *Psychoneuroendocrinology* 2015, **58**, 46-50.
30. Murgatroyd C, Quinn JP, Sharp HM, Pickles A, Hill J (2015): **Effects of prenatal and postnatal depression, and maternal stroking, at the glucocorticoid receptor gene.** *Transl Psychiatry* 2015, **5**:e560.
31. Weinstock M: **Sex-dependent changes induced by prenatal stress in cortical and hippocampal morphology and behaviour in rats: an update.** *Stress* 2011, **14**(6):604-613.

534 32. Love OP, Williams TD: **Plasticity in the adrenocortical response of a free-living**
535 **vertebrate: the role of pre- and post-natal developmental stress.** *Horm Behav* 2008,
536 **54**(4):496-505.
537 33. Love OP, Williams TD: **The adaptive value of stress-induced phenotypes: effects of**
538 **maternally derived corticosterone on sex-biased investment, cost of reproduction,**
539 **and maternal fitness.** *Am Nat* 2008, **172**(4):E135-149.
540 34. Braithwaite EC, Kundakovic M, Ramchandani PG, Murphy SE, Champagne FA:
541 **Maternal prenatal depressive symptoms predict infant NR3C1 1F and BDNF IV**
542 **DNA methylation.** *Epigenetics* 2015, **10**(5):408-17.
543 35. Sharp H, Pickles A, Meaney M, Marshall K, Tibu F, Hill J: **Frequency of infant**
544 **stroking reported by mothers moderates the effect of prenatal depression on infant**
545 **behavioural and physiological outcomes.** *PLoS One* 2012, **7**(10):e45446.
546 36. Noble M, Wright G, Dibben C, Smith G, McLennan D, Anttila C, *et al.*: The English
547 Indices of Deprivation 2004 (revised). Report to the Office of the Deputy Prime
548 Minister; 2004 (Neighbourhood Renewal Unit., London).
549 37. Cox JL, Chapman G, Murray D, Jones P: **Validation of the Edinburgh Postnatal**
550 **Depression Scale (EPDS) in non-postnatal women.** *J Affect Disord* 1996, **39**(3):185-
551 189.
552 38. Rescorla LA, Achenbach TM, Ivanova MY, Harder VS, Otten L, Bilenberg N, *et al.*:
553 **International comparisons of behavioral and emotional problems in preschool**
554 **children: parents' reports from 24 societies.** *J Clin Child Adolesc Psychol* 2011,
555 **40**(3):456-467.
556 39. Achenbach TM, Rescorla LA: Manual for the ASEBA Preschool Forms & Profiles.
557 Burlington, VT: University of Vermont, Research Center for Children, Youth, &
558 Families; 2000.

559 40. Moffitt TE, Caspi A, Krueger RF, Magdol L, Margolin G, Silva PA, *et al.*: **Do partners**
560 **agree about abuse in their relationship? A psychometric evaluation of interpartner**
561 **agreement.** *Psychological Assessment* 1997, **9**(1):47-56.
562 41. Knopik VS, Maccani MA, Francazio S, McGeary JE: **The epigenetics of maternal**
563 **cigarette smoking during pregnancy and effects on child development.** *Dev*
564 *Psychopathol* 2012, **24**(4):1377-1390.
565 42. Beck JE, Shaw DS: **The influence of perinatal complications and environmental**
566 **adversity on boys' antisocial behavior.** *J Child Psychol Psychiatry* 2005, **46**(1):35-46.
567 43. Sterba SK, Prinstein MJ, Cox MJ: **Trajectories of internalizing problems across**
568 **childhood: heterogeneity, external validity, and gender differences.** *Dev*
569 *Psychopathol* 2007, **19**(2):345-366.
570 44. Jaffe AE, Irizarry RA: **Accounting for cellular heterogeneity is critical in epigenome-**
571 **wide association studies.** *Genome Biology* 2014, **15**(2):R31.
572 45. Tylee DS, Kawaguchi DM, Glatt SJ: **On the outside, looking in: a review and**
573 **evaluation of the comparability of blood and brain "-omes".** *American Journal of*
574 *Medical Genetics. Part B, Neuropsychiatric Genetics : the official publication of the*
575 *International Society of Psychiatric Genetics* 2013, **162b**(7):595-603.
576 46. Conradt E, Lester BM, Appleton AA, Armstrong DA, Marsit CJ: **The roles of DNA**
577 **methylation of NR3C1 and 11β-HSD2 and exposure to maternal mood disorder in**
578 **utero on newborn neurobehavior.** *Epigenetics* 2013, **8**(12):1321-9.
579 47. Weaver IC, Cervoni N, Champagne FA, D'Alessio AC, Sharma S, Seckl JR, *et al.*:
580 **Epigenetic programming by maternal behavior.** *Nat Neurosci* 2004, **7**: 847–854.

581

582 **Figures**

583 Figure 1 Scheme of the human NR3C1 gene analyzed by bisulfite pyrosequencing. The 5′-

584 end of the human NR3C1 gene contains multiple first exons, with multiple transcriptional
585 start sites and mRNA splice variants. The region analyzed by bisulfite pyrosequencing
586 (primer sequences are in bold) contains 29 CpGs and encompasses exon 1-F, which is the
587 human homolog of the rat exon 1–7, previously shown to be differentially methylated [47]
588
589 Figure 2 Structural equation model fitted to *NR3C1* 1-F promoter methylation at 14
590 months and CBCL anxious-depressed scores at ages 2.5, 3.5, and 5 years
591
592 Figure 3 Regression lines for the interaction between pre- and post-natal depression and
593 child anxious-depressed symptoms, showing the effect of postnatal depression at the
594 mean and one standard deviation either side of the mean
595
596 Figure 4 Regression lines for the interaction between pre- and post-natal
597 depression and child NR3C1 1-F promoter methylation, showing the effect of
598 postnatal depression at the mean and one standard deviation either side of the mean
599

Table 1 Summary statistics for outcomes, predictors and variables included as potential confounders for the modelled sample (I = measure based on intensively assessed sub-sample only)

	Girls			Boys		
	N	Mean	S		Mean	SD
Child anxious-depressed symptoms 2.5 years(I)	125	1.54	1.77	120	1.27	1.61
Child anxious-depressed symptoms 3.5 years	387	1.60	1.64	366	1.59	1.70
Child anxious-depressed symptoms 5 years	372	1.76	1.96	347	1.78	2.01
Child *NR3C1* methylation(I)	89	3.42	1.85	87	3.55	1.96
Prenatal EPDS maternal depression scores	412	6.94	4.74	382	7.42	4.54
Mean postnatal EPDS maternal depression scores	412	5.24	3.92	382	5.79	4.35
Stratum low	412	77%		382	75%	
Stratum mid		8%			7%	
Stratum high		16%			18%	
Maternal age <21 years	412	10%		382	12%	
Maternal age 21-30 years		56%			56%	
Maternal age >30 years		34%			32%	
No maternal education	412	62%		382	67%	

beyond age 18						
Smoking – none	412	62%		382	64%	
Smoking before pregnancy		21%			19%	
Smoking during pregnancy		17%			18%	
No partner	412	17%		382	19%	
Most Deprived Quintile	412	37%		382	36%	
Obstetric risk index	412	2.20	1.18	382	2.20	1.19
Birthweight/gestation (gm/wk)	412	83.6	11.9	382	86.5	11.4
1 Minute Apgar score	412	8.95	1.60	382	8.86	1.76

Table 2 Summary of SEM analyses predicting *NR3C1* 1-F promoter methylation and child anxious depressed symptoms

	Female (n=412)		Male (n=382)	
	Std Coeff [p-value]	95% C.I	Std Coeff [p-value]	95% C.I
Effects on child symptoms of anxiety-depression				
Prenatal maternal depression	-0.06	-0.23, 0.11	0.16	-0.00, 0.33
Postnatal maternal depression	0.21	0.05, 0.38	0.17	0.03, 0.31
Prenatal-postnatal interaction	-0.19 [p=.011]	-0.34,-0.05	0.01 [p=.920]	-0.11, 0.12
Effects on child *NR3C1* 1-F promoter methylation				
Prenatal maternal depression	-.02	-0.28, 0.24	-0.11	-0.34, 0.12
Postnatal maternal depression	0.45	0.16, 0.75	0.38	0.11, 0.65
Prenatal-postnatal interaction	-0.39 [p=.00001]	-0.56, -0.21	-0.21 [p=.003]	-0.32. -0.08
Child anxious-depressed symptoms factor loadings				
2.5 years	0.81		0.72	
3.5 years	0.80		0.67	
5 years	0.57		0.81	

The table shows standardized factor loadings of child CBCL anxious-depressed symptoms at ages 2.5, 3.5 and 5 years, and main effects and effects of interaction of

prenatal and postnatal depression in the prediction of the anxious-depressed factor and the *NR3C1* 1-F promoter methylation (effects of stratification factors and confounders not shown). Anxious-depressed symptoms and methylation are analysed together as correlated outcomes in an SEM. Coefficients for the effects of confounders and stratification factors are not shown (stratum, maternal age, maternal smoking, maternal education, no partner, neighbourhood deprivation).

1 2 3 4 5 6 7 8 9 10 11

CACTTCACGCAACTCGGCCCGGCGGCGGCGGCGCGGGCCACTCACGCAGCTCAGCCGCGGGA

12 13 14 15 16 17 18 19

GGCGCCCCGGCTCTTGTGGCCCGCCCGCTGTCACCCGCAGGGGCACTGGCGGCGCTTGCCGC

20 21 22 23 24 25

CAAGGGGCAGAGCGAGCTCCCGAGTGGGTCTGGAGCCGCGGAGCTGGGCGGGGGCGGGAAGG

26 27 28 29

AGGTAGCGAGAAAAGAAACTGGAGAAACTCGGTGGCCCTCTTAACGCCGCCCCAGAGAGACC

Figure 1 Scheme of the human NR3C1 gene analyzed by bisulfite pyrosequencing. The 5′-end of the human NR3C1 gene contains multiple first exons, with multiple transcriptional start sites and mRNA splice variants. The region analyzed by bisulfite pyrosequencing (primer sequences are in bold) contains 29 CpGs and encompasses exon 1-F, which is the human homolog of the rat exon 1–7, previously shown to be differentially methylated [47]

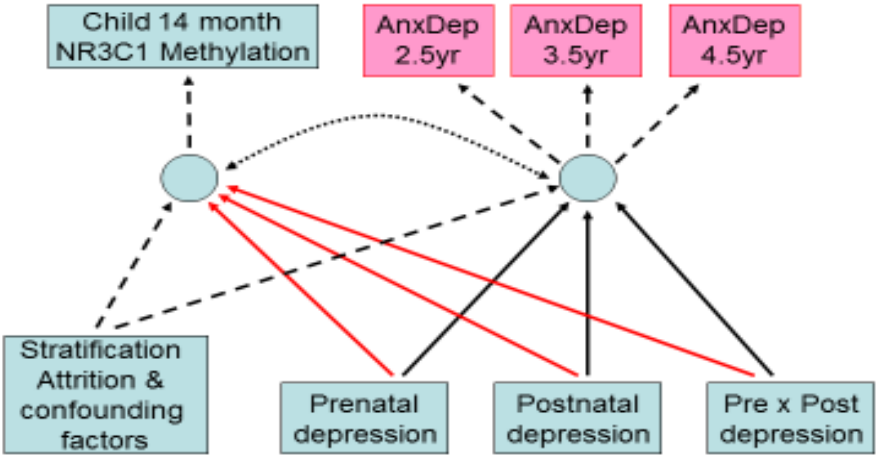

Figure 2 Structural equation model fitted to *NR3C1* 1-F promoter methylation at 14 months and CBCL anxious-depressed scores at ages 2.5, 3.5, and 5 years

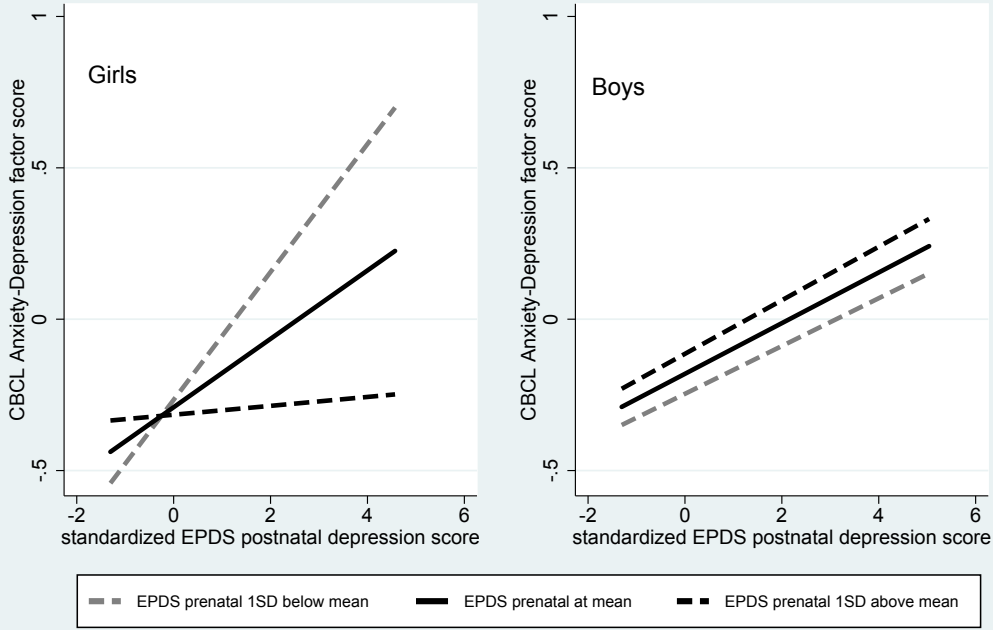

Figure 3 Regression lines for the interaction between pre- and post-natal depression and child anxious-depressed symptoms, showing the effect of postnatal depression at the mean and one standard deviation either side of the mean

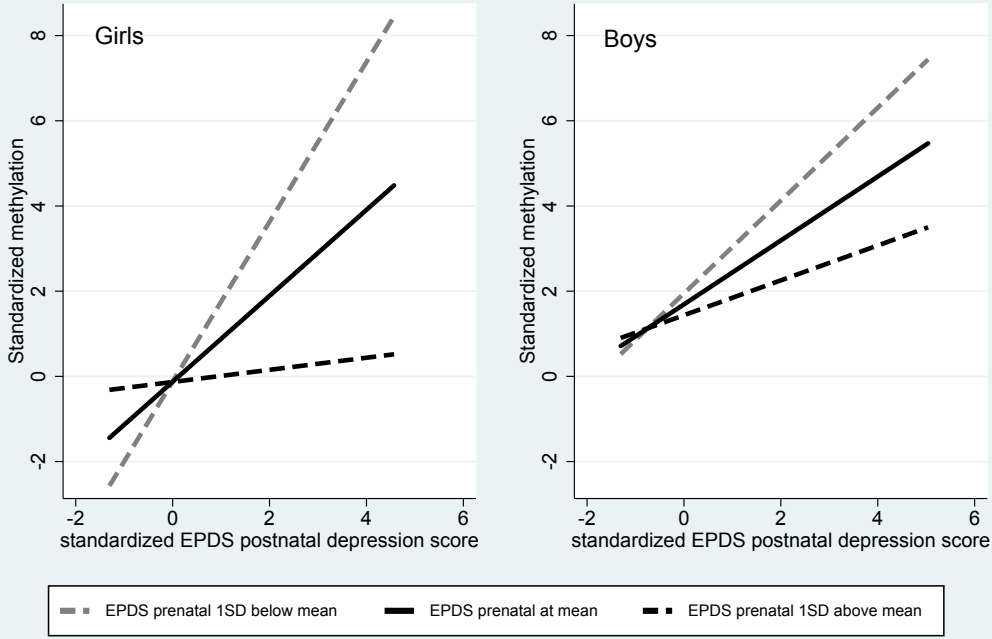

Figure 4 Regression lines for the interaction between pre- and post-natal depression and child NR3C1 1-F promoter methylation, showing the effect of postnatal depression at the mean and one standard deviation either side of the mean